PLENARY ABSTRACTS

1

MOLECULAR CLOCKS IN MAMMALIAN CIRCADIAN SYSTEM

H. Okamura

Division of Molecular Brain Science, Department of Brain Sciences, Kobe University Graduate School of Medicine, Kobe, Japan.

Rhythmic change in the internal milieu corresponding to the environmental cycle is universally observed in almost all living organisms. Among these periodical cycles, the 24-hr rhythm corresponding to the light-dark cycles elicited by the rotation of earth affects temporal patterns of gene expression, cellular activities, and the function and behavior of organs. Many of these daily variations arise from an endogenous mechanism that is autonomously oscillating with a period of 24 hr. The discovery of clock genes and a general principle of their oscillation have taken the research on biological clocks to an interesting field of life science. In mammals, as in other species, the mammalian circadian core oscillator is composed of an autoregulatory transcription-(post)translation-based feedback loop involving a set of clock genes. Production, phosphorylation, ubiquitination and proteasome-dependent degradation of clock proteins have a key role for generating the clock oscillation. The signal transduction cascade originating from this core oscillatory loop induces the expression of a variety of genes in a circadian fashion. The generation of internal clock time occurs in the hypothalamic suprachiasmatic nucleus (SCN), where multiphased cell-clock rhythms is coupled and amplified among communications of thousands of cell-clocks. The strong rhythmic expression of gene was detected in the living animals through the inserted optical fiber just above the SCN of the transgenic mice carrying mPer1 promoter driven luciferase reporter. Clock signals were transmitted from the SCN to peripheral tissues through oscillation conducting systems. Arrived clock signals entrain the intracellular oscillating loop in the peripheral cells which may coordinate the timing of the expression of a variety of tissue-specific genes. Investigations of biological clocks open a fascinating perspective to analyze the integration mechanism of "time" from genes to the living organism.

2

THE MAKING OF AN EMBRYO: SHORT-TERM GOALS AND LONG-TERM IMPLICATIONS

Tom P. Fleming

School of Biological Sciences, University of Southampton, Southampton SO16 7PX, UK.

At first sight, the construction of the preimplantation embryo in eutherian mammals seems such a simple process: the generation of a hollow ball of cells comprising an outer trophectoderm epithelium (TE) layer and enclosing an inner cell mass (ICM) with placental and fetal fates, respectively. However, the fascination is in the detail and the subtle mechanisms utilising cell-cell interactions, differentiative cell divisions and an inherent gene expression programme to guide the formation, segregation and relative size of these two critical cell populations. The mouse embryo has become an ideal model for understanding how an epithelium forms in a step-wise manner in a 'real' tissue *in situ* engaging temporally controlled gene activity. We have

focused on the mechanisms coordinating biogenesis of intercellular adhesion and multi-protein membrane junction complexes in the TE and these will be discussed. With the advent of reproductive biotechnologies, it has become apparent that these short-term goals of lineage formation and diversification prior to implantation may have more lasting consequences. The mammalian early embryo is sensitive to its environment which may influence both early morphogenesis but most significantly later fetal and postnatal growth and physiology. Thus, in two models we have developed in rodents, (i) maternal low protein diet fed exclusively during the preimplantation period and (ii) in vitro culture followed by embryo transfer, both have been shown to alter postnatal growth, systolic blood pressure and organ allometry in a gender-specific manner. Such potential 'programming' during early development has clear healthcare implications. To unravel mechanisms of dietary influence upon embryos, analysis of maternal serum, uterus or uterine fluid composition indicates potential roles for amino acid and growth factor environments in the mediation of programming. Analysis of embryo responses to adverse conditions indicate subtle changes occur in lineage allocation and gene expression potential, in particular associated with imprinted genes. The legacy of such early changes is under current investigation. For example, in one direction, we find that post-implantation nutritional support provided by the rodent visceral yolk sac becomes compromised. Thus, we consider that embryonic programming involves a combination of interacting processes operating at metabolic, genetic, cellular and physiological levels. The fascination with simple embryos continues!

4

STUDYING THE GH/IGF/INSULIN AXIS USING THE KNOCKOUT AND TRANSGENIC APPROACHES

Derek Le Roith

Diabetes Branch, NIH, Bethesda, MD, USA.

Insulin-like growth factors (IGFs) are involved in normal growth and development. Initially, it was thought that IGF-I was produced solely by the liver in response to growth hormone (GH), and that this liver-produced, circulating IGF-I was the mediator of GH-effects on pubertal growth. More recently, it has been established that virtually all tissues produce IGF-I. Thus two distinct systems were recognized, the "endocrine" circulating IGF-I, and the locally produced "autocrine/paracrine" IGF-I. Since the endocrine form of IGF-I is extremely sensitive to GH, it was believed that GH stimulates growth via the circulating IGF-I and locally produced IGF-I has some local tissue-specific effects.

We have re-examined this hypothesis by ablating the liver production of IGF-I in a tissue-specific manner (LID mice), utilizing the cre/loxP system. Circulating IGF-I levels are significantly reduced (by 70%) and GH levels are markedly elevated; proving that circulating IGF-I is primarily derived from the liver and controls GH secretion. Suprisingly the (LID) mice had normal growth and development. However, when we crossed these mice with acid-labile subunit knockout (ALSKO) mice we were able to reduce circulating IGF-I levels even further and these mice showed postnatal growth retardation and osteopenia, suggesting that circulating IGF-I is important for post-natal growth and development.

Circulating GH levels were elevated in the LID mice and this was associated with insulin resistance as determined by hyperinsulinemia in the face of normoglycemia and using the hyperinsulinemic-euglycemic clamp technique. The insulin resistance could be corrected by injecting rhIGF-I, a GHRH antagonist and most convincingly by crossing these animals with a

GH antagonist transgenic mouse. Thus it appears that, at least in the case of this mouse model, GH is the most proximal cause for the insulin resistance seen in the face of low circulating IGF-I levels.

5

RISK FACTORS IN AUTOIMMUNE THYROID DISEASE

Terry F. Davies

Division of Endocrinology, Diabetes and Bone Disease, Mount Sinai School of Medicine, New York, New York, USA.

A variety of new information has been generated which sheds light on susceptibility to the autoimmune thyroid diseases. Major risk factors include both genetic and environmental factors which are under intensive investigation. To date at least four susceptibility genes have been characterized including HLA, CTLA-4, CD40 and thyroglobulin as well as a number of disease linked loci with genes still to be identified. Environmental interactions with these genes are very likely with the role of pregnancy on the immune system, and the generation of maternal microchimerism, as well as the role of iodine influencing the antigenicity of thyroglobulin, being primary examples. Polymorphisms in these known susceptibility genes, along with a life and environmental history, will soon be used to develop a clinical risk profile which will allow precise disease prediction.

6

THE REGULATION OF PHOSPHOINOSITIDE SIGNALLING BY LIPID PHOSPHATASES

C.A. Mitchell, L. Ooms, J. Dyson, A. Kong, R. Gurung, H. Nandurkar

Department of Biochemistry and Molecular Biology, Monash University, Clayton, Vic 3800, Australia.

Signalling pathways generated by phosphoinositides regulate insulin action, cell proliferation, differentiation and survival, vesicular trafficking and cytoskeletal rearrangement. Phosphoinositides recruit proteins containing discrete phosphoinositide-binding domains, thereby localizing signalling proteins to specific membrane compartments and activating down-stream signalling cascades. Phosphoinositides are respectively synthesised and in turn metabolised by specific lipid kinases and phosphatases, which phosphorylate and dephosphorylate the inositol ring. Eight phosphoinositide signalling molecules including PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 have been identified in mammalian cells. The inositol polyphosphate 5-phosphatases (5-phosphatases) hydrolyse the 5-position phosphate from both inositol phosphates and phosphoinositides and share the same catalytic mechanism as the apurinic/apyrimidinic (AP) endonucleases. Ten mammalian enzymes have been cloned and characterized and four homologues have been identified in Saccharomyces cerevisiae. Gene-targeted deletion of these enzymes in yeast and mice have demonstrated the 5-phosphatases regulate many essential processes including neuronal signalling, endocytosis, insulin signalling, and hematopoietic cell proliferation. Mice lacking SHIP-2 demonstrate insulin hypersensitivity, resulting from enhanced transport of the glucose transporter (GLUT-4) to the plasma membrane. We have investigated the

intracellular localization, substrate specificity and cellular function of both yeast and mammalian 5-phosphatases to determine the sites at which these enzymes hydrolyse specific phosphoinositide signalling molecules and thereby regulate secretion, cytoskeletal rearrangement and GLUT-4 translocation. We have shown SHIP-2 localizes and forms a complex with the actin–binding protein filamin and may thereby regulate submembraneous actin. This may facilitate GLUT-4 translocation. We have also localized and characterised other recently identified 5-phosphatases including SKIP and the 72 kDa 5-phosphatase, which also regulate insulin signalling and GLUT-4 translocation to the plasma membrane.

7

INSULIN SIGNALING IN TIME AND SPACE

Alan R. Saltiel
University of Michigan

Despite remarkable progress in dissecting the signaling pathways critical for the metabolic effects of insulin, the molecular basis for the specificity of the cellular actions of the hormone is not fully understood. One clue to this dilemma might lie in the spatial and temporal aspects of signaling. Recent evidence suggests that signaling molecules and pathways are localized to discrete compartments in cells, due to specific protein interactions. One example is the lipid raft, a presumed site for the segregation of insulin receptor and a subset of its substrates. Recent evidence suggests that part of the insulin signaling pathway is segregated into these microdomains of the plasma membrane. Additionally, the reception of signals from the insulin receptor also occurs in compartments, due to the interaction of some of the targets of insulin action with scaffolding proteins. The duration of signals is also likely to be important in defining specificity. The rapid termination of tyrosine or lipid phosphorylation by phosphatases or serine kinases may tightly control the strength of a signaling pathway, thus determining its relative impact on growth, differentiation and metabolism. Thus, mechanisms exist to restrict insulin action in time and space, explaining the unique and specific actions of the hormone.

8

PPARS AND THE COMPLEX JOURNEY TO OBESITY

Ronald M. Evans, Chih-hao Lee, Yongxu Wang, Ruth Yu, Andrea Hevener*, Weimin He*, Peter Olson and Jerry Olefsky*
Gene Expression Laboratory, The Salk Institute /Howard Hughes Medical Institute, La Jolla, CA 92037

****Dept. of Medicine, Univ. of Calif. San Diego, La Jolla, CA, Gene Expression Laboratory, The Salk Institute /Howard Hughes Medical Institute, La Jolla, CA 92037***

Obesity is a primary risk factor for insulin resistance, hypolipidemia and atherosclerosis, collectively known as Syndrome X. Thiazolidinediones (TZDs) are insulin-sensitizing drugs which function as highly selective and potent agonists of PPARg, while the fibrates treat hyperlipidemia by promoting PPARa activation in the liver.

In contrast to the well-established roles of PPARg and a in lipid metabolism, little is known about PPARd in these processes. Macrophages derived from PPARd deficient bone marrow show highly compromised capacity to convert to atherogenic foam cells. This appears to reflect its ability to control the inflammatory status of the macrophage. Gain of function studies show that PPARd synthetic agonists are able to inhibit inflammation b regulating the availability of BCL-6, a mediator of Nf-kB signalling. In contrast to the macrophage, targeted activation of PPARd in adipose tissue induces fatty acid oxidation and energy dissipation. In mice, this leads to improved lipid profiles and a markedly lean phenotype. Importantly, these animals are completely resistant to both high fat diet-induced and genetically predisposed db/db obesity. Mice exposed to a PPARd drug show increased generation of type I muscle fibers associated with mitochondrial proliferation and increased respiration. Together, these studies suggest that PPARd activation may both suppress inflammation and at the same time, promote a lean phenotype by metabolic activation. This pathway offers a new and specific metabolic approach to the treatment of obesity related disease such as Syndrome X.

SYMPOSIUM ABSTRACTS

30

THE CURRENT ROLE OF HORMONE THERAPY IN THE PREVENTION AND TREATMENT OF OSTEOPOROSIS

Helena Teede

Monash University Dept Medicine, Dandenong Hospital, Dandenong, Victoria.

Hormone therapy, formally referred to as HRT, has been used to alleviate menopausal symptoms for four decades. Over this time, it was recognized that the estrogen receptor was ubiquitous and that estrogen had wide spread effects, not only on the reproductive system, but on bone, the cardiovascular system, the coagulation system, the brain and the colon along with many others.

Observational data strongly suggested that HT use reduced fractures. Extensive interventional data also demonstrated improvement in bone density with estrogen. This data, combined with the cost of other agents used to prevent fractures, lead to a significant increase in the prescription of HT for fracture prevention in the 1990s. The efficacy of HT was finally confirmed with a reduction in fractures noted in the Women's Health Initiative. This randomized placebo controlled trial of oral combined continuous HT, was completed in16, 808 women over 5.2 years. In the current session, the efficacy of HT in the treatment of osteoporosis will be discussed, along with that of Bisphosphonates and Selective Estrogen Receptor Modulators.

As with all mediations used for prevention, efficacy in fracture prevention needs to be balanced against the risks. This is especially the case for HT. To this end, the findings for all major end-points from the WHI study will be reviewed, including a perspective on the small absolute risk of adverse effects, but also the small absolute benefits achieved in the primary prevention setting. The implications for secondary prevention of osteoporosis will also be discussed, focusing on the critical issue of risk versus benefit for all agents available for fracture prevention.

31

THE ROLE OF SELECTIVE OESTROGEN RECEPTOR MODULATORS (SERMS) IN DISEASE PREVENTION

Susan R. Davis

The NHMRC Centre for Clinical Research Excellence at the Jean Hailes Foundation, Clayton.

SERMs are compounds that mimic oestrogen in some tissues and act as anti-oestrogens in others. Like oestradiol, SERMs bind to the ligand binding domain (LBD) of the receptors ERα and β. Although agonists and antagonists bind at the same site within the core of the LBD, each induces specific conformations in the transactivation domain of the receptor, known as AF-2 and this influences whether specific co-activator or co-repressor proteins are recruited to the complex. Recent research has provided insight into when SERMs act as agonists or antagonists. In breast tissue, where tamoxifen and raloxifene act as oestrogen antagonists, the SERM–oestrogen receptor complexes bind directly to oestrogen response elements and recruit co-repressor proteins. In contrast, in the endometrium, SERMs appear to influence genes through an indirect mechanism and show differential actions: tamoxifen recruits co-activators (and thus mimics

oestrogen), whereas raloxifene does not and consequently has no effect at this site. Tamoxifen, the first SERM, is extensively used as adjuvant treatment for ER(+) breast cancer, but in primary prevention is associated with significantly increased risk of venous thromboembolic events (VTE) and endometrial cancer. Raloxifene exhibits oestrogen agonist activity on bone and lipids, and antagonist activity in breast and the endometrium. Earlier studies confirmed the positive effects of raloxifene versus placebo on increasing bone mineral density. The 'Multiple Outcomes of Raloxifene Evaluation' study has translated this benefit on BMD into data on fractures in post-menopausal, osteoporotic women. This trial demonstrated a significantly reduced risk of vertebral fractures in women receiving two doses of raloxifene vs placebo. Risk of non-vertebral fractures did not differ significantly, and those women receiving raloxifene had an increased relative risk of VTE of 3.1 (95% CI 1.5-6.2). In the analysis of 3 and 4-year data, women receiving the two doses of raloxifene had a 72 percent reduction in the risk of invasive breast cancer, as compared to placebo equating to 1.3 vs 4.7 breast cancers per 1000 women-years. Among women at high risk of coronary heart disease, those taking raloxifene had statistically significant reductions in the risk of any cardiovascular event (28 events in 359 women treated with raloxifene [60 mg] versus 41 events in 317 women treated with placebo) and of stroke (six events in 359 women treated with raloxifene [60 mg] versus 14 events in 317 women treated with placebo). With regard to cognition, there was no difference between cognitive scores in women treated with raloxifene versus placebo after 3 years. These findings must be confirmed by an adequately powered, randomised trial, with breast cancer and cardiovascular events as predefined outcomes. Unfortunately both tamoxifen and raloxifene have the tendency to cause rather than alleviate hot flushes and vaginal dryness, which limits their acceptability in post-menopausal women. Several new SERMs are currently undergoing clinical trials. A future alternative also being studied is the use of both an oestrogen and a SERM concurrently.

32

THE CYP19 TTTA REPEAT POLYMORPHISM IS ASSOCIATED WITH REDUCED BONE MINERAL DENSITY AND FRACTURE

R.L. Prince, A. Devine, I.M. Dick, S.S. Dhaliwal

School of Medicine and Pharmacology, University of WA and Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, Western Australia.

The human Aromatase (CYP19) gene, localized on 15q21.2, catalyzes the conversion of testosterone to estradiol, and androstenedione to estrone. A variable number of tandem repeat (VNTR) polymorphism of the CYP19 gene has been associated with reduced bone mineral density (BMD) and increased fracture in early postmenopausal women but the importance of this has not been determined in older women.

We examined the CYP19 TTTA VNTR in a population based study of 1257 Caucasian women over age 70. DXA bone mineral density (BMD) data and calcaneal quantitative ultrasound (QUS) data was obtained. The number of atraumatic prevalent fractures at entry into the study and the number of atraumatic incident fractures during the following three years was assessed. Genotyping of the CYP19 TTTA VNTR was done by PCR amplification.

Genotyping resulted in 10 different PCR fragments ranging in size from 142 to 182 bp, designated A1 to A11. The data was stratified based on the presence or absence of A4 (158 bp, number of TA repeats=9). A4 was present in 27% of subjects and was associated with higher BMD at all sites of the hip (3.8% total hip, 2.6% femoral neck, 3.8% intertrochanter, 4.5%

trochanter) and the lumbar spine (14.7%) compared to the absence of A4. A4 was also associated with higher values for the calcaneal quantitative ultrasound (QUS) parameters BUA (1.8%), SOS (0.5%) and stiffness (4.5%) compared to the absence of A4. A4 was associated with a decreased risk of osteoporosis as defined by WHO criteria (OR 0.56, 95% CI 0.34-0.93). A4 was associated with a decrease in the deoxypryridinoline creatinine ratio (DpdCr) (30.3 ± 10.6 vs 26.9 ± 8.9, $p=0.02$) and an increase in the Free Estradiol Index (FEI) (0.64 ± 0.57 vs 0.71 ± 0.59, $p=0.049$). Despite the association of A4 with increased BMD and QUS, there was no decrease in prevalent fractures or incident fractures recorded over 3 years observed. An interaction between BMD and the presence or absence of A4 was observed, indicating that subjects with low BMD (osteopenia or osteoporosis) who had a A4 where protected against incident fracture compared to subjects with a low BMD who did not have an A4 allele (R.R. 3.2, 95% CI 1.0-10.0).

33

NEW CONSENSUS GUIDELINES FOR MANAGEMENT OF ASYMPTOMATIC PRIMARY HYPERPARATHYROIDISM (PHPT)

Peter R. Ebeling

Departments of Diabetes and Endocrinology, and Medicine, The Royal Melbourne Hospital, Parkville 3050, Victoria.

In vitro, continuous exposure to PTH results in preferential stimulation of osteoclasts, whereas discontinuous exposure results in osteoblast proliferation. Despite intermittent recombinant human PTH (1-34) (Teriparatide) being the most potent anabolic agent yet used to treat osteoporosis, sustained elevations of PTH in PHPT result in accelerated bone loss and increases in vertebral, forearm and hip fractures. Effects of PHPT differ according to proportions of cortical and trabecular bone. Trabecular sites are somewhat protected. There is an increase in the activation frequency of bone remodelling units, while erosion depth is reduced so the risk for trabecular perforations is reduced. Bone width and bone area are also increased. At cortices, increased endosteal resorption results in cortical thinning and cortical porosity is increased. By contrast, intermittent PTH therapy increases bone mineral content, BMD, cortical thickness and cortical porosity.

Initial evaluation should include serum calcium corrected for albumin, dual energy x-ray absorptiometry (DXA) of the spine, proximal femur and distal one-third radius, 24 h urine collection for calcium excretion and creatinine clearance, and renal imaging with ultrasound and/or abdominal x-rays. The majority of patients with PHPT are asymptomatic, so decreased bone density is an important indication for surgery. One of the two main changes in the new 2002 NIH-sponsored guidelines for surgery in asymptomatic PHPT reflects the significant association of PHPT with osteoporosis. This is a liberalisation in BMD thresholds to a T-score of < -2.5 at any site (spine, proximal femur or distal one-third radius). Two caveats are that areal BMD, measured by DXA, underestimates the geometric changes present in bone in PHPT and second that the relationship between T-score and fracture risk in PHPT has not yet been prospectively studied. The other change is a decrease in the threshold for serum corrected calcium to 0.25 mmol/L above the upper limit of normal range. The other indications for surgery based on age $<$ 50 years, urine calcium $>$ 100 mmol/day and Cr clearance $<$ 70% of normal, all remain unchanged.

It is therefore likely that the presence of osteoporosis on bone densitometry will increase surgical treatment of asymptomatic PHPT. This recommendation for surgery based on T-score could be modified when specific medical therapies are shown to increase BMD in PHPT.

34

TYPE 2 DIABETES TRANSGENIC MOUSE MODEL

Derek Le Roith
Diabetes Branch, NIH, Bethesda, MD, USA.

A model of type 2 diabetes was created by the transgenic approach. The dominant negative IGF-I receptor was overexpressed in skeletal muscle of mice under the muscle-specific creatine kinase promoter (MKR mice). The abnormal receptors formed hybrids with both the endogenocus IGF-I receptors as well as the insulin receptors in skeletal muscle only. At 3 weeks of age the mice demonstrated severe insulin resistance with elevated serum insulin levels and normal glucose levels. At six weeks they were diabetic with glucose levels 3-times normal. Using the hyperinsulinemic-euglycemic clamp technique it was shown that these mice have insulin resistance in liver, fat and muscle and at the time of the development of diabetes, first phase insulin secretion is defective, while second phase is prolonged. Thus they represent a model of insulin resistance progressing to full-blown type 2 diabetes.

To investigate the cause for the progression of the disorder we fed the animals a fibrate (WY, 14643) which within a week resulted in reversal of the diabetes with a marked reduction in insulin resistance and a more normal pancreatic insulin secretory response to glucose. Interestingly, both in liver and muscle, the triglyceride levels fell supporting the concept that intramyocellar fat is adding to the insulin resistance in those organs.

To further establish the role of tissue triglycerides in this model we crossed these mice with the skeletal muscle CD36 transgenic and this also resulted in a reversal of the diabetic phenotype. Muscle CD36 not only increased TG uptake but also increased fatty acid oxidation resulting in reduced tissue TG levels.

Thus a significant effect on insulin resistance and the progression of the disorder in this model is seen with high tissue TG levels and reversal of this "lipotoxicity" had dramatic effects.
We are currently studying the potential role of "glucotoxicity", metformin, PPARg agonists and exercise to further undertsand the pathophysiology.

35

SPATIAL COMPARTMENTALIZATION IN INSULIN ACTION

Alan R. Saltiel
University of Michigan

Although the effects of insulin on glucose and lipid metabolism are well documented, gaps remain in our understanding of the precise molecular mechanisms of signal transduction. Recent evidence suggests that compartmentalization of signaling molecules and metabolic enzymes may explain the unique cellular effects of the hormone. Signal initiation from the insulin receptor is restricted in part to caveolae microdomains of the plasma membrane. A fraction of the insulin

receptor directly interacts with caveolin, thus directing the protein to caveolae. Following its activation by insulin, the receptor recruits a series of adapter proteins, resulting in the activation of the G protein TC10, which also resides in caveolae. TC10 can influence a number of cellular processes, including changes in the actin cytoskeleton, recruitment of effector including the adapter protein CIP4, and assembly of the exocyst complex. These events play crucial roles in the trafficking, docking and fusion of vesicles containing the insulin-responsive glucose transporter Glut4 at the plasma membrane.

36.1

THE 72 KDA 5-PHOSPHATASE PROMOTES GLUT4 PLASMA MEMBRANE TRANSLOCATION

Anne Kong, Kristy Horan, Luke Collyer, Christina A. Mitchell

Department of Biochemistry, Monash University, Clayton Vic 3800.

The inositol polyphosphate 5-phosphatases remove the 5-position phosphate from phosphoinositide signalling molecules. The 72 kDa 5-phosphatase contains an N-terminal proline rich domain, a central 5-phosphatase domain and a C-terminal CAAX motif. The 72 kDa 5-phosphatase (also called the Type IV 5-phosphatase) hydrolyses phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P_3) and phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) forming phosphatidylinositol 3,4 bisphosphate (PtdIns (3,4)P_2) and PtdIns 4-P respectively. The enzyme is highly expressed in adipocytes, brain and testis and localizes to the cytoplasmic Golgi membrane surface and thereby may regulate Golgi-vesicular trafficking. Over expression of wild type 72 kDa 5-phosphatase, or a catalytically inactive mutant did not affect constitutive secretion from the Golgi as assessed by the trafficking of VSVG to the plasma membrane. However, overexpression of the wild type, but not the catalytically inactive mutant 5-phosphatase in 3T3L1 adipocytes promoted translocation of the glucose transporter GLUT-4 from the peri-Golgi region via recycling endosomes to the plasma membrane, in the absence of insulin stimulation. The enzyme had no effect on insulin-stimulated GLUT-4 translocation. To investigate the signalling pathways mediating 72 kDa 5-phosphatase induced translocation of GLUT-4 to the plasma membrane, in the absence of insulin, we investigated the Golgi-endosomal trafficking pathway. Inhibition of endosome recycling to the plasma membrane via co-expression of a constitutively inactive Rab11(S25N) mutant, inhibited 72 kDa mediated translocation of GLUT-4, as did blockage of endosomal recycling by incubation of adipocytes at 16^{o}C. We determined which phosphoinositide signalling molecules hydrolysed by the 72 kDa 5-phosphatase regulated GLUT-4 translocation. Inhibition of PI 3-kinase synthesis of PtdIns(3,4,5)P_3 by wortmannin treatment, did not block 72 kDa 5-phosphatase induced GLUT-4 translocation however; sequestering PtdIns(4,5)P_2 using neomycin inhibited 72 kDa enzyme induced insulin-independent GLUT-4 translocation. Collectively these studies demonstrate the 72 kDa 5-phosphatase regulates the endosomal recycling of GLUT-4 via regulation of PtdIns(4,5)P_2.

36.2

IMPROVED GLUCOSE HOMEOSTASIS AND ENHANCED INSULIN SIGNALLING IN GRB14-DEFICIENT MICE

Roger J. Daly

Garvan Institute of Medical Research, Sydney, NSW 2010, Australia

Growth factor receptor bound (Grb)14 is a member of the Grb7 family of src homology 2 domain-containing proteins, and associates with the insulin receptor (IR). To characterize the physiological role of Grb14, we abolished its expression by gene targeting. Adult male $Grb14^{-/-}$ mice displayed improved glucose tolerance despite lower circulating insulin levels due to tissue-specific effects on insulin signalling. In liver and skeletal muscle from insulin-stimulated animals, Grb14 ablation was associated with increased tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and recruitment of the p85 subunit of phosphatidylinositol 3' kinase to this docking protein. Activation of protein kinase B was also significantly enhanced in these tissues. However, insulin-induced signalling was not enhanced in white adipose tissue. Consistent with these observations, insulin-induced glucose transport was enhanced in soleus muscle isolated from Grb14-deficient mice, but not in epididymal adipose tissue. These findings demonstrate that Grb14 functions *in vivo* as a tissue-specific inhibitor of insulin action, most likely via repression of IR-mediated IRS-1 tyrosine phosphorylation, and highlight this protein as a potential target for therapeutic intervention.

37

HEALTHY AGEING FOR MEN, THE MALE MENOPAUSE AND THE EUROPEAN MALE AGEING STUDY

F.C.W. Wu

Andrology Research Unit, Department of Endocrinology, University of Manchester, U.K.

Ageing is the consequence of inefficiencies in the body's maintenance and repair processes resulting in the stochastic accumulation of detrimental free radicals and toxins. The life - long build up of cellular and tissue damage give rise to loss of physiological functions, mutations in nuclear and mitochondrial DNA and increased susceptibility to chronic degenerative diseases – the overall condition of senescence. However, this cascade of changes is neither invariable nor inevitable but highly amenable to individual and population modifications. Thus preventing or deferring the disabilities associated with ageing and compression of morbidities to extend healthy lifespan of the expanding elderly population has become an increasingly pressing healthcare priority in the 21st century.

In men, one aspect amongst many of these ageing-related changes is the gradual and variable decline of circulating testosterone into the low normal (rather than the truly hypogonadal) range. Causal relationships between the various well recognised ageing-associated functional decline and decreased testosterone, and thus a rational basis for a 'clinical syndrome of androgen insufficiency ' in elderly men, has not been established. Further observational studies in the general population are required to investigate the predisposing variables (genetic and acquired) underlying hormonal dysregulation, their relationships to health status, to define thresholds at which and identify individuals in whom various interventions including hormone replacement

may prevent disabilities and improve quality of life. These objectives are currently being addressed in the multi-national, multi-disciplinary prospective cohort European Male Ageing Study (EMAS) involving 8 countries and 3,400 men aged 40 – 79 yr.

Short-term interventional trials (in highly selected clinic populations) with testosterone have shown that hormone supplementation can increase lean body mass and decrease fat mass. But, as yet, significant improvements in function (including fracture incidence), muscle strength and performance, activities of daily living and quality of life have not been demonstrated. So far, the limited studies are not powered to assess longer-term risks of testosterone treatment. They include exacerbation of occult prostate cancer, polycythaemia, sleep apnoea, fluid retention. Accurate risk-benefit assessment of hormone replacement in ageing men is currently not possible. Large-scale prospective randomised placebo-controlled trials, along the lines of those conducted for female hormone replacement, are required.

Currently, there is insufficient evidence to support the routine practice of hormone replacement for elderly men without a clear diagnosis of hypogonadism. Emphasis should also be placed on active prevention from earlier years to achieve healthy ageing.

38

AMELIORATING SARCOPENIA: STRATEGIES FOR IMPROVING MUSCLE FUNCTION IN THE FRAIL ELDERLY

Gordon S. Lynch

Muscle Mechanics Laboratory, Department of Physiology, The University of Melbourne, Victoria, 3010, Australia.

Some of the most serious consequences of aging are its effects on skeletal muscle structure and function. Aging is associated with a progressive loss of muscle mass (sarcopenia), a slowing of movement, and a decline in strength. These changes contribute to a gradual loss of functional independence and an increased incidence of fall-related injuries. Progressive muscle fibre denervation and subsequent motor unit remodelling have been implicated in the loss of force and power output with aging.(1) However, the age-related slowing of contraction has been reported to occur before the onset of severe muscle wasting, indicating that changes intrinsic to skeletal muscle fibres cannot be excluded. A decrease in circulating anabolic hormones and growth factors also contributes to the loss of muscle size and strength.(2) Some of these growth factors are responsible for the repair of muscles injured during everyday "wear and tear". From a public health perspective, sarcopenia has widespread clinical implications. Diminished strength increases the dependence of the frail elderly on assistance for performing even basic tasks of everyday living. It is generally agreed that age-related changes in skeletal muscle are inevitable, although debate exists as to whether these changes can be delayed or reversed. A number of strategies are being investigated that can improve muscle repair following injury and restore muscle size and strength, factors that can potentially offset the effects of aging on skeletal muscle, and improve quality of life for the frail elderly. Exercise, particularly high-intensity resistance training is effective as an intervention for improving muscle strength, but not everyone (e.g. frail elders) can perform these exercises. Some beta-adrenoceptor agonists have powerful muscle anabolic effects and we are investigating their use for ameliorating sarcopenia.(3) Combining exercise with safe, low-dose treatments of muscle anabolic agents may be effective in

combating sarcopenia, but much research is needed before such strategies can be recommended for implementation.

(1) Plant DR, Lynch GS. Journal of Physiology 2002;543:169-176.
(2) Lynch GS. Expert Opinion on Therapeutic Patents 2002;12:11-27.
(3) Ryall JG, Gregorevic P, Plant DR, Sillence MN, Lynch GS, American Journal of Physiology 2002; 283:R1386-R1396.

39

CURRENT CONCEPTS OF HORMONAL MANIPULATION IN PROSTATE CANCER

Robin M.L. Murray

Peter MacCallum Cancer Centre, East Melbourne

Hormonal manipulation has been the mainstay of treatment for advanced prostate cancer for 60 years, but attempts to improve the remission rate of around 80% have been largely unsuccessful. Thus an meta-analysis of trials comparing single therapy (medical or surgical orchidectomy) with combined therapy (maximum androgen blockade – orchidectomy plus an antiandrogen) showed differences in survival of only 2.7% at 5 and 2% at 10 years. (Prostate Cancer Trialists' Collaborative Group. Lancet 2000; 355: 1491-1498) The timing of initiation of treatment of advanced disease is still the subject of controversy, however re analysis of the early Veterans studies and the MRC trial of immediate versus delayed intervention have made persuasive arguments for early intervention (MRC Research Council Prostate Cancer Working Party Investigators Group 1997). There is now evidence that in some instances of so called hormone refractory prostate cancer (cancer that has progressed on first line hormone treatment) the tumour cells are exquisitely sensitive to stimulation by testosterone – This forms the basis for some second line therapies – adrenal blockade with aminoglutethimide or ketoconazole.

Results of studies using neoadjuvant hormonal therapy prior to radiotherapy or radical prostatectomy have shown that, in disease with a high risk of recurring, the use of an LHRH analogue for 3 to 6 months prior to radiotherapy is associated with a prolonged disease free survival rate and prolonged overall survival. In the case of radical prostatectomy the use of neoadjuvant therapy is associated with an increase in the percentage of patients whose prostate is free of tumour at the surgical margins, but this has not translated into a survival benefit.

Adjuvant therapy with either an LHRH analogue or an anti androgen following radical prostatectomy or radiotherapy has also been shown to be associated with improved disease free survival and cause specific survival in some studies.

Zincke et al. J Urol. 166(6): 2208-2215, 2001.
See et al. J. Urol. 168(2): 429-435, 2002

40

GROWTH HORMONE, AGEING AND EXERCISE

Ross C. Cuneo

Metabolic Research Unit, University of Queensland and Department of Diabetes and Endocrinology, Princess Alexandra Hospital, Brisbane.

Apparently normal ageing results in changes in body composition, physical function and metabolism that adversely affects quality and duration of life. Losses of skeletal muscle strength and endurance and neuromuscular co-ordination lead to falls and, combined with progressive osteopaenia, bony fractures. Increasing fat mass contributes to the insulin resistant syndrome, with hypercholesterolaemia, hypertension, diabetes mellitus and atherosclerotic disease.

Rudman first proposed that falling Growth Hormone (GH) secretion caused many of these changes. Cross-sectional studies in ageing adults show substantial reductions in GH secretion and serum IGF-I concentrations. There are many similarities between the syndrome of GH deficiency in adults, described in patients with pituitary disease, and "normal" ageing. Recombinant human GH treatment in GH deficient adults reverses abnormalities in body composition, muscle function, bone density, and some (but not all) of the metabolic derangements. Similarly, deficiencies in sex-steroids in both males and females may contribute to age-related dysfunctions.

Recent clinical trials of GH and sex-steroid treatment in healthy ageing individuals have shown some improvement in body composition and exercise capacity. Unfortunately the magnitude of the changes and adverse effects of treatment have not led to universal acceptance of this strategy to improve quality of life during the advanced years. Comparison is made with benefits that can be achieved with physical training, which result in more impressive changes in strength and endurance exercise capacity. This area of research retains potential for future developments that may benefit our expanding ageing population.

41

COHESINS: LINKING CANCER AND MEIOSIS

Huiling Xu, Matthew Beasley, Sandra Verschoor, Jonathan Tomaszewski, Michael J. McKay

Research and Radiation Oncology Divisions, Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, Vic.

Improper transmission of genetic information from a cell to its daughters results in aneuploidy, a hall marker of cancer. Genes involved in the maintenance of genome stability are thus of great interest, and are potential targets for future therapies. The recently discovered multi-protein complex, called 'cohesin' serves as a chromosomal glue which plays important role in chromosome segregation and DNA repair, guarding the cell against malignant transformation (2). In mitotic cells, cohesin complex comprises at least four proteins: RAD21 (also known as SCC1), SCC3, SMC1 and SMC3. All these cohesin subunits except RAD21 are required for meiosis in yeast (1). The RAD21 subunit is replaced by its meiosis-specific paralogue, REC8.

Abundant evidence suggests that cohesin defects may have detrimental consequences for genome stability, such as aneuploidy as seen in the cells of malignant tumors and chromosomal birth disorders (eg., Down's syndrome). To investigate the role of cohesins in chromosome stability and segregation in mammals, our laboratory cloned human and mouse homologs of yeast *Rad21* and *Rec8* genes. We recently produced the first cohesin mutant mice by targeted deletion of the *Rec8* gene. Abrogation of *Rec8* gene function results in sterility in both males and females, confirming that the essential role of *Rec8* in meiosis is conserved in mammals. Analysis of chromosome spreads of spermatocytes revealed that *Rec8* mutant cells display aberrant meiotic chromosomal structures. These studies may provide insight into chromosome segregation defects contributing to human disorders such as infertility and pathogenesis of cancer.

(1) Nasmyth, K. Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. *Annu Rev Genet* **35**, 673-745 (2001).
(2) Michaelis, C., Ciosk, R. & Nasmyth, K. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**, 35-45 (1997).

42

THE PSA-RELATED KALLIKREIN ENZYMES AND HORMONE-DEPENDENT CANCERS

Judith Clements, Tara Veveris-Lowe, Loan Bui, Rachael Collard, Mitchell Lawrence, Dimitri Odorico Frank Gardiner, David Nicol, Adrian Herington, Ying Dong

Hormone Dependent Cancer Program, School of Life Sciences & Cluster for Molecular Biotechnology, Science Research Centre, Queensland University of Technology and University of Queensland Department of Surgery, Royal Brisbane and Princess Alexandra Hospitals, Brisbane, QLD.

The human tissue kallikreins are a multigene family of 15 serine proteases, which are expressed in hormone-dependent cancers. Prostate-specific antigen or PSA, a member of this family, is the current serum biomarker for detection and monitoring of prostate cancer. Other kallikreins are being investigated as potential diagnostic/prognostic markers, particularly for prostate (KLK2) and ovarian cancer (KLKs 4-11 & 14, 15), the latter specifically as biomarkers for serous epithelial ovarian carcinomas. Interestingly, some ovarian KLKs (8, 9, 14 & 15) appeared to be useful indicators of good prognosis whereas others (KLKs 4, 5, 6, 7, 10 & 11) indicated a poor outcome. One novel aspect of the human KLKs is the number of differentially-spliced transcripts that could encode truncated proteins devoid of protease activity. We have shown that some variant transcripts (for PSA, KLK2, KLK4, KLK5 & KLK7) are more highly expressed than full length transcripts in malignant compared to benign tissues suggesting they may have potential as more cancer-specific biomarkers. Intriguingly, some KLK4 variants are localized to the nucleus which is not a typical intracellular site for a protease that is normally secreted, emphasizing possible non-proteolytic functions of these variants. From biochemical studies, it appears that kallikreins are involved in a range of functional activities via the degradation of polypeptides (extracellular matrix proteins, IGFBPs) or polypeptide activation via hydrolysis of the pro-peptide (uPA, TGFβ). Although not yet proven, these observations suggest that kallikreins play a role in events associated with tumour progression. We have recently developed prostate cancer PC3 cell lines stably-transfected with prepro-PSA, -KLK2 and -KLK4. We have

shown that PSA and KLK4 over-expression, but not KLK2, elicits a morphological change, increased migration to various chemo-attractants and increased attachment to extracellular matrix components (KLK4 only). The mechanisms underlying these changes are currently under investigation.

43

STEROID REGULATION OF BREAST CANCER CELL PROLIFERATION

Robert L. Sutherland, Jason S. Carroll, C. Marcello Sergio, Alexander Swarbrick, Elizabeth A. Musgrove

Cancer Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Darlinghurst, NSW 2010.

Estrogens are potent mitogens in a number of target tissues including the mammary gland where they play a pivotal role in the development and progression of mammary carcinoma. The demonstration that estrogen-induced mitogenesis is associated with an increased rate of progression through G_1 phase of the cell cycle has focussed attention on the estrogen regulation of molecules in the cyclin/CDK/pRb pathway that controls G_1 to S phase progression. Steroid-responsive breast cancer cells pretreated with a pure estrogen antagonist arrest in quiescence i.e. G_0 and respond to estrogen treatment with synchronous progression into S phase. Entry into S phase is preceded by increased expression of c-Myc and cyclin D1, activation of cyclin D1-Cdk4 and cyclin E-Cdk2 complexes and phosphorylation of the retinoblastoma gene product, pRb. Activation of cyclin D-Cdk4 is due predominantly to estrogen-induced transcriptional activation of cyclin D1. In contrast, cyclin E-Cdk2 activation does not involve major changes in cyclin E expression but rather redistribution of the p21 CDK inhibitor away from cyclin E-Cdk2 complexes. This is mediated by two distinct mechanisms: sequestration into newly-formed cyclin D1-Cdk4-p21 complexes and transcriptional inhibition of p21 gene expression.

In the same model progestins are growth inhibitory and arrest cells in G_1 phase. Growth arrest is accompanied by decreased expression of both cyclin D1 and cyclin E and induction of the CDK inhibitor $p18^{INK4C}$. These changes lead to reassortment of cyclin-CDK-CDK inhibitor complexes and increasing availability of p27 to form inhibitory cyclin E-Cdk2-p27 complexes. Thus, both cyclin D-Cdk4 and cyclin E-Cdk2 activities are inhibited, resulting in decreased pRb phosphorylation and arrest in G_1 phase. These data indicate that steroid hormones stimulate or inhibit cell cycle progression through effects on multiple targets in the pRb pathway. The aberrant expression of several of these targets in breast cancer i.e. overexpression of c-Myc, cyclin D1 and cyclin E and loss of expression of p27, potentially contributes to the loss of steroid sensitivity and endocrine resistance associated with the progression of breast cancer.

44

HORMONAL INFLUENCES IN HEREDITARY BREAST CANCER

Kelly-Anne Phillips

Peter MacCallum Cancer Centre East Melbourne, 3002, Victoria.

Approximately 5 to 10% of all breast cancer (BC) cases are hereditary. Germline mutations in the tumour suppressor genes, BRCA1 and BRCA2 account for up to 40% of hereditary cases. Carrying a mutation in either of these genes confers a high lifetime risk for female BC. Hormonal factors may modify BC risk in BRCA1 and BRCA2 carriers. Recent studies of BRCA1 and BRCA2 carriers show that (premenopausal) prophylactic oophorectomy decreases BC risk by about 50%. Conversely, use of the oral contraceptive pill (particularly for greater than 5 years or if started before age 30) may be associated with a significantly increased risk for BC in BRCA1 carriers. Also, BRCA1 or BRCA2 mutation carriers who have had a full term pregnancy may be more likely to develop BC by age 40 than nulliparous carriers. Unfortunately, data on the role of the anti-oestrogen Tamoxifen for BC prevention in mutation carriers is limited and conflicting. Importantly, the results of most studies to date of BC risk modifiers (including hormonal factors) in BRCA1 and BRCA2 carriers must be interpreted with caution. Most have been retrospective prevalent case-control studies using living cases of BC drawn from families with multiple cases of the disease. Data collection has thus usually been restricted to survivors and their living relatives, and data on exposures to potential risk modifiers have been based on recall of life events many decades earlier. These have been opportunistic, rather than designed, research studies and thus there is a high likelihood of systematic biases. Randomised controlled trials of most potential modifiers (eg prophylactic surgery, use of the oral contraceptive pill, parity) are not feasible. Thus it is recognized that long-term prospective, systematic follow-up of large BC family cohorts, will provide better information. Such a study is currently underway in Australasia as part of the Kathleen Cuningham Foundation for Research into Familial Breast Cancer (kConFab). Such studies are essential in order to optimise the clinical risk management strategies of individuals attending Family Cancer Centres, and hence reduce the morbidity and mortality of hereditary BC.

45

CENTRAL PLAYERS IN THE DEVELOPMENT OF OBESITY

Denis G. Baskin

University of Washington and VA Puget Sound Health Care System, Seattle, WA, USA.

A fundamental paradigm in the field of obesity research is the hypothesis that CNS control of energy homeostasis is regulated by adiposity signals - leptin and insulin - that circulate in proportion to fat mass, and that genetic and ontogenetic conditions causing deficits of leptin or insulin action in the CNS result in obesity. While a large variety of neurons produce peptides and neurotransmitters that are capable of altering food intake and energy metabolism, hypothalamic neurons that express POMC and NPY/AGRP peptides are primary players for transducing adiposity signaling into altered energy homeostasis. Leptin and insulin stimulate POMC neurons that secrete aMSH (anorexic peptide) and inhibit expression of NPY/AGRP (orexigenic peptides). The ability of insulin and leptin to cause changes in food intake is mediated in part by

signal transduction pathways that involve IRS2 and phosphatidylinositol-3-kinase in arcuate nucleus neurons. Leptin-sensitive POMC neurons in the arcuate nucleus are hypothesized to project to the paraventricular nucleus (PVN), where aMSH is released to act on neurons that project to brainstem regions that regulate meal size. A subset of PVN neurons that express oxytocin has direct connection to the nucleus tractus solitarius (NTS) of the brainstem, which receives vagal input from neural and hormonal satiety signals (e.g., gastric distension, CCK), resulting in meal termination. Recent findings show that leptin stimulates Fos expression in PVN oxytocin neurons that project to NTS subdivisions activated by CCK, potentiates the satiety effect of CCK, and increases CCK-induced Fos expression in the NTS. Pharmacological blockade of oxytocin receptors reverses the anorexic effect of leptin on food intake and also prevents the leptin-stimulated potentiation of CCK-induced Fos expression in the NTS. Thus, changes in leptin signaling to the hypothalamus appear to be relayed to the NTS in part via oxytocin neurons. We hypothesize that leptin signaling through this oxytocin circuit increases the efficacy of satiety signals received in the NTS, resulting in smaller meals. Therefore, these central players comprise components of an adaptive mechanism for adjusting the number of calories consumed in individual meals to changes in adipose tissue mass. Dysfunction of this energy homeostatic mechanism would result in increased meal sizes and increased cumulative food intake over time, ultimately leading to obesity.

46

NEUROPEPTIDE Y AND ENERGY HOMEOSTASIS: INSIGHTS FROM Y RECEPTOR KNOCKOUT MODELS

Herbert Herzog

Neurobiology Program, Garvan Institute of Medical Research.

A complex system has evolved to regulate food intake and to maintain energy homeostasis. A series of short-term hormonal and neural signals that derive from the gastrointestinal tract, such as cholecystokinin (CCK), pancreatic polypetide (PP) and the recently discovered PYY(3-36) regulate meal size. Others such as ghrelin initiate meals, and insulin and leptin, together with circulating nutrients, indicate long-term energy stores. All these signals act on central nervous system sites which converge on the hypothalamus, an area that contains a large number of peptide and other neurotransmitters that influence food intake with neuropeptide Y (NPY) being one of the most prominent ones. Five Y receptors are known which mediate the action of NPY and its two other family members, PYY and PP. Elevated NPY expression in the hypothalamus leads to the development of obesity and its related phenotypes, Type II diabetes and cardiovascular disease. The limited availability of specific pharmacological tools and the considerable large number of Y receptors has made it difficult to delineate their individual contributions to the regulation of energy homeostasis. However, recent studies analysing transgenic and knockout NPY and Y receptor mouse models have started to unravel some of the individual functions of these Y receptors potentially also helping to develop novel therapeutics for a variety of physiological disorders including obesity.

NEURAL PATHWAYS INVOLVED IN FOOD INTAKE AND ENERGY EXPENDITURE

B.J.Oldfield
Howard Florey Institute, University of Melbourne

Obesity and its allied disorders of cardiovascular disease and type 2 diabetes have now reached epidemic proportions in all industrialised countries. It has recently become apparent that therapeutic strategies that target energy expenditure may be more effective in the control of body weight than those directed at food intake. It is necessary therefore to develop a more complete understanding of the CNS pathways that are involved in the control of the function of key themogenic tissues such as the brown adipose tissue (BAT) prominent in rodents and other small mammals. We have utilised the retrograde transynaptic transport of pseudorabies virus to define the CNS polysynaptic projections from the hypothalamus to the BAT then, in conjunction with procedures to identify a range of peptides, determined the neurochemical profile of these pathways. Functional approaches have also been established using telemeters to measure temperature of the BAT and therefore thermogenic activity subsequent to the central blockade of peptides shown to be prominent in thermogenic pathways.

After inoculation of the interscapular BAT of Sprague Dawley rats with pseudorabies virus, infected neurons were detected at different levels of the neuraxis at different survival times. For example, virus infected neurons were initially detected in the stellate ganglia and thereafter subsequent orders of neurons were found in the spinal cord, medulla and hypothalamus at approximately 24 hour intervals. Third order (premotor) neurons were found coincidentally in discrete regions of the medulla and in the paraventricular nucleus and lateral aspects of the hypothalamus. After an additional 24 hours, presumptive 4th order neurons were found in the mediobasal hypothalamus. Of the range of orexigenic and anorectic peptides considered, those found most prominently in hypothalamic neurons shown to project polysynaptically to the BAT were MCH, the orexins A and B, and CART. In additional experiments, the central actions of each of these peptides has been blocked either with antisera to CART or specific non peptide antagonists of orexin A or MCH1 receptors introduced intracerebroventricularly and the impact on thermogenesis evaluated. These studies have provided the beginnings of a blue print of the neural pathways and chemical substrates that are involved in the CNS mediation of thermogenic activity.

ORAL PRESENTATION ABSTRACTS

100

METFORMIN RAPIDLY INCREASES INSULIN RECEPTOR ACTIVATION IN HUMAN LIVER AND SIGNALS PREFERENTIALLY THROUGH INSULIN-RECEPTOR SUBSTRATE-2

Jenny E. Gunton [1], Patric J. D. Delhanty[1], Shin-Ichiro Takahashi[2], And Robert C. Baxter [1]

1. ***Kolling Institute Of Medical Research, University Of Sydney, Royal North Shore Hospital, St. Leonards, Sydney, New South Wales 2065 Australia***
2. ***2Graduate School Of Agriculture And Life Sciences, University Of Tokyo, Tokyo 113-8657, Japan***

Metformin was first used in clinical practice in the 1950s, but its mechanism of action remains uncertain. Metformin decreases endogenous glucose production by the liver. In diabetes mellitus, the major effect is to decrease fasting glucose, with a smaller effect on post-prandial glucose. Few studies have examined the effect of metformin upon the insulin-signaling pathway in liver, and none have presented data on effects in normal human liver. Huh7 human hepatoma cells, primary human hepatocytes and normal human liver tissue were used. Huh7 cells are insulin-responsive at roughly physiological levels. Insulin receptor (IR) and IR substrates (IRS)-1 and IRS–2 were assessed by immunoprecipitation and immunoblot. Normal human liver was used to measure IR kinase activity (IR-KA). Tyrphostin AG1024, a selective inhibitor of IR-KA, was used to examine effects downstream from IR. GLUT1 translocation was assessed using the plasma membrane lawn technique. Metformin treatment (1μg/ml, 30 minutes) increased IR-β tyrosine phosphorylation by 78% ($p< 0.0007$) in primary human hepatocytes and Huh7 cells. The effect persisted to 24-hours. Metformin did not augment maximal or sub-maximal insulin-stimulated IR-β activation. Using IR isolated from normal human liver for an *in vitro* kinase assay, metformin increased IR-KA by 150% ($p< 0.0001$). Increased IR-β activation was accompanied by increased tyrosine phosphorylation of IRS-2 (70% increase, $p<0.0001$), but no change in IRS-1. Metformin treatment led to a 65% increase in deoxyglucose uptake in Huh7 cells ($p<0.0001$). Increased deoxyglucose uptake was mediated via translocation of the glucose transporter GLUT-1 to the plasma membrane (50% increase, $p<0.0000001$). Tyrphostin AG1024 inhibited basal, insulin-stimulated and metformin-stimulated IR-β phosphorylation in a concentration-dependent manner. IR-inhibition with AG1024 abolished metformin-induced IRS-2 phosphorylation and abolished metformin-induced 2-deoxyglucose uptake. These studies demonstrate that the mechanism of action of metformin in liver involves rapid IR-β activation, followed by selective IRS-2 activation, and increased glucose uptake via increased GLUT-1 translocation to the plasma membrane. The effects of metformin were completely blocked by selective inhibition of the insulin receptor.

Acknowledgements: Jenny Gunton was supported by a NH&MRC post-graduate medical and dental scholarship.

PROSTAGLANDIN SYNTHETIC ENZYMES IN OVINE MYOMETRIUM, ENDOMETRIUM AND PLACENTA DURING INDUCED-PRETERM AND SPONTANEOUS LABOUR

H.K. Palliser[1], J.J. Hirst[1], G.E. Rice[3], N.L. Dellios[1], R.M. Escalona[2], G.T. Ooi[2], I.R. Young[1]

[1] Physiology Department, Monash University, Victoria, [2] Prince Henrys Institute of Medical Research, Victoria, [3] Department of Obstetrics and Gynecology, University of Melbourne, Mercy Hospital, East Melbourne, Victoria.

Preterm labour remains the leading cause of perinatal mortality and morbidity. Current tocolytic therapies are relatively ineffective or pose risks to the fetus. This situation reflects our inadequate understanding of the mechanisms controlling uterine contractility. Prostaglandins (PGs) play an essential role in the contraction of the uterus however the regulation of the synthesis of stimulatory PGs remains uncertain. A labour-associated increase in PGH synthase-2 (PGHS-2) expression leads to a global elevation in PGs in both fetal and maternal plasma, with an increased $PGF_{2\alpha}$: PGE_2 ratio. The aim of this study was to determine the role of specific synthases that direct the synthesis of the labour-associated PGs $F_{2\alpha}$ and E_2 from PGH_2. For the first time changes in mRNA expression of PGHS-2, PG E synthase (PGES) and PG F synthase (PGFS) were simultaneously characterized in ovine myometrium, endometrium and placentome following dexamethasone-induced preterm and spontaneous labour onset using real-time polymerase chain reaction. We hypothesized that PGHS-2 and PGFS would increase in all tissues during labour while PGES would remain unchanged. Three groups of 4 pregnant ewes were killed either during spontaneous labour, dexamethasone-induced labour or after saline treatment (late gestation, which act as controls). As hypothesized PGHS-2 mRNA expression was increased in all tissues following dexamethasone-induced and spontaneous labour (P<0.005). PGFS mRNA expression showed a marked increase following dexamethasone-induced and spontaneous labour onset in placentome (P<0.005) but not in the myometrium or endometrium. PGES mRNA expression decreased in dexamethasone-induced labour in the placentome but was unchanged in the myometrium and endometrium. PGES mRNA did not change in any tissue during spontaneous labour. Consistent with previous reports the ratio of plasma $PGF_{2\alpha}$: PGE_2 increased markedly after labour onset (P<0.05). The increased PG production resulting from PGHS-2 expression may be preferentially directed to $PGF_{2\alpha}$ production via elevated expression of PGFS. Thus in addition to the established role of PGHS-2, we have found that PGFS may play a significant role in the onset of term and preterm labour and hence provide a possible target for a new tocolytic therapy.

Support NH&MRC.

102

SEXUAL DIMORPHISM IN THE EFFECT OF FETAL GROWTH RESTRICTION ON THE DEVELOPMENT OF THE INSULIN RESISTANCE SYNDROME IN THE AGED GUINEA PIG

Prema Thavaneswaran[1], Dane M. Horton[1], Karen L. Kind[2], Jeffrey S. Robinson[2], Julie A. Owens[1]

Discipline of Physiology[1] and Department of Obstetrics and Gynaecology[2], University of Adelaide, Adelaide, South Australia, 5005.

Epidemiological studies have demonstrated that impaired growth in early life, as indicated by being light or thin at birth, is associated with an increased risk of developing the Insulin Resistance Syndrome (IRS) in adult life. The aim of the current study was to determine whether the guinea pig that undergoes spontaneous fetal growth restriction subsequently develops the IRS as an adult. Pregnant guinea pigs were allowed to deliver and size at birth was measured in offspring, which then had vascular catheters inserted into the carotid artery and jugular vein under general anaesthesia and strict asepsis at 400 days of age. All studies were approved by the Animal Ethics Committee of the University of Adelaide. Simple correlation analysis and multiple linear regression analysis were used to determine the relationship between parameters of adult function and measures of size at birth. Fasting plasma glucose concentrations increased with decreasing weight at birth (B_W) in females ($r=-0.70$, $p<0.001$), but not males. Glucose tolerance, calculated as the area under the glucose concentration curve during an intravenous glucose tolerance test (IVGTT) (0.5g/kg dextrose), increased with decreasing B_W in females ($r=-0.45$, $p<0.05$), but not males. Whole body insulin sensitivity of glucose metabolism, as indicated by the steady state glucose infusion rate achieved during the hyperinsulinaemic euglycaemic clamp (HEC) (120 minutes, 7.5mU insulin/min/kg), decreased with decreasing B_W in females ($r=-0.61$, $p<0.05$), but not males. Systolic blood pressure increased with decreasing abdominal circumference at birth (B_{AC}) ($r=-0.67$, $p<0.05$) in females, but not males. Heart rate increased with decreasing B_{AC} ($r=-0.56$, $p<0.05$) and head width at birth (B_{HW}) ($r=-0.57$, $p<0.05$) in females, but not males. Pulse pressure increased with decreasing B_{AC} ($r=-0.59$, $p<0.05$) and B_{HW} ($r=-0.61$ $p<0.05$) in females, but not males. In conclusion, the effect of fetal growth restriction on the development of the IRS in the aged guinea pig exhibits a sexually dimorphic pattern, producing female offspring that are insulin resistant, diabetic, and hypertensive and male offspring with unaltered insulin sensitivity, glucose tolerance and blood pressure.

103

INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 (IGFBP-3) INHIBITS ADIPOCYTE DIFFERENTIATION

Sophie S.Y. Chan, Stephen M. Twigg, Robert C. Baxter

Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, NSW 2065, Australia.

At a cellular level, IGFBP-3 regulates bioactivity of IGFs and exerts IGF-independent actions. IGFBP-3 is a binding partner for the retinoid X receptor-α (RXR-α). Peroxisome proliferator activated receptor gamma receptor (PPAR-γ) is a crucial transcription factor in adipocyte

differentiation. As RXR-α is an obligatory dimerization partner for PPAR-γ, we hypothesized that IGFBP-3 may interfere with adipocyte differentiation through competitive partnering with RXR-α. We aimed to examine the effects of IGFBP-3 on adipogenesis by studying adipocyte gene expression using real-time RT-PCR. PPAR-γ and resistin mRNA were used as markers of the adipocyte phenotype. Plasminogen activator inhibitor-1 (PAI-1) was used as a marker of preadipocyte phenotype. Exogenous fluorescently labelled IGFBP-3 translocated into the nucleus of 3T3-L1 adipocytes by 2 h. After incubation of 3T3-L1 adipocytes with 300ng/mL of IGFBP-3 in SF conditions for 24 h, there was a significant down-regulation of PPAR-γ (by 68%) and resistin (by 56%) and up-regulation of PAI-1 (by 352%). The effect of IGFBP-3 in shifting the adipocyte towards a more de-differentiated phenotype appears to be independent of IGF-1 as IGFBP-2 (50 – 1000ng/mL) did not have an effect on the genes studied. When IGFBP-3 (500ng/mL) was present during the process of differentiation, there was a retardation of differentiation as assessed by reduced PPAR-γ (by 72%) and resistin mRNA (by 78%) and an increase in PAI-1 mRNA (by 110%) by day 9. 3T3-L1 preadipocytes were stably transfected with IGFBP-3 cDNA resulting in secretion of approximately 20ng/mL of IGFBP-3 into condition media over 24 h as determined by RIA. Preadipocytes transfected with IGFBP-3 showed inhibition of differentiation as assessed by reduced PPAR-γ and resistin mRNA compared to vector transfectants at days 4, 7 and 10. In contrast preadipocytes transfected with a mutated nuclear localization signal (NLS) form of IGFBP-3 which is unable to translocate to the nucleus or bind RXR-α had no effect on differentiation of preadipocytes. In conclusion, short-term IGFBP-3 treatment shifts the mature adipocyte towards a more de-differentiated state and exposure to IGFBP-3 during differentiation retards this process. This is consistent with IGFBP-3 interacting with RXR-α as the NLS mutant form does not inhibit adipocyte differentiation.
(S. Chan is supported by a NHMRC Medical and Dental postgraduate scholarship)

104

THE EXPRESSION OF CD151 IN PRIMARY PROSTATE CANCER AND ITS CLINICAL SIGNIFICANCE

Jian Ang , Albert G. Frauman

Clinical Pharmacology And Therapeutics Unit, Austin & Repatriation Medical Centre, Department Of Medicine, University Of Melbourne.

CD151 has been described as a tumour associated gene based on cell culture experiments (1,2). However, its biological function and expression phenotype among different tumours has not yet been well investigated. Our research has been assessing the expression of CD151 protein by immunohistochemistry in primary human prostate cancer of varying histological grades. The value of CD151 as a prognostic factor in predicting the clinical behaviour of prostate cancer was evaluated as well. Tissue specimens of 76 primary prostate cancers and 30 benign prostate hyperplasia (BPH) controls were obtained from the Austin and Repatriation Medical Centre from 1984 to 1993. We used quantitative immunohistochemical analysis to measure CD151 protein expression (M2 program of Microcomputer Imaging Device, Imaging Research Incorporated, St. Catharine's, Canada). The Kaplan-Meier method was used to estimate the probability of overall survival. CD151 expression was found to be significantly higher in prostate cancer specimens compared to BPH specimens ($P<0.0001$). Poorly differentiated cancers expressed the strongest staining while well differentiated cancers expressing the weakest staining for CD151. The

difference between each of the two groups was significant (P<0.0001 respectively). The overall survival rate for cases in which CD151 expression was reduced was significantly higher than for cases in which CD151 was highly expressed (P = 0.0023). Our data show that CD151 has an increasing expression pattern in prostate cancer progression and higher levels of CD151 are associated with poorer prognosis. Although its mechanism in tumour progression is still unknown, CD151 could be a valuable biological marker for the prognostication of prostate cancer.

(1) Kohno M et al (2002) Int J Cancer, 97, 336-43.
(2) Testa JE et al (1999) Cancer Res, 59, 3812-20.

105

GHRELIN AND A NOVEL GHRELIN ISOFORM HAVE POTENTIAL AUTOCRINE/PARACRINE ROLES IN HORMONE-DEPENDENT CANCER

Penny L. Jeffery, Adrian C. Herington, Lisa K. Chopin

Hormone-dependent cancer group, Queensland University of Technology.

Ghrelin, a newly discovered and unique peptide hormone activates the growth hormone secretagogue receptor (GHS-R) to potently induce growth hormone release. Ghrelin treatment rapidly increases serum growth hormone concentrations, as well as stimulating food intake *in vivo*. New evidence has emerged suggesting that ghrelin also participates in the growth of peripheral tissues. For example, ghrelin stimulates the growth of adipocytes and kidney cells *in vitro*. Our laboratory was the first to demonstrate that ghrelin increases the proliferation of the prostate cancer cell lines PC3 and LNCaP *in vitro* and that these cells express the GHS-R at the mRNA and protein level. Since our discovery, other groups have shown that other cancer cell lines including the HepG2 hepatoma cell line display increased proliferation after ghrelin treatment. We also report the identification of a new exon 3-deleted proghrelin human mRNA variant and an equivalent homologue in the mouse. The deletion of exon 3 results in a frameshift and the generation of a unique C-terminal peptide sequence. Using RT-PCR and immunohistochemistry, we have shown that this variant is expressed by a wide range of hormone-dependent carcinoma cell lines and cancer tissues including prostate, breast and endometrial cancers. By employing an antibody generated against the unique exon 3-deleted proghrelin peptide sequence, we have demonstrated immunoreactivity for this variant in normal and malignant prostate glands. Staining intensity in the malignant prostate glands is greater than in normal prostate tissue. This peptide is also expressed in the glands and stroma of breast cancer tissues, with high-grade carcinoma specimens exhibiting the most intense staining. Normal breast sections displayed weak glandular staining and no stromal staining. In conclusion, ghrelin and the novel exon 3-deleted isoform are expressed in hormone-dependent cancers and we have discovered that ghrelin increases the proliferation of prostate cancer cells *in vitro*. Ghrelin potentially plays an autocrine/paracrine mitogenic role in these tissues and may be a target for therapeutic blockade. The fact that ghrelin and the exon 3-deleted proghrelin isoform are differentially expressed in normal and cancer tissues could provide an avenue for the development of new diagnostic and prognostic markers for hormone-dependent cancer.

106

ESTROGEN DEFICIENCY RESULTS IN APOPTOSIS IN THE HYPOTHALAMUS AND PONS REGION OF MALE MICE: ESTROGENIC ACTIONS IN THE HYPOTHALAMUS MAY REGULATE SEXUAL BEHAVIOUR AND AGGRESSION?

Rachel A. Hill[1], Margaret E.E. Jones[1], Evan R. Simpson[1], Wah Chin Boon[1]

[1]Prince Henry's Institute of Medical Research, Clayton, VIC, Australia, 3168.

Estrogenic effects in the brain have been widely studied in females, with results implying a neuroprotective role for estrogen. However, the effects of estrogen on the male brain are still controversial. The presence of estrogen receptors and aromatase (the enzyme that synthesizes estrogen) in specific areas of the brain suggest a role for estrogen in the male brain. In order to study the physiological effects of estrogen, our laboratory generated an aromatase knockout mouse. Here, exon IX of the *Cyp19* (aromatase), gene was replaced with a *neomycin* resistance cassette, rendering the gene non-functional. Hence, the aromatase knockout mouse (ArKO) is deficient in estrogen. TUNEL staining was performed on male ArKO and wild type (WT) brains and apoptosis was detected in the hypothalamus and pons region of the brain of one year-old (1yo) male ArKO mice but not in WT littermate controls. This was further confirmed by performing RNase protection assay on total RNA extracted from ArKO and WT hypothalamus and pons regions. Transcript levels from the death receptor pro-apoptotic genes; *caspase-8*, *FASL* and *FADD* were up-regulated in the hypothalamus of 1yo mice, whilst in the pons region the anti-apoptotic gene *bfl-1* was significantly down regulated and the pro-apoptotic gene *bak* was significantly up regulated in 1yo ArKO compared to WT. Furthermore transcript levels from pro-apoptotic genes *bax*, *bcl-x*$_{(S)}$, and *bad* were all up regulated in the ArKO compared to WT 20 wk-old hypothalamic RNA. The hypothalamus has been observed to regulate sexual behaviour, aggression, and grooming behaviour. Interestingly, we have previously reported that male ArKO exhibit a severe disruption in sexual behaviour with increased latency to mount receptive females, and a lack of aggression toward male intruders. In addition we have observed that the male ArKO displays a significant increase in grooming behaviour when compared to WT. Hence, we have provided the first evidence to suggest that estrogen may be involved in regulating sexual behaviour, aggression and grooming in males via actions in the hypothalamus in addition to its neuroprotective effect.

107

REDUCED CIRCULATING PROGESTERONE DURING LATE PREGNANCY IN THE RAT DECREASES FETAL AND PLACENTAL GROWTH

Peter J. Mark, Jeremy T. Smith, Brendan J. Waddell

School of Anatomy & Human Biology, The University of Western Australia, Crawley, W.A., 6009.

Growth and development of the rodent fetus is critically dependent upon adequate functioning of the placenta, the control of which is regulated by a number of endocrine signals. While the role of progesterone in ensuring conceptus survival and pregnancy maintenance is clearly established,

progesterone has also been proposed as a trophic factor supporting placental and consequently fetal growth. Thus, complete progesterone withdrawal at mid-pregnancy has been shown to dramatically reduce placental protein synthesis, but whether more physiological reductions in progesterone compromise placental growth is unknown. In this study we developed a rat model to reduce circulating levels of progesterone during the final stages of rat pregnancy and assessed the impact on placental and fetal growth. Rats were ovariectomized on day 16 of pregnancy (term = day 23) and estradiol replaced by injection and infusion to mimic normal levels. Twice-daily injections of progesterone were administered to restore circulating progesterone levels to normal (progesterone-restored; n = 7) or to one third of normal (progesterone-reduced; n = 12). Preliminary studies established that the latter was sufficient to maintain pregnancy. Because progesterone can antagonize glucocorticoid actions, we also assessed whether the effects of progesterone reduction were altered by co-treatment with the glucocorticoid synthesis inhibitor, metyrapone (n = 6). On day 22, fetuses and placentas were removed and weighed (three from each mother) and each placental zone (basal and labyrinth) dissected and weighed separately. Progesterone reduction resulted in lower fetal (89%; $P<0.01$), placental (77%; $P<0.001$), basal zone (65%; $P<0.001$) and labyrinth zone (88%; $P=0.05$) weights in comparison to the sham-control group (n = 7). Fetal and placental weights (both zones) were similar in sham-control and progesterone-restored animals. Co-treatment with metyrapone appeared to counter the effects of progesterone reduction in the labyrinth zone, although fetal, total placental and basal zone weights did not differ significantly from either sham-control or progesterone-reduced animals. These results indicate that progesterone provides trophic support for the placenta and fetus, and suggest that the effect of progesterone, at least in the labyrinth zone, may involve antagonism of glucocorticoid action. Further studies are required to determine the mechanism/s by which progesterone supports placental and fetal growth.
Supported by NHMRC Project Grant 139104.

108

POST-TRANSCRIPTIONAL DOWN-REGULATION OF PGDH MESSENGER RNA IN THE CHORION LAEVE BEFORE TERM LABOUR ONSET

C. Mitchell[1], R. Johnson[1], C. Evans[1], V. Clifton[1], T. Zakar[1,2]

[1] Mothers and Babies Research Centre, University of Newcastle and [2] Division of Obstetrics and Gynaecology, John Hunter Hospital, Newcastle. NSW, Australia.

We have reported recently that mRNA for 15-hydroxyprostaglandin dehydrogenase (PGDH, the main prostaglandin metabolic enzyme) is transiently down-regulated in the chorion membrane at term, increasing labour-promoting prostaglandin levels in the uterus before labour. Here we examined the possibility that the PGDH mRNA down-regulation involves a post-transcriptional mechanism.

Chorion membranes were isolated after elective Cesarean section at term (CS, n=27) and pre-term (PCS, n=17) and after spontaneous labour at term (SL, n=24). Abundance of the functional and two non-functional splice variants of PGDH mRNA as well as of total PGDH mRNA (based on a sequence common to all splice variants) were determined by quantitative real-time RT-PCR (Q-RT-PCR). PGDH gene activity was assessed by measuring PGDH heterogeneous nuclear (hnRNA) using Q-RT-PCR.

The levels of the non-functional PGDH mRNA variants and of total PGDH mRNA did not differ between groups. The level of the functional PGDH mRNA variant, however, was lower in the CS group ($p<0.05$ Kruskal-Wallis and Tukey's tests) than in the PCS and SL groups. In individual tissues, PGDH hnRNA levels predicted total PGDH mRNA abundance in all groups and functional PGDH mRNA abundance in the PCS and SL groups ($p<0.05$, regression), but did not predict functional PGDH mRNA abundance in the CS group. Thus, PGDH mRNA expression is controlled by PGDH gene activity before term and during labour, but not before term labour onset, when PGDH mRNA abundance is decreased predominantly by post-transcriptional process(es). Preliminary experiments with short-term chorion tissue incubations indicated that a short-lived message-destabilizing factor contributes to PGDH mRNA degradation at term prior to labour.

The post-transcriptional down-regulation of PGDH expression in the chorion membrane is potentially a critical event in the regulatory cascade leading to term labour onset.

109

THE ROLE OF CORTICOTROPIN RELEASING HORMONE IN THE MICROVASCULAR PHYSIOLOGY OF PREGNANCY

Lynda Dierkx, Vicki Clifton, Ian Wright, Mark Read, Roger Smith

Mothers and Babies Research Centre, Hunter Medical Research Institute, John Hunter Hospital, University of Newcastle, Newcastle. NSW. Australia.

We have previously found that corticotropin-releasing hormone (CRH) is a potent vasodilator in the non-pregnant female microvascular skin circulation. Furthermore there was a positive correlation between CRH-induced dilation and circulating estrogen concentrations during the menstrual cycle. Since both CRH and estrogen concentrations rise during gestation, we questioned whether there were any alterations in CRH-induced vasodilation as pregnancy progressed. Furthermore CRH levels are known to be significantly increased in pregnant women with pre-eclampsia and thus there may be alterations in the response to CRH in the microvascular circulation of this group. We hypothesised CRH-induced dilation would be altered in normal and pre-eclamptic pregnant women as gestation progressed. CRH (1nM) was administered transcutaneously by iontophoresis to a small area of skin in the female forearm and blood flow in the same area was measured simultaneously by laser Doppler. Human CRH (1nM) caused vasodilation in the skin circulation of non pre-eclamptic women (n=88) at around 16, 25, and 35 weeks gestation. CRH-induced dilation increased as gestation progressed. In women with pre-eclampsia (31-40 weeks gestation, n=18) CRH-induced dilation was significantly reduced when compared to non-pre-eclamptic, pregnant women (n=35, GEE $P<0.05$). CRH-induced dilation was compared between non-pregnant, midcycle females (n=8) and pregnant women at around 35 weeks gestation (n=35, GEE $P<0.05$). There was a significant reduction in CRH-induced dilation in the presence of pregnancy. These data indicate that CRH induced dilation is down regulated in the presence of high circulating concentrations of CRH during pregnancy and further decreased in the presence of pre-eclampsia. However these data suggest that changes in maternal blood flow as gestation progresses could be partially dependent upon CRH-induced dilation and the presence of pre-eclampsia is associated with a decreased ability of maternal blood vessels to respond to vasodilator stimuli.

110

DIRECTIONAL SECRETION AND TRANSPORT OF LEPTIN BY HUMAN PLACENTAL BeWo CELLS AND THE EFFECTS OF SYNCYTIUM FORMATION AND GLUCOCORTICOIDS

Caitlin Wyrwoll, Peter J. Mark, Brendan J. Waddell

School of Anatomy & Human Biology, The University of Western Australia, Nedlands, W.A., 6009.

Leptin, the 16 kDa product of the *ob* gene, has been proposed as an important regulator of fetal growth and development, but the control of fetal leptin exposure remains unclear. Fetal leptin synthesis appears to be relatively low for much of pregnancy, suggesting that the placenta and/or the mother may provide important sources of leptin to the fetus. In this study we investigated the transport of exogenous leptin across monolayers of the human placental BeWo cell line as an index of transplacental leptin passage. In addition, we measured directional secretion of endogenous leptin by BeWo cells and assessed the effects of syncytium formation and glucocorticoid treatment. BeWo cells were seeded onto the polycarbonate membrane of cell culture inserts at a density of 18,000 cells per cm^2. Bidirectional transfer of ^{125}I-leptin was very low (<1%) but exceeded that of ^{3}H-inulin, indicative of specific transcellular passage of leptin. Transport of ^{125}I-leptin from the basal to apical chamber (0.88 ± 0.02 % after 6h) exceeded ($P<0.05$) its apical to basal transfer (0.70 ± 0.01 %). Secretion of endogenous leptin into the basal compartment (35 ± 4 pg/6h) exceeded ($P<0.05$) apical secretion (13 ± 2 pg/6h), and both were increased (by up to 45%, $P<0.05$) with dexamethasone treatment (0.1 μM) for 72h. Incubation of BeWo cells with forskolin (20 μM) induced formation of syncytial clumps and the loss of monolayer integrity, thereby preventing separate analysis of apical-basal secretion. Nevertheless, total leptin secretion increased markedly (5-fold; $P<0.01$) in response to syncytium formation, and this effect was further enhanced (55% increase) by dexamethasone (1 μM; $P<0.05$). In conclusion, our data show that human placental BeWo cells show a slight preference for secretion leptin from their basal surface, but their transport of exogenous ^{125}I-leptin favors passage from the basal to apical side. In addition, glucocorticoids and syncytium formation are both potent stimulators of leptin secretion by BeWo cells.

Supported by NHMRC of Australia (Project Grant 139104).

111

EFFECT OF SIZE AT BIRTH ON SALIVARY CORTISOL IN THE GUINEA PIG FROM BIRTH TO OLD ADULTHOOD

S. Grover, M.R. Walker, N.K. Lloyd, C.L. Coulter, J.A. Owens

Research Centre for the Physiology of Early Development, School of Molecular and Biomedical Science, Discipline of Physiology, University of Adelaide, Adelaide, South Australia, 5005.

Studies in humans suggest that small size at birth is associated with increased hypothalamic pituitary adrenal (HPA) axis activity in later life, which could in turn contribute to the associated increased risks of developing diabetes, hypertension and cardiovascular disease (1). The precise

timing and extent of such prenatally induced alterations in HPA axis activity, in terms of cortisol secretion and circulating cortisol, in the adult human, is unclear however. We hypothesised that restriction of fetal growth programmes the activity of the HPA axis postnatally, increasing circulating and hence salivary cortisol in the guinea pig.

Saliva was sampled every 2 hours (8am-4pm) in males and females of known size at birth from 10 to 400 days of age. The effects of age, gender and birth weight on mean salivary cortisol concentrations were examined by ANOVA and correlation analysis.

Mean salivary cortisol varied with age and gender and their interaction ($p>0.05$ for all). Mean salivary cortisol was highest in males at 90, 120 and 300 days of age and correlated negatively with birth weight at 90 and 120 days. In females, the highest mean salivary cortisol occurred at 300 days of age and correlated negatively with birth weight at this age ($p>0.05$ for all).

In conclusion, restricted fetal growth increases HPA axis activity postnatally in the guinea pig, as indicated by increased salivary cortisol concentrations. The presence of this prenatally induced increase in cortisol varies with age and gender however, and consequently so may any contributions to prenatally induced insulin resistance, glucose tolerance or hypertension.

(1) Phillips D.I. et al. (1998) Journal of Clinical Endocrinology and Metabolism, 83(3): 757-760.

112

BIRTHWEIGHT AND FUNCTION OF THE HPA AND GH/IGF-1 AXES IN ADULT LIFE

Tien-Ming Hng, N. Wah Cheung, Mark McLean

Department of Diabetes and Endocrinology, Westmead Hospital, Westmead, NSW 2145 Australia.

OBJECTIVE: To examine the effect of birthweight (BW) on dynamic function of the hypothalamic–pituitary–adrenal axis and growth hormone–IGF-1axes using a twin model to control for confounding genetic factors.

DESIGN AND METHODS: 19 sets of adult same sex twins were recruited via the Australian Twin Registry. Consent and birth records were obtained. A 75-gram oral glucose tolerance test (GTT) was performed after obtaining baseline blood samples, including plasma IGF-1. After the GTT, 1 μg Synacthen was administered intravenously and a brief intense exercise stimulation was performed. Plasma cortisol and GH samples were obtained before and 30 min after the stimulation tests. Results were analysed by paired t-test, given as mean ± SE (or SD where stated).

RESULTS: The mean age was 38.9 ± SD10.1 years. There were 17 female and 2 male twin sets. 13 sets were monozygotic (1 male). Mean BW for all individuals was 2496.6 ± SD503.9 grams. Mean intra-pair BW difference was 311 ± SD47.4 grams. There were no significant differences in plasma IGF-1, or glucose levels during the GTT. At baseline the heavier BW twin had higher plasma cortisol levels (321.0 ± 26.3 vs 261.9 ± 20.6 nmol/L, $p<0.05$). Synacthen stimulation produced a greater cortisol increment in the lower BW twins (428.5 ± 28.6 vs 349.8 ± 30.2 nmol/L, $p<0.01$). Exercise caused a rise in plasma GH in all subjects, but with a greater increment in the heavier BW twin (14.9 ± 2.7 vs 6.5 ± 2.4 mU/L, $p=0.01$)

CONCLUSION: In twin pairs, lower BW is associated with lower baseline cortisol levels but an augmented response to HPA stimulation. Lower BW is also associated with reduced GH response to stimulation. These results suggest that BW is associated with long-term programming of the HPA and GH-IGF axes, which may influence susceptibility to disease in adult life.

113

INTRA-FETAL IGF-I INFUSION IN THE SHEEP – DISSOCIATION BETWEEN ADRENAL GROWTH AND STEROIDOGENESIS BEFORE BIRTH

J.T. Ross, I.C. McMillen, F. Lok[1], A.G. Thiel, J.A. Owens, C.L. Coulter

Discipline of Physiology, School of Molecular and Biomedical Sciences, [1]Dept of Obstetrics & Gynecology, University of Adelaide, Adelaide, SA, Australia 5005.

It has been demonstrated that ACTH 1-39 plays a major role in the stimulation of growth and steroidogenesis of the immature adrenal cortex. There is also evidence that intra-adrenal growth factors such as the insulin-like growth factors (IGFs) mediate the actions of ACTH 1-39 on adrenal growth. It is unclear, however, whether IGFs mediate both the growth-promoting and steroidogenic actions of ACTH on the fetal adrenal. The aim of this study therefore was to investigate the effects of an intra-fetal infusion of IGF-1 on adrenal growth and steroidogenesis. Fetal sheep were infused for 10 days with either IGF-I (26μg/kg/h; n=14) or saline (n=10) between 120d and 130d gestation and then adrenal glands were collected, weighed and either fixed for morphological analysis or frozen for determination of the mRNA expression of the steroidogenic enzymes P450 side change cleavage (CYP11A1), P450 17 hydroxylase (CYP17) and 3β hydroxysteroid dehydrogenase (3βHSD). Fetal plasma cortisol concentrations were also determined. Fetal adrenal weight was significantly increased following IGF-1 infusion (414.3 ± 30.0 mg vs 301.4 ± 19.2mg). This was as a consequence of a significant increase in cell size within the zona glomerulosa, zona fasciculata and the adrenaline-synthesizing zone of the adrenal glands in the IGF-infused group. There was no difference in fetal plasma cortisol concentrations between the IGF-I (10.0 ± 3.21 nmol/L) and vehicle infused groups (7.0 ± 1.31 nmol/L). In addition, there was no effect of IGF-1 infusion on the adrenal expression of CYP11A1, CYP17 or 3βHSD mRNA. In summary, we have shown that elevated levels of plasma IGF-1 concentrations in late gestation markedly stimulated growth of the adrenal cortex and of the adrenaline-synthesizing cells of the adrenal medulla. There was no effect however, on adrenal steroidogenic enzyme mRNA expression or on fetal plasma cortisol concentrations. Therefore, these *in vivo* experiments have demonstrated a dissociation of adrenal growth and steroidogenesis. These data suggest that ACTH may mediate its action on the fetal adrenal via two distinct pathways. One via intra-adrenal growth factors, such as IGF, to stimulate adrenal growth and the second specifically activates the steroidogenic enzymes, to increase cortisol synthesis.

114

LOCALISATION OF PROSTAGLANDIN H SYNTHASE (PGHS) TYPES-1 AND –2 IN THE GESTATIONAL TISSUES OF THE GUINEA PIG

T. Zakar[1,2], T. Welsh[1], S. Mesiano[1,2]

[1]Mothers and Babies Research Centre, University of Newcastle and [2]Division of Obstetrics and Gynaecology, John Hunter Hospital, Newcastle, NSW, Australia.

Prostaglandins (PGs), generated by PGHS-1 and –2, are important paracrine mediators of parturition. We have shown previously that PGHS-1 mRNA is induced in the guinea pig amnion and placenta with advancing gestation and falls during labour. PGHS-2 mRNA, on the other hand, is induced in the placenta and myo-endometrium, but not in the fetal membranes, during labour. Here we have determined the cellular localisation of PGHS-1 and –2 proteins in the guinea pig gestational tissues at late pregnancy and at labour.

Amnion, chorion, placenta and myo-endometrium were collected between 45d gestation and term (62-70d) and in labour. Tissues were fixed in formalin and blocked in paraffin before being sliced (5μm) and mounted on slides. Sections were probed with antibodies against PGHS-1 or –2, and binding was detected using the avidin-biotin-peroxidase system. Controls were probed with non-immune serum.

PGHS-1 protein was expressed pervasively in the amnion, with the most intense staining at term. In chorion, myometrium and endometrium, PGHS-1 was present in sporadic single cells. PGHS-1 protein was present in the parietal yolk sac membrane, which overlays the guinea pig placenta, and in sporadic cells throughout the placenta. The yolk sac membrane exhibited intense staining for PGHS-2 protein. PGHS-2 was localised to the syncyciotrophoblast at 45d, but not later. Weak staining for PGHS-2 was seen in amnion, but PGHS-2 protein was undetectable in chorion. Within the myo-endometrium, PGHS-2 was localised only to endometrium and was not present in myometrium at any stage.

Thus, PGHS-1 is induced in the amnion and the parietal yolk sac membrane before labour likely producing PGs involved in labour onset. PGHS-2 is induced in the endometrium and the parietal yolk sac membrane during labour, probably producing PGs involved in labour progression. PGHS-1 appears to localise to sporadic immune-type cells in the chorion, placenta and myo-endometrium.

115

ANDROGENS EXERT DISTINCT EFFECTS ON GH SIGNALLING VIA THE JAK2/STAT5, MAPK AND PI3K PATHWAYS

Gary M. Leong[1], Kin-Chuen Leung[1], Nathan Doyle[1], Cathy Choong[2], Wayne D. Tilley[2], Ken K.Y. Ho[1]

[1]Pituitary Research Unit, Garvan Institute of Medical Research, St Vincent's Hospital, Sydney, NSW 2010; [2]Dame Roma Mitchell Cancer Research Laboratories, Hanson Institute, Adelaide, SA 5000.

Androgens positively modulate growth hormone (GH) actions on body growth and body composition, yet the molecular mechanisms underlying this regulation is unclear. The aim was to investigate the effects of androgens on GH signalling via the Janus kinase 2/signal transducer and

activator of transcription 5 (JAK2/STAT5), mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3'-kinase (PI3K) pathways. Human embryonic kidney (HEK293) cells stably expressing human GH receptor were transiently transfected with human androgen receptor (AR) (1-100ng) and STAT5 (100ng) expression plasmids with a luciferase reporter containing the STAT5 binding element. Cells were treated for 24 hr with 100ng/ml GH and/or 1nM dihydrotestosterone (DHT) after which luciferase activity was measured. Phosphorylation of JAK2, STAT5, ERK1/2 (for the MAPK pathway) and Akt (for the PI3K pathway) in the cell lysates was quantified by Western analysis. GH induced phosphorylation of JAK2 and of STAT5, ERK1/2 and Akt. DHT alone had no effect, but enhanced the effect of GH on ERK1/2 phosphorylation, but not that of JAK2, STAT5 or Akt. In the reporter assay, DHT decreased GH-induced luciferase activity of the STAT5 reporter by more than 80% in an AR-concentration-dependent manner. Similar inhibitory effects of androgens on GH-dependent reporter activities were observed in human hepatoma cells (HuH7) and foreskin fibroblasts. In summary, signalling activation by GH of the MAPK pathway is enhanced while that of the JAK/STAT pathway is inhibited by androgens. Thus, androgens exert selective and distinct effects on GH signalling. Amplification of GH signalling via the MAPK pathway may be one mechanism by which androgens enhance the mitogenic effect of GH.
Supported by the NHMRC and Vincent Fairfax Family Foundation.

116

EFFECTS OF SHORT TERM (1H) AND LONG TERM (24H) GHRH AND GHRP-2 TREATMENT ON THE GENE EXPRESSION OF GH, GHRH-R, GHRP-R, PIT-1 AND SSTR1-5 ON RAT PITUITARY CELLS IN VITRO

M. Hernandez, M. Yan, C. Chen

Prince Henry's Institute of Medical Research, Melbourne.

Growth Hormone (GH) secretion from pituitary somatotropes is controlled by the interaction between GH-releasing hormone (GHRH), recently discovered endogenous GH secretagogues (GHS)-ghrelin, and somatostatin (SRIF). GHRH and GHSs stimulate whereas SRIF inhibits GH secretion. Pituitary somatotropes are not only passively regulated by these factors, but the sensitivity of cells to each regulatory hormone is also constantly changed according to environmental conditions. The balance of sensitivities of somatotropes to releasing and inhibitory hormones is also regulated by regulatory hormones and metabolic factors. In order to clarify the effect of GHRH and GHSs on somatotropes, in vitro treatment of rat pituitary cells with GHRH and GHRP-2 (a potent GHS) was used to investigate the synthesis of GH, GHRH-R, GHS-R, pituitary specific transcription factor (Pit-1), and SRIF-Rs (sst-R1-5). Enzyme-dissociated rat pituitary cells were seeded at 800,000 cells/well in culture medium containing 10% inactivated FCS and incubated for 72h before treatment. The cells were then subjected to short term (1h), and long term (24h) GHRH/GHRP-2 treatment in fresh culture medium. Total RNA was then extracted and semi quantitative PCR was performed to determine the levels of mRNA encoding for each of the above mentioned genes. Culture medium was also collected and stored at –20°C for GH assay with RIA kits from NIH. After 1h treatment of cells with GHRH (10 nM) + GHRP-2 (100 nM), the mRNA levels of GHS-R, Pit-1, and sst-R1, 3, 4 were increased compared with that in untreated cells at the same time point. Levels of GH mRNA and sst-R2, 5 mRNAs showed

no change whereas GHRH-R mRNA was decreased slightly. GH levels in culture medium were significantly increased by GHRH/GHRP-2 treatment. After 24h treatment with GHRH/GHRP-2, the mRNA levels encoding for GHS-R, sst-R1, 3, 4 were increased, whereas Pit-1, sst-R2, 5, GH, and GHRH-R mRNA levels were slightly decreased. Accumulated GH in cultured medium was increased by GHRH/GHRP-2 treatment. These results indicate that GHRH and GHS act directly on somatotropes to regulate the synthesis of receptors and GH, leading to a modification of sensitivities of somatotropes to releasing and inhibitory hormones.
Supported by NHMRC.

117

NOVEL IGF COMPLEXES FOR APPLICATIONS IN TISSUE REPAIR AND REGENERATION

Z. Upton, B. Hollier, C. Hyde, R. Dawson, P. Gillies, J. Kricker, C. Towne, D. Leavesley, D. Harkin

Tissue BioRegeneration and Integration Program, Queensland University of Technology, Brisbane, Queensland.

We recently discovered novel links between IGFs, IGFBPs and vitronectin (VN) and have demonstrated that these complexes significantly enhance migration and proliferation in a diverse range of cells including skin and corneal epithelial cell lines. Importantly, studies in primary cultures of keratinocytes derived from adult skin and cornea have validated these findings. Furthermore, our functional assays using IGF analogues with reduced affinity for the IGF receptor (IGF-IR) (but which retain binding to IGFBPs and VN) and a function-blocking antibody against the VN-binding α_v integrins, indicate that these complexes enhance cell migration through coordinate activation of both the IGF-IR and VN-binding integrins. Recognising the key significance of our discoveries, and realising that this phenomenon of matricellular signalling could be exploited as a biological system to deliver growth factors in situations where cell proliferation and migration are wanted, led us to examine the potential of the complexes in *ex vivo* expansion of progenitor cells for tissue regeneration. Thus we recently commenced studies investigating the potential of IGF:IGFBP:VN complexes to replace serum and feeder cells used in current best clinical practice for *ex vivo* expansion of keratinocytes for autografting. These studies revealed that cells seeded at low density on IGF:IGFBP:VN-coated dishes were not only able to survive, but also, to expand more rapidly than those grown using current clinical protocols (ie. seeded at the same density and grown in the presence of serum and growth-arrested murine feeder cells). The use of this technology for *ex vivo* keratinocyte expansion is being further developed in collaboration with the Queensland Skin Bank/Australian Red Cross to provide an improved, safe, animal-product free, autologous cell-based therapy for burns patients. Indeed, patient studies using skin grafts with cells cultivated using this technology are scheduled to commence in 12 months. Moreover we anticipate that a similar approach will also be useful to developing safe cell-based wound therapies for diabetic and venous ulcers.

EPIDERMAL EQUILIBRIUM: A FUNCTIONAL ROLE FOR IGFBP-3 *IN VIVO*

Stephanie Edmondson[1], Susan Thumiger[1], Brian Loh[1], Rachel Koelmeyer[2], Amy Li[2], Josef Silha[3], Liam Murphy[3], George Werther[1], Christopher Wraight[1], Pritinder Kaur[2]

[1]Cutaneous Biology Group, Dermal Therapeutics, Centre for Hormone Research, Murdoch Childrens Research Institute, Parkville, 3052, Victoria. [2]Peter MacCallum Cancer Institute, East Melbourne, 3002, Victoria. [3]Dept Medicine & Physiology, University of Manitoba, Winnipeg, Manitoba, R3E 3P4, Canada.

Epidermal balance requires an exquisite process of keratinocyte growth and terminal differentiation. Basal keratinocyte stem cells (KSC) evolve into transit amplifying (TA) and ultimately post-mitotic differentiating (PMD) cells that exit the germinative layer and continue to metamorphose through upper epidermal layers. IGF-IR -/- mice confirm the importance of the IGF system in epidermal homeostasis. Our studies indicate that IGFBP-3 expression is tightly linked to selected basal keratinocytes, is reduced on differentiation and is grossly altered in hyperplastic epidermis. These studies aimed to (i) clarify the basal keratinocyte sub-type expression of IGFBP-3 and IGF-IR mRNA and (ii) utilise transgenic mice to elucidate the functional role of IGFBP-3 in epidermal homeostasis.

Human basal keratinocytes were FACS fractionated on the basis of α6 integrin (α6) and transferrin receptor (CD71) abundance (KSC: $\alpha6^{bright}$/$CD71^{dim}$; TA: $\alpha6^{bright}$ /$CD71^{bright}$; PMD: $\alpha6^{dim}$). Real time-PCR revealed that IGFBP-3 mRNA abundance was significantly higher in TA cells (5.9 +/-0.7 SE) when compared with KSCs (1.0 +/-0.002 SE) and PMD (1.0 +/- 0.2 SE). In contrast, IGF-IR mRNA levels were similar in KSC (1.0 +/- 0.002 SE) and TA (1.3 +/- 0.3 SE) and significantly elevated in PMD (2.8 +/- 0.7 SE) keratinocytes.

To further clarify the role of IGFBP-3 in keratinocyte proliferation, we examined the effect of IGFBP-3 over-expression in a transgenic mouse model. When compared with wild-type, back, belly and tail skin of transgenic mice (CMV-human IGFBP-3 cDNA) exhibited (i) over-expression of human IGFBP-3 cDNA and protein in epidermal keratinocytes without grossly altered morphology and (iii) a significant reduction of proliferative (Ki67 positive) keratinocytes (mean normal: transgenic; 8.2+/-1.2 SE : 1.5+/-0.3 SE).

In conclusion, these data confirm that in skin (i) IGFBP-3 mRNA is primarily expressed by proliferative keratinocytes (TA), and (ii) IGFBP-3 over-expression inhibits the proliferative capacity of epidermal keratinocytes. Additionally, elevated IGF-IR mRNA in PMD keratinocytes supports a role for the IGF system in the epidermal differentiation. Finally, these findings provide the first functional evidence for epidermal IGFBP-3 and strongly support a role for IGFBP-3 in modulating keratinocyte proliferation *in vivo*.

SITE DIRECTED MUTAGENESIS TO ALTER HIGFBP-2 CELL SURFACE ASSOCIATION AND PROTEOLYTIC PROCESSING

S.I. Ymer[1], D.G. Cossens[1], E. Andaloro[1], B. Schutt[1], L.A. Bach, G.A. Werther[1], V.C. Russo
[1]Centre for Hormone Research, Murdoch Childrens Research Institute, Dept. of Paediatrics University of Melbourne, Parkville 3052. [2]Dept. of Medicine, University of Melbourne, Austin & Repatriation Medical Centre, Heidelberg 3084, Victoria.
(russov@cryptic.rch.unimelb.edu.au)

Modulation of IGF action by the IGFBPs occurs in the pericellular space involving interactions with matrix proteins including proteoglycans. IGFBP/IGF interaction might be further modulated by specific proteases, which cleave IGFBPs to fragments with reduced affinity for IGF-I. We have demonstrated IGFBP-2 binding to brain proteoglycans (PGs) and in neuronal cells we have shown that FGF-2 induced proteolysis of soluble and cell surface associated IGFBP-2. Of interest was that one of the IGFBP-2 fragments retained the ability to complex with IGF-I on the cell surface. However, the role of IGFBP-2 cell surface association and the biological consequences of IGFBP-2 proteolytic processing in IGF action remain unclear.

We therefore aimed to alter hIGFBP-2 cell surface association and proteolytic processing by mutating the putative HBD and proteolytic cleavage sites of hIGFBP2 by mutagenesis.

HBD-IGFBP-2 mutant: The putative HBD of hIGFBP2, ($_{218}$PKKLRP$_{223\text{-}}$) was mutated to ($_{218}$PNNLAP$_{223\text{-}}$). Mutations were introduced in hIGFBP-2 by overlap extension PCR. Both cDNA HBD-IGFBP-2 mutant and WT-IGFBP-2 control were transiently expressed in SK-N-SHEP neuroblastoma cells (IGFBP-2 negative) and as His6-tagged HBD- or WT- IGFBP-2 in E-coli. The expressed HBD/WT-IGFBP2 were purified from conditioned medium by IGF-I affinity chromatography and the purity was verified by SDS-PAGE followed by silver staining and immunoblotting. In both mammalian and bacterial systems HBD- and WT-hIGFBP-2 maintained the ability to bind ^{125}I-IGF-I as determined by WLB and cross-linking analysis. Scatchard analysis revealed that the binding affinity of the HBD-mutant for IGF1 was not significantly different from WT- IGFBP-2 or commercial IGFBP-2. However, the HBD-IGFBP-2 mutant showed reduced binding to heparin, thus suggesting that the predicted HBD at $_{218}$PKKLRP$_{223}$ is involved in this interaction.

Protease resistant IGFBP-2: The published sequence of IGFBP2 fragments has suggested the presence of two potential proteolytic cleavage sites **(**$_{207}$KGGK$_{210\text{-}}$**)** and **(**$_{219}$KKLR$_{222\text{-}}$**)**. We have mutated both of these sites and made various mutants using the methodology described above for HBD-mutants. The identification of protease resistant IGFBP-2 and characterisation of these mutants is in progress.

The generation of IGFBP-2 with altered functional domains is central to understanding the role of IGFBP2 in modulation of IGF action.

120

THREE-DIMENSIONAL STRUCTURE OF THE C-DOMAIN OF IGFBP-6 IN SOLUTION USING NUCLEAR MAGNETIC RESONANCE (NMR)

Leon A. Bach[1], Stephen J. Headey[1, 2], Shenggen Yao[2], David W. Keizer[2], Nigel J. Parker[1], Phillip Kantharidis[1], Raymond S. Norton[2]

[1] University of Melbourne, Department of Medicine, Austin & Repatriation Medical Centre, Heidelberg, Victoria 3084, [2] Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050.

IGFBPs 1-6 share a three-domain structure. The N- and C-domains are highly conserved and contain internal disulfide linkages, and they are joined by a non-conserved linker domain. Both the N- and C-domains contribute to high affinity IGF binding. In addition, binding to glycosaminoglycans and nuclear localization of IGFBPs –3 and –5 are dependent on C-domain residues. To date, the only three-dimensional structure of any IGFBP is confined to a ~50-residue IGF-binding region in the N-domain of IGFBP-5. We therefore expressed the C-domain (residues 161-240 based on proIGFBP-6) of IGFBP-6 as a His_6-tagged protein in *E. coli* and purified it by Ni-NTA chromatography followed by SP-FF Sepharose cation-exchange chromatography (pH 4.5). Its three-dimensional structure was determined using NMR on protein uniformly labelled with ^{15}N and ^{13}C. Assignment of NOEs and structure calculations were performed using the CANDID module of CYANA. The structure reveals the presence of an α-helix-β-strand at the beginning of the C-domain followed by a two-stranded β-sheet linked by less well-defined loops. This fold is similar to that of the type 1 thyroglobulin domain. The C-terminal region (residues 224-240) remains relatively mobile. The structural core of the C-domain consists of Tyr^{186}, Pro^{188}, Tyr^{196}, Gln^{200}, Trp^{203} and Val^{205}. A putative IGF-binding surface was identified and is currently being evaluated by coincubation of labelled C-domain with unlabelled IGF-II. Residues homologous with those involved in nuclear localization of IGFBP-3 and –5 form a distinct surface adjacent to and overlapping that involved in glycosaminoglycan binding. Availability of the three-dimensional structure of the C-domain of an IGFBP for the first time will accelerate our understanding of the structure-function relationships of IGFBPs.

121

FSH-REGULATED GENES IDENTIFIED BY MICROARRAY ANALYSIS OF AN ACCUTE FSH WITHDRAWL MODEL IN JUVENILE RATS

[1]Kate Loveland, [2]Michael Skinner, [3]Sarah Meachem

[1]Monash Institute of Reproduction & Development and [1]The Australian Research Council Centre of Excellence in Biotechnology & Development, Melbourne; [2]Centre for Reproductive Sciences, Univ of Washington, Pullman USA; [3]Prince Henry's Institute for Medical Research, Melbourne, Victoria, Australia

Follicle stimulating hormone (FSH) affects testicular development and function, acting through receptors on Sertoli cells. We are interested in events that occur during the first wave of spermatogenesis, as during this period, adult sperm output is fixed by factors, including FSH, that influence the total number of Sertoli cells available to support developing germ cells. Acute FSH withdrawal was achieved by immunoneutralisation of FSH in rats for 4 days, and testes were

collected from animals at 18 days postpartum (dpp). We previously observed that Sertoli cell proliferation and apoptosis were unaffected by this treatment, as was germ cell proliferation. However, a significant increase in apoptotic germ cell numbers was revealed by TUNEL staining. To identify genes regulated by FSH at this developmental interval, we isolated RNA from these samples and performed microarray analysis on Affymetrix rat genomic U34A chips. Two individual animals were examined for both control and FSH neutralised samples. Genes with at least a 1.5-fold level of expression difference between groups (using GeneSifter analysis program) were considered as significant candidates. Our study identified genes found in a similar analysis of cultured Sertoli cells ± FSH from 20 dpp rats (McClean 2003 Mol Endo). The steroid acute-regulatory (STAR) protein and endothelin genes were down- and up-regulated, respectively, in the absence of FSH. We will next examine other time points following *in vivo* FSH withdrawal and during other stages of testis development when the cellular response to FSH is known to differ.

122

FSH REGULATES SERTOLI CELL AND SPERMATOGONIAL POPULATIONS IN THE ADULT DJUNGARIAN HAMSTER

Sarah J. Meachem [1,2] **and Stefan Schlatt** [2]

[1] ***Prince Henry's Institute of Medical Research, Clayton, Victoria, Australia and*** [2] ***Institute of Reproductive Medicine, University of Münster, Germany.***

The hormones that regulate spermatogonial (Sg) development are ill defined. In part due to lack of appropriate Sg enriched experimental models. The photo-inhibited hamster model provides a rich source of Sg, thus making it an ideal model to study their control. This study aimed to assess the effects of FSH and testosterone on the re-initiation of Sertoli cell and Sg development in the gonadotrophin deplete Djungarian hamster, as induced by photo-inhibition. Long day (LD) photoperiod (16L:8D) adult hamsters were exposed to a short day (SD) photoperiod (8L:16D) for 11 weeks to suppress gonadotrophins, resulting in a Sg only testis. Animals then received FSH alone or in combination with either testosterone or the anti-androgen, flutamide for 7 days. Another group received testosterone alone. Bouin's fixed testes embedded in resin were used for the determination of Sertoli and early germ cell number using the optical disector stereological technique. The number of Sertoli cells, type A Sg, type B Sg/preleptotene spermatocytes (S'cytes) and leptotene/zygotene S'cytes were suppressed in SD controls, to 66%, 34%, 19% and 10% (all $P<0.01$) of LD control values, respectively. Later germ cell types were not observed. FSH treatment, in the absence/presence of testosterone increased Sertoli cell number ($P<0.01$) to normal LD values. Similarly, FSH treatment in the absence/presence of testosterone increased type A Sg, type B Sg/preleptotene S'cytes and leptotene/zygotene S'cytes to ~85%, 69% and 80% (all $P<0.001$) of LD controls, respectively. Testosterone alone did not effect Sertoli and germ cell numbers and remained at SD controls values. This data demonstrates that the re-initiation of Sg is dependent on FSH, with testosterone playing no role. Surprisingly the adult Sertoli cells population in this model is hormonally dependent. This naturally occurring model now provides an extraordinary opportunity to understand the mechanism (apoptotic and or proliferative) by which FSH regulates Sertoli and germ cell development.

123

PHYTOESTROGEN EXPOSURE REDUCES FERTILITY OF MALE RATS

Amy Glover, Helen Nicholson, and Stephen Assinder
Department of Anatomy and Structural Biology, University of Otago, New Zealand

Phytoestrogens are plant-derived compounds able to bind to and activate oestrogen receptors α (ERα) and β (ERβ). Exposure to phytoestrogens, in particular soy, through diet is very common. ERα and ERβ are present throughout the male reproductive tract, however the exact role of oestrogen in male reproductive biology is unclear. Male and female Wistar rats used for this study were offspring of female rats maintained on a low soy diet (containing 112μg/g isoflavanoid) prior to conception through to weaning. After weaning, the juvenile rats were fed the same low soy diet into adulthood. Six adult male rats were transferred to a high soy diet (containing 465μg/g isoflavanoid) (n=6); the remaining male rats were continued on the low soy control diet (n=8). On days 3, 6, 12 and 25 following the commencement of the high soy diet, the male rats were housed overnight with pro-estrus female rats (1:1). The female rats were housed separately until parturition. The size and sex ratio of the litters were recorded. After the final mating, the male rats were killed and the epididymides were removed. Sperm counts were performed on the initial segment, caput, corpus and cauda of one epididymis from each rat. Sperm counts showed fewer sperm in the initial segment ($P<0.05$), corpus ($P<0.05$) and cauda ($P<0.01$) epididymides of high soy rats, as compared to the low soy rats. The litter sizes for the treatment groups showed an exposure-dependent response. The litter sizes of the day 3 ($P<0.01$) and day 6 ($P<0.05$) high soy groups was significantly lower than the low soy group, while litter sizes of the day 12 and 25 groups were not significantly different to low soy fed animals. The sex ratios of the litters from both groups were not significantly different. In conclusion, short-term exposure to high phytoestrogen levels reduces male fertility. The mechanisms involved in these changes are being investigated.

124

LEYDIG CELL RESPONSE TO HCG IN AGEING MEN

C.A. Allan[1,2], E.A. Forbes[1], H.G. Burger[1], R.I. McLachlan[1,2]
[1]Prince Henry's Institute of Medical Research, Monash Medical Centre, Clayton, [2]Dept of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia, 3168.

Serum total testosterone (TT) levels decline by ~1% per year from the third decade. The associated rise in serum LH suggests an underlying primary testicular defect but changes in the hypothalamo-pituitary axis have also been suggested. In this study we aimed to characterise further the effects of ageing on testosterone production in 108 non-obese men aged ≥55 years, and with symptoms suggestive of androgen deficiency, taking part in studies of testicular function and testosterone replacement. hCG (5000 IU IM), as an LH substitute, was administered to assess testicular reserve; TT was measured at baseline and on day 3. Ten reproductively healthy young men (23-35 years) acted as controls (to date 6 have completed testing). Compared to younger men, older men had a lower baseline TT (15.5±0.5nM vs. 21.0±1.1nM, mean±SEM; p=0.004) and higher LH levels (5.3±0.5IU/L vs. 3.0±0.4IU/L; p=0.004). Following hCG the older men achieved a TT of 28.0±0.9nM and the younger men 36.2±2.8nM (p=0.01). The

absolute increase in TT was of borderline significance (12.5±0.7nM vs. 15.2±0.5nM; p=0.059) but the percentage rise from baseline was not different. When older men with baseline TT<15nM (n=55; TT 11.8±0.3nM) were compared to those with baseline TT>15nM (n=53; TT 19.5±0.5nM) no difference was seen in the absolute rise in TT achieved (12.5±0.7nM vs. 12.7±1.3nM) while the percentage rise was greater in the lower TT group (112 vs. 67%, p<0.0001). Across all older men the % rise in TT was greater with lower baseline TT levels (p<0.0001). We conclude (1) Overall older men have baseline features of Leydig cell dysfunction (lower TT, higher LH) and a borderline diminished absolute secretory response to hCG compared to younger men, (2) Older men with lower baseline T levels did not have increased LH levels and showed a comparable absolute Leydig cell response to hCG leading to a greater percentage rise in TT. These data may indicate a relative paucity of LH secretion in these older men consistent with a degree of hypothalamo-pituitary dysfunction. Further studies of the hypothalamo-pituitary-testicular axis are in progress.

125

IDENTIFICATION AND CELLULAR LOCALISATION OF CYCLOOXYGENASE-1 AND -2 IN THE ADULT RAT TESTIS

Wendy Winnall, Moira O'Bryan, Ugur Ali, Julie Muir, Mark Hedger

Monash Institute of Reproduction and Development, Monash University.

Although the testis undergoes qualitatively normal inflammatory responses to infection and other stimuli, it is also considered to be an immune privileged site supporting prolonged graft survival. The unique immune environment of the testes appears to be due, in part, to suppression of the pro-inflammatory functions and up-regulation of anti-inflammatory functions of the testicular macrophages. In macrophages from other tissues, regulation of this phenotype has been shown to be a consequence of prolonged prostaglandin (PG) synthesis by the macrophages itself. Although PGs are present at significant levels in the testis even under normal conditions, little is known about their cellular origin or regulation. Synthesis of PGs involves one of two distinct forms of cyclooxygenase (COX): the constitutively expressed COX-1, and the inducible COX-2, which promotes inflammation. Expression of COX-1 and –2 was examined in cultures (3h, 37°C) of isolated rat testicular cells (macrophages, Sertoli cells, Leydig cells), seminiferous tubules, whole testis fragments and peritoneal macrophages (as control) with or without lipopolysaccharide (LPS; 10μg/ml), using real-time PCR and/or Western blot analysis. Both COX-1 and -2 were detected in all testicular cells and fragments. However, following stimulation with LPS, COX-2 was significantly up-regulated in testicular and peritoneal macrophages only. As expected, COX-1 showed no response to LPS in any cell type. These data describe, for the first time, the cellular distribution of both COX forms in the rat testis. Both COX forms are expressed in a wide range of testicular cell types, including the testicular somatic cells, macrophages and germ cells, but only the macrophages show an increase in the inflammatory COX form in response to LPS-stimulation. These data provide an explanation for the endogenous levels of PGs in the normal testis, and suggest that production of PGs by testicular cells other than the macrophage may influence the anti-inflammatory/immunosuppressive phenotype of the testicular macrophage.

126

PENILE DEVELOPMENT IN TAMMAR WALLABIES: A CONTINUING ENIGMA

Michael W. Leihy, Geoffrey Shaw, Marilyn B. Renfree, Jean D. Wilson
Department of Zoology, University of Melbourne, Victoria 3010

Androgens from the developing testes induce differentiation of the phallus in all mammals. In keeping with this concept, administration of androgen to (1) or transplantation of testes into (2) female tammar wallaby pouch young (PY) causes development of a male phallus. Phallic development in tammar males begins relatively late (3) and at a time (after day 60 of pouch life) when there is no sexual dimorphism in levels of plasma androgens (4). To address this dichotomy, we performed two studies. To determine if the late onset of phallic development is due to inactivation of testosterone (by conversion to androstenedione) at earlier stages, we compared the effects of methyltestosterone enanthate (MTE) (which cannot be oxidized to methylandrostenedione) and testosterone enanthate (TE) in female PY beginning at day 20. MTE did not accelerate phallic development whereas TE accelerated phallic growth, suggesting that the delay in male phallic development is due to limiting levels of endogenous androgens. Secondly, to address the fact that levels of androgens [testosterone (T), dihydrotestosterone (D), and androstanediol (A)] are not different in male and female PY between days 60 and 150, we considered the possibility that these hormones are released in a pulsatile fashion missed on routine sampling. Administration of a GnRH analogue to day 104 PY caused only a 55% increase in plasma T with no change in D or A, and we found no evidence of diurnal or pulsatile secretion (levels of T, D, and A averaged 58, 17, and 8 ng/dL) in day 90-115 male PY bled hourly around the clock. In summary, the mechanism by which androgens virilize the phallus is unclear. Possible explanations include the presence of some unidentified androgen in plasma (such as a conjugate), or action of unbound testosterone (circumventing the absence of a high affinity transport protein in this species) that is difficult to measure with available techniques.

1. Leihy *et al.* (2003) *Endocr* **143**: 2643-51.
2. Tyndale-Biscoe & Hinds (1989) *Reprod Fertil Dev* **1**:243-54.
3. Butler *et al.*, (1999) *Anat Embryol* **199**: 451-457.
4. Wilson *et al.* (1999) *Biol Reprod* **61**: 471-475.

127

INVESTIGATION OF ANDROGEN ACTION IN SKELETAL MUSCLE GROWTH AND FUNCTION

A. Axell[1], H.E. MacLean[1], D. Plant[2], M. Jimenez[3], D.J. Handelsman[3], G. Lynch[2], J.D. Zajac[1]

[1]Department of Medicine, Austin Health, Heidelberg VIC, [2]Department of Physiology, University of Melbourne, Parkville VIC, [3]ANZAC Research Institute, Sydney NSW.

The anabolic effects of androgens on skeletal muscle have long been recognised and exploited by athletes, however the definitive mechanism of androgen action in muscle growth and maintenance remains poorly understood. Our aim is to investigate the physiological effect of

androgens on skeletal muscle growth and function using an *in vivo* androgen withdrawal/replacement mouse model. Orchidectomised (Orx) male mice received 3 intraperitoneal 0.1mg testosterone (T) implants (Orx+T, n = 6) or vehicle (Orx, n=5) for 11 weeks. Serum testosterone levels were measured and the following muscles were excised and weighed: extensor digitorum longus (EDL), soleus (SOL), quadriceps, plantaris, gastrocnemius, tibialis anterior and levator ani (LA). LA muscle sections were assessed for fibre cross-sectional area. Following 11 weeks treatment Orx mice had negligible serum T levels (mean ± SE; 0.4nM ± 0.1) compared to Orx+T mice (31nM ± 7.1, $p<0.001$). Orx+T mice showed a statistically significant increase in muscle mass compared to controls for fast-twitch (EDL; Orx=9.1mg ± 0.3, Orx+T=11.4mg ± 0.5, $p<0.005$) and slow-twitch (SOL; Orx=7.8mg ± 0.3, Orx+T=10mg ± 0.5, $p<0.01$) muscle types, as well as all other muscle groups analysed. These mice also displayed a marked increase in LA fibre area (81% increase compared to Orx, $p<0.001$). We have observed significant muscle degeneration upon androgen withdrawal *in vivo* as reflected by a decrease in muscle mass and fibre size. Androgen replacement via testosterone implant prevented muscle atrophy by promoting hypertrophy of fibres. We are currently assessing the impact of androgen withdrawal/replacement on muscle function parameters such as maximum force, power output and fatigue to determine the relationship between androgen-induced muscle hypertrophy and strength.

128

TEMPORO-SPATIAL ALTERATIONS IN PROSTATE BRANCHING MORPHOGENESIS IN ESTROGEN DEFICIENT AROMATASE KNOCKOUT (ARKO) MICE

Ghanim Almahbobi[1], Shelley Hedwards[1], Vikrant Reddy[1], Stephen McPherson[1], Gabriel Fricout[2], Gail P. Risbridger[1]

[1]Monash Institute of Reproduction and Development, Monash University, Melbourne; [2]Centre de Morphologie Mathematique, Ecole des Mines de Paris, France.

The development and growth of the prostate gland requires branching morphogenesis, a process that continues during the neonatal period. Estrogen is involved in prostate development but its temporo-spatial effects during early development are not understood. Using new methods of confocal microscopy and volume rendering image analysis, the aim was to detect and quantify the temporo-spatial changes in prostate branching morphogenesis in neonatal estrogen deficient ArKO mice. In wild type animals, the lateral and medial ducts of anterior prostatic lobes reveal developmental asymmetry with significant increases in branching morphogenesis of the lateral duct, which is retained during day 0-3. In ventral lobes, similar asymmetrical growth is observed but increases in branching occur randomly in lateral or medial ducts. At day14, knockout ArKO mice exhibit prostatic hypertrophy and significant increases in the stromal, epithelial and lumenal volume. To determine whether estrogen deficiency has specific immediate effects on neonatal prostate branching morphogenesis, prostatic lobes were analysed at days1 and 3. At day1, anterior lobe from knockout mice exhibits significant increase in the duct volume only. At day3, all events of branching morphogenesis are significantly accelerated in both knockout and heterozygous mice. In the ventral lobe, the effect of estrogen deficiency results in significant increases in some branching parameters in knockout animals at day3 only. To determine the spatial distribution of these alterations within prostatic lobes, branching events were assessed in

individual ducts. In the anterior lobe, all parameters are significantly increased in both lateral and medial ducts in knockout animals with significantly pronounced increases in the medial ducts, resulting in less asymmetry. In the ventral lobe however, no significant alteration is found in either duct. In summary, estrogen deficiency in ArKO mice leads to significant neonatal age-related lobe and duct-specific alterations; being more detrimental in anterior than in ventral lobes and more pronounced in medial than in lateral ducts. We conclude that estrogen is a critical regulator of prostate gland development, exerting significant early effects on gland growth. Therefore, the detection and quantification of aberrant branching morphogenesis within the first 4 days of neonatal life predicts prostate pathology, that is known to occur in adulthood.

129

TREATMENT OF ACROMEGALY WITH ORAL OESTROGEN

A.G. Nugent, J. Gibney, G. Johannsson, K.C. Leung, K.K.Y. Ho

Pituitary Research Unit, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, NSW 2010, Australia.

Oral oestrogen reduces circulating IGF-I and attenuates the metabolic effects of GH (1,2). To assess its therapeutic potential in acromegaly, we undertook an open label two period study of oral oestrogen treatment in 13 women (age 48±3 y) with persistent disease after surgical treatment. Either ethinyl oestradiol (30-50µg/d) or conjugated oestrogens (0.625-1.25mg/d) was used with the dose titrated until IGF-I fell into normal range. Biochemical (GH, IGF1, GHBP, glucose), body composition (fat mass [FM], lean body mass [LBM], extracellular water [ECW], total body nitrogen [TBN]) and metabolic (fat oxidation [Fox] during a GTT) measurements were performed before, after 4 months of oestrogen and of control (no oestrogen) treatments. Four were studied in the reverse sequence. Body composition was measured by dual Xray absorptiometry, ECW by bromide dilution and TBN by neutron activation.

	No treatment	Oestrogen	p value
IGF-I nmol/L	46±3	22±3	0.0001
Fat mass	30.8±4.5	32.5±5.1	0.055
LBM kg	45.9±2.3	44.1±2.2	0.005
ECW L	21.1±2.5	19.1±2.5	0.02
Fox GTT(mg/min)	34.4±2.2	31.5±3.4	0.07

IGF-I (mean±SE) levels fell uniformly into the normal range in all subjects. Oestrogen treatment increased mean GHBP ($p<0.001$) but had no effect on GH levels. Body weight did not change. FM was higher while LBM, TBN and ECW were lower during the oestrogen phase although the difference in TBN did not achieving statistical significance. Glucose tolerance profiles were not different between the two periods. Fox in response to oral glucose was suppressed to a greater degree during the oestrogen phase. Changes in IGF-I, GHBP, LBM and ECW were highly significant ($P<0.05$) between treatments while TBN and Fox showed strong trends. No major side effects were encountered. In the doses used, oral oestrogen normalised IGF without affecting GH levels and induced significant body composition and metabolic changes consistent with reduced GH action. Oral oestrogen is potentially an economic, effective and safe form of adjunctive treatment for acromegaly in women.

Supported by the NHMRC of Australia

(1) O'Sullivan et al, JCI 1998;102:1035-1040

(2) Wolthers et al, Am J Physiol 2001;281:E1191-1196

SAFETY AND EFFICACY OF CONVERTING FROM LONG-ACTING OCTREOTIDE (LAR) TO PEGVISOMANT IN PATIENTS WITH ACROMEGALY

Peter J. Trainer[1], Ariel Barkan[2], Mary Lee Vance[3], Pia Burman[4], Philip Harris[4], Keith E. Friend[4]

[1]Christie Hospital, Manchester, UK; [2]University of Michigan, Ann Arbor, MI, USA; [3]University of Virginia, Charlottesville, VA, USA, [4]Pfizer Corporation, New York, NY, USA.

We report the efficacy, safety, and consequence on glucose homeostasis of converting acromegaly patients from LAR (treated for ≥3 months) to pegvisomant, a GH receptor antagonist. 52 patients (mean age 49, range 23-81, 13 diabetic) who had previously participated in a pegvisomant clinical trial and then received LAR were enrolled in a 32-week, open-label, multicenter trial. Pegvisomant 10 mg/d was started 4 weeks after the last dose of LAR (week 0) and the dose was adjusted based on serum IGF-I levels (Nichols acid extraction, obtained monthly) at weeks 12, 20 and 28. Assessments included a signs & symptom score (SSS), ring size, fasting plasma glucose (FPG), HbA1c, insulin, and glucose tolerance (assessed at weeks 4 and 32). During treatment with pegvisomant (mean dose 16 mg/day), IGF-I was normalized in 85% of patients compared to 46% normalized on LAR at week 0. Normalization of IGF-I was paralleled by improvement in the SSS. Median FPG decreased significantly with pegvisomant in diabetics (week 4 7.4 v 4.9 mmol/l week 32, P=0.0015) and non-diabetics (5.6 to 4.6 mmol/l, P<0.0001) as did HbA1c (7.1% v 6.3%, P=0.0068 in diabetics, and 5.8% v 5.4%, P<0.0001 in non-diabetics). In non-diabetic patients, the oGTT glucose AUC was significantly lower at week 32 v 4 (1284.4 v 1510.8 mmol x h/L, P=0.0001). In 15 patients with normal IGF-I on LAR, FPG (-1.4 mmol/L; P=0.006) and HbA1c (-0.2%; P=0.09) improved with pegvisomant therapy in the absence of any additional reduction in IGF-I. The pharmacological effect during the 16-week overlap of LAR and pegvisomant was minimal; 8% of patients had an IGF-I that dropped below the normal range. No other adverse effects due to the overlapping effects were apparent. In summary, converting patients from LAR to pegvisomant with no washout period was safe and pegvisomant was effective in normalizing IGF-I. Pegvisomant improved glucose homeostasis in both diabetic and non-diabetic patients, which may be an important consideration for appropriate therapy for acromegaly.

131

ETHNICITY AND THE IGF SYSTEM IN ELITE ATHLETES: IMPLICATIONS FOR DETECTING GROWTH HORMONE DOPING IN SPORTS

Chris Howe[1], Kevin Hardman[2], Sridevi Meka[2], Anne Nelson[4], Tuan Nguyen[3], Kin Leung[4], Graham Trout[1], Robert Baxter[2], David Handelsman[3], Minoru Irie[5], Ray Kazlauskas[1], Ken Ho[4]

[1]ASDTL, Pymble; [2]Kolling Institute, St Leonards; [3]ANZAC Research Institute, Concord; [4]Garvan Institute, Sydney; and the [5]Japan Institute of Sports Science, Japan.

IGF-I and its binding partners IGF binding protein-3 (IGFBP-3) and acid-labile subunit (ALS) are potential markers of GH doping as they are GH-regulated. How demographic factors influence these components of the IGF-I system has not been systematically studied. This study examined the relationship of IGF system components to ethnicity, age, gender and body mass index (BMI) in a multi-ethnic population of elite athletes. Serum IGF-I, IGFBP-3, and ALS concentrations were measured by radioimmunoassay in 1087 elite athletes, aged 22±5 yr, from 14 countries comprising 573 Caucasians, 349 East Asians, 109 Africans, and 56 of other ethnicity. Blood samples were obtained on at least three successive occasions, separated by an average of 5 days. And measured in duplicate in the same assay. A random-effects analysis of variance was used to estimate the effects ethnicity adjusted for age, BMI and sex. IGF-I, IGFBP-3, and ALS levels decreased with age, with each positively associated with BMI. Age- and BMI-adjusted IGF-I, IGFBP-3, and ALS levels in females were 3.5%, 9.0%, and 16%, respectively, higher than in males ($p<0.01$). The proportion of variance accounted for by the three factors (age, sex and BMI) ranged from 8% (IGFBP-3) to 21% (IGF-I and ALS). After adjusting for age, BMI, and sex, IGF-I levels in Caucasians were 20% lower than in East Asians ($p=0.03$), but 20% higher than in Africans ($p=0.02$). Adjusted IGFBP-3 levels in Caucasians was also lower than in East Asians but 18% higher than in Africans ($p=0.008$). There were no significant differences in ALS between ethnic groups. These data show, for the first time, that ethnicity significantly influences serum IGF-I and IGFBP-3 levels, independent of age, sex, and BMI. These results underscore the importance of ethnicity as an important factor in defining reference ranges for GH doping in sports, and in the clinical utilisation of these markers for diagnostic purpose.

Supported by the World Anti Doping Agency.

132

PETROSAL SINUS SAMPLING IN ACTH-DEPENDENT CUSHING'S SYNDROME

Sue Mei Lau, Mark McLean

for the Pituitary Clinical Research Group at Westmead, Dept of Diabetes and Endocrinology, Westmead Hospital, Westmead NSW 2145.

We retrospectively analysed our experience with 25 inferior petrosal sinus sampling (IPSS) procedures performed in 23 patients (1990-2002). 14 procedures (since 1996) included CRH stimulation, earlier studies were unstimulated (9) or used naloxone (2). There were 2 failed cannulations (jugular samples collected) and 3 complications (transient loss of taste, DVT, and

femoral artery puncture). 18 patients had verified Cushing's disease (CD), 2 had confirmed and 1 had probable ectopic ACTH secretion, 2 proved not to have ACTH dependent Cushing's syndrome and were excluded from further analysis. Of the 18 patients with Cushing's disease, 5/18 had a positive MRI, 6/6 had a positive CRH stimulation test and 13/14 suppressed on the high dose dexamethasone suppression test (HDDST). 16 showed a significant central:peripheral gradient ($\geq$2:1 at baseline or $\geq$3:1 with stimulation), and 13 of these also had a lateralising gradient ($\geq$2:1 gradient). None of these lateralising results were contradicted by surgical findings. 2 patients had false negative IPSS (1 naloxone, 1 unstimulated). 1 false positive IPSS result occurred in a patient with empty sella at operation; no source of ACTH secretion was identified. In CRH stimulated studies all but 1 had a central:peripheral gradient evident on the pre-stimulation samples, but CRH stimulation produced lateralising gradients not evident at baseline in 4 cases.

IPSS had a sensitivity of 89%, specificity of 67%, positive predictive value (PPV) of 94% and negative predictive value (NPV) of 50%. Of the 19 HDDST performed, 16 correctly suggested CD. There was 1 false positive and 1 false negative. This test had a sensitivity of 97%, specificity of 100%, PPV of 100% and NPV of 67%. 22 MRIs were performed and only 6 revealed a pituitary lesion. In conclusion, the accuracy of IPSS improved with operator experience, with a 100% sensitivity and specificity in the 14 procedures performed in the later 5 years. Lateralisation appeared to be accurate, with CRH improving lateralisation rates. HDDST and CRH testing also had high accuracy in differentiating pituitary from ectopic ACTH production, but MRI scanning has poor discrimination.

133

A CASE OF NON *MEN1* GENE MULTIPLE ENDOCRINE NEOPLASIA

Karen McNeil[1], John Cardinal[3], George Jerums[1], Albert Frauman[2]

[1]Endocrinology Unit and [2]Clinical Pharmacology, Austin Health Vic, [3]Diabetes and Endocrinology ,Princess Alexandra Hospital Brisbane.

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterized by tumours predominantly affecting the parathyroid gland, pancreatic islet cells and pituitary. The *MEN1* gene is localised to a region on the long arm of chromosome 11 (11q13) and encodes a 610 amino acid nuclear protein called menin. Loss of menin function leads to loss of tumour supression. *MEN1* gene mutations have been documented in 47/63 (75%) of unrelated MEN1 kindreds (1). We report a new kindred of Afghanis that has hypercalcaemia and pancreatic lesions without the MEN 1 gene mutation. A 39 year old Afghani woman with bone pain and a right ureteric calculus was found to have an elevated serum corrected calcium of 2.98mmol/L (2.1-2.6), lowered phosphate of 0.75mmol/L (0.80-1.4) and elevated parathyroid hormone at 39.5pmol/L (0.8-8.0). Her mother aged 68 years at the time was also known to have primary hyperparathyroidism. Screening for MEN1 revealed a raised gastrin, and MRI imaging revealed a 1.5cm lesion contiguous with the tail of the pancreas and the duodeno-jejunal flexure. Pituitary testing revealed a normal prolactin level with a 3mm hypointense region suspicious for pituitary adenoma. Subtotal parathyroidectomy, thymectomy, distal pancreatectomy and duodenotomy with excision of lesions presumed to be gastrinomas were performed. Subsequent histopathology showed these lesions to have microscopic characteristics of islet cell tumours. Immunohistochemical staining on the largest of these tumours was positive for glucagon. *MEN1* mutation detection was performed by DNA sequencing for all coding regions and splice sites and

no mutations were detected. This analysis does not exclude the possibility of causative sequence variations in the promoter and the untranslated regions or the presence of a very large deletion. To date no sequence variations have been reported in the promoter region. These findings indicate this woman to have a MEN1 syndrome that is not the result of a *MEN1* coding sequence mutation and further gene linkage analysis will be performed in order to identify the underlying genetic defect.

(1) Bassett, et al., Am J Hum Genet 1998; 62:232.

134

BRONCHOPULMONARY CARCINOID IN MEN 1

N. Sachithanandan, J. Burgess, * R. Hale

Departments of Endocrinology and *Radiology, Royal Hobart Hospital.

Multiple endocrine neoplasia 1 (MEN 1) is an autosomal dominant syndrome associated with tumours of pituitary, pancreas, parathyroid and foregut lineage neuroendocrine tissue.

Whilst enteropancreatic foregut carcinoid is well described in MEN1 (>30%), bronchopulmonary carcinoid has been thought rare, although the prevalence has not been systematically evaluated. The aim of this study is to determine the prevalence of bronchopulmonary carcinoids in patients with MEN 1 who underwent routine MEN 1 screening, including chest CT imaging.

The records of 31 patients (21 female, 10 male; age 18-80) with MEN 1 who had CT imaging of the chest were evaluated and the results of chest CT imaging were reviewed by a radiologist.

Variable		Nodules n=11	No Nodules n=20	P value
Female		9 (81.8%)	12 (60%)	0.214
Age (mean; SEM)		35-80 (53.3; 4.8)	18-77 (41.4; 3.7)	0.063
HPT		11 (100%)	18 (90%)	0.279
ZES		6 (54.5%)	2 (10%)	0.007
Nodules	Pancreas	10 (90.9)	12 (60%)	0.070
	Pituitary	6 (54.5%)	5 (25%)	0.100
	Adrenal	4 (36.4%)	7 (35%)	0.940
Thymic carcinoid		2 (18.2)	0	0.049
Gastric carcinoid		2 (18.2%)	1 (5%)	0.235

ZES = Zollinger Elison syndrome, HPT = hyper parathyroidism

Overall 35% of subjects had pulmonary nodules on CT scan. Four (36%) patients with large lesions underwent surgical resection and carcinoid was confirmed on histopathology. Of these patients 75% were women and 25% were men, all had pancreatic nodules, 50% had ZES and 75% had pituitary tumours. One patient exhibited lymph node metastases but no deaths or distant metastasis have occurred despite long term follow up of all patients.

Conclusion Bronchopulmonary carcinoid is frequently identified in MEN1, particularly in those patients with neuroendocrine neoplasms at other foregut sites.

135

CO-REGISTRATION OF COMPUTERISED TOMOGRAPHY AND OCTREOTIDE SCINTIGRAPHY FOR LOCALISATION AND STAGING OF PANCREATIC ENDOCRINE TUMOURS

T. Quach, G. Lewis, J. Bear, R. Smith, M. Epstein

Endocrinology, Radiology and Nuclear medicine department of the John Hunter Hospital.

Aims: After biochemical diagnosis of pancreatic, gut or carcinoid endocrine tumours, localisation and staging can be difficult as the primary tumours are often small and their metastases are often widespread. CT scan is not sensitive in detecting small lesions. Radioactive labeled octreotide is taken up avidly by most pancreatic endocrine tumours or metastases, but the scan often provides poor anatomical localisation of the lesion. We combined the two types of scans to improve anatomical localisation.

Method: Two patients with sporadic gastrinomas, five patients with Multiple Endocrine Neoplasia type 1, two with gastrinomas, three with non-functioning tumours (high chromogranin and/ or pancreatic polypeptide) were studied. We co-registered the two types of scans by using propriety software from the nuclear medicine department. We compare the findings of CT scan, octreotide scan and the combined studies.

Results: Thirteen lesions were identified by octreotide scanning. CT alone identified only four of these lesions. Co-registration permitted anatomical localisation of all thirteen lesions.

Conclusion: Octreotide scan was poor in anatomical localisation of the tumours. CT scan of the pancreas alone was extremely poor in the identifying pancreatic endocrine tumours. However, after co-registration with octreotide scan, CT scan was able to anatomically identify both pancreatic and non-pancreatic lesions. Accurate anatomical localisation greatly facilitated management especially surgical approaches.

136

INCREASED DOSAGE OF THE TRANSCRIPTION FACTOR *SOX3* IS ASSOCIATED WITH X-LINKED HYPOPITUITARISM (XH)

Nicola Solomon[1,2], Shelley Ross[1], Thomas Morgan[3], Garry Warne[4], Susan Forrest[2,5], Paul Thomas[1]

[1]Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, VIC, 3052, Australia; [2]Department of Paediatrics, University of Melbourne, Melbourne, VIC, 3052, Australia; [3]Yale University School of Medicine, Department of Genetics, 333 Cedar Street, New Haven, CT 06520 USA; [4]Department of Endocrinology, Royal Children's Hospital, Melbourne, VIC, 3052, Australia; [5]Australian Genome Research Facility, Flemington Road, Parkville, Melbourne, VIC, 3052, Australia.

The severe pituitary disorder X-linked hypopituitarism (XH) affects males and is characterised by anterior pituitary hormone deficiencies and mild mental retardation. Hypopituitarism in XH males is congenital and ranges from isolated GH deficiency to panhypopituitarism, and may result in neonatal death if untreated. Using cytogenetic and molecular analysis of affected males

from a three generation XH family, we have identified a 9Mb interstitial duplication at Xq26-q27 that cosegregates with the disorder (1). To facilitate the detection of duplications in additional XH families, we have developed an array-Comparative Genomic Hybridisation (CGH) technique using selected BAC clones from Xq26-q27. Using this approach, we have identified different Xq26-q27 duplications in three additional XH families. Together, these data define a 4Mb XH critical region at Xq27.2-q27.3 and, as each of the duplications is different, strongly support increased gene dosage as the mechanism for this disorder. To identify candidate genes, we performed extensive expression analysis of all annotated genes within the critical region. We showed that the transcription factor *SOX3* is specifically expressed in the developing pituitary gland and CNS, indicating that it may be causative for this disorder. This was recently confirmed by the identification of a poly-alanine expansion mutation in *SOX3* in an additional XH family (2). Currently we are generating a transgenic mouse model for this disorder by adding extra copies of *SOX3* to the genome. Detailed characterisation of the pituitary and CNS abnormalities in these mice will provide a greater understanding of the molecular pathology of XH and may ultimately lead to the development of more effective therapies with which to treat this disorder.

(1) Solomon, N. M., Nouri, S., Warne, G.L., Lagerstrom-Fermer, M., Forrest, S.M. and Thomas, P.Q. Increased gene dosage at Xq26-q27 is associated with X-linked hypopituitarism (XH). *Genomics* **79**, 1-7

(2) Frédéric Laumonnier, Nathalie Ronce, Ben C.J. Hamel, Thomas, P.Q., James Lespinasse, Martine Raynaud, Christine Paringaux, Helger Yntema, Vera Kalscheuer, Jean-Pierre Fryns, Jamel Chelly, Claude Moraine and Sylvain Briault. Transcription factor *SOX3* is involved in X-linked mental retardation with growth hormone deficiency. *American Journal of Human Genetics* **71**, 1450-5

138

DIFFERENT LEPTIN RESPONSES TO FASTING IN MERINO WETHERS

Kate G. Kauter, Tim O'Shea, James R. McFarlane

Physiology, University of New England, Armidale, NSW.

Leptin is produced primarily by adipose tissue regulating food intake and energy expenditure (1,2) together with a role in immune system (3). Nutritional status of humans and rodents is reflected in changes in circulating leptin concentrations, with leptin decreasing with food deprivation (4,5). This study describes changes in circulating leptin in Merino wethers during a 72 hour fast and after reintroduction of food. A group of mature Merino wethers (n=10, body weight 52.6±1.2 kg) were housed in a group pen with free access to both water and chaff prior to the fasting period when the chaff was withdrawn. Blood samples were obtained via jugular venipuncture prior to fasting (baseline) and then every 24 hours of the fast and 6 hours after reintroduction of chaff. Plasma leptin and glucose were determined using an ovine leptin ELISA (6) and a Dade Clinical Analyser, respectively.

As a group, plasma leptin decreased from 4.4±0.6 ng/ml (baseline, 100%) to 64.8±7.8% of baseline after 48 hours of fasting ($P<0.05$), reaching 108.3±13.3% six hours after reintroduction of chaff. Plasma glucose decreased from 4.3±0.1 mmol/L (baseline) to 2.7±0.1 mmol/l at 72

hours of fasting (P<0.05). The data were then arbitrarily divided into animals where plasma leptin decreased to less than (responders, R, n=6) or greater than (nonresponders, NR, n=4) 75% of baseline after 48 fasting. In the R group plasma leptin was 53.9±6.0% of baseline at 48 hours of fasting (P<0.05), however at 72 hours was no different to baseline. In the NR group, plasma leptin was no different to baseline during the fast. Plasma glucose was similar in the 2 groups during the fast. At 6 hours after the reintroduction of chaff, plasma leptin levels were elevated in the NR group compared to the R group (P<0.05).

The apparent differential leptin response to a fast may impact on the coping mechanisms of animals during times of both nutritional stress and during challenges to the immune system, possibly making the NR group more resilient in these circumstances.

(1) Zhang *et al* 1994. *Nature* 372 425-432.
(2) Campfield *et al* 1995. *Science* 269 546-549.
(3) Borstein *et al* 1998. *J C E & M* 83 280-283.
(4) Sinha *et al* 1996. *J Clin Invest* 98 1277-1282.
(5) Mizuno *et al* 1999. *Endocrinology* 140 4551-4557.
(6) Kauter KG *et al.* 2000. *J Endo* 166 127-135.

139

LEPTIN RECEPTOR (OB-RB) AND GROWTH HORMONE RELEASING PEPTIDE GHRP/GHRELIN RECEPTOR (GHRP-R) GENE EXPRESSION IN THE ARCUATE NUCLEUS WITH LONG-TERM MANIPULATION OF ADIPOSITY

A. Rao, Y. Kurose, Y. Murata, J. Iqbal, I.J. Clarke

Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton VIC 3168.

Alterations in body weight change plasma levels of leptin and ghrelin, but signalling may be affected by differences in expression of the leptin receptor (Ob-Rb) and growth hormone releasing peptide/ghrelin receptor (GHRP-R). Our aim was to examine whether alterations in body weight affect the level of gene expression for Ob-Rb and GHRP-R in the arcuate nucleus (ARC) of the sheep. Ovariectomised Corriedale ewes were divided into three groups with mean (±SEM) body weights of 33±1.1kg (Lean, n=4), 50±1.6kg (Normal, n=5), or 75±1.3kg (Fat, n=5). Compared to control, plasma GH levels were lower (P<0.01) in Fat animals (4.5±3.8 ng/ml) and higher in Lean animals (50±16 ng/ml). Plasma insulin and leptin levels were consistent with the metabolic status of the animals. The sheep were killed and brains were collected and snap frozen. Hypothalamic sections (100μm) were cut and the ARC was microdissected and extracted to obtain RNA. GHRP-R and Ob-Rb mRNA expression was analysed using real-time PCR with cyclophilin as a control. The level of expression of transcripts for both GHRP-R and Ob-Rb was higher in Lean animals than in Fat animals, with no difference between Fat and Normal animals. We conclude that the expression of GHRP-R and Ob-Rb is increased by lowered adiposity but is not affected by increased adiposity. Upregulation of Ob-Rb and GHRP-R in lean animals could be an adaptive mechanism to allow increased sensitivity of neuroendocrine systems to leptin and the stimulation of GH secretion respectively.

TABLE 1. GHRP-R and Ob-Rb mRNA levels in Lean, Normal and Fat ewes

	Lean	Normal	Fat
GHRP-R mRNA (fg/fg cyclophilin)	0.246±0.044 [a]	0.0796±0.02 [b]	0.091±0.02 [b]
Ob-Rb mRNA (fg/fg cyclophilin)	0.10±0.025 [c]	0.0638±0.012	0.0426±0.01 [d]

(a vs b: P<0.001; c vs d: P<0.05).

140

NEURO-PROTECTIVE ACTION OF LEPTIN IN NEURONAL CELLS

S. Metaxas, M. Harris, K. Kobayashi, G.A. Werther, V.C. Russo

Centre for Hormone Research, Murdoch Childrens Research Institute, Dept. of Paediatrics University of Melbourne, Royal Children's Hospital, Parkville 3052, Victoria.

The processes involved in early brain development and postnatal maturation of the nervous system are largely unknown. However a number of growth factors and hormones are known to be involved in the modulation of neurotrophic, neurogenetic and neuroprotective events that take place within the brain. Much interest has been recently devoted to the role of leptin in the brain. Leptin, mainly known for its key roles in nutrition and energy balance, is found expressed in neuronally rich and plastic regions such as the hypothalamus, hippocampus and cerebellum in early life, and therefore, in addition to its known metabolic activity, it is likely to be also involved in neuro-development processes. Leptin is a 16kDa protein encoded by the obese gene (*ob*), which regulates metabolic, neuroendocrine, reproductive, haematopoietic and immune functions. Leptin action is mediated by Ob-Rb receptor which mainly signals via the JAK/STAT pathway but also utilises other common substrates such as p38 MAPK, IRS-1 and PI3K. Whether leptin exerts exclusively trophic functions or might also have neurogenic and neuroprotective activity it is not known, and therefore we aimed to investigate these functions in neuronal cells. Human neuroblastoma cells *SK-N-SH*-SY5Y (Ob-Rb +/+) were cultured for up to 72h in serum free (SF) conditions +/- leptin (100nM), and cell viability determined. IGF-I, a potent growth and survival agent in these cells was used as a control. Leptin (0-72 hours) prevented SF-induced cell loss by increasing cell number to 2-fold over that seen in SF cultured cells ($p<0.01$). By comparison, IGF-I at3nM) increased cell number 4 fold, ($p<0,001$). Apoptotic DNA fragmentation, as detected in SF cultured cells, was reduced by 30% in the presence of leptin and by 70% by IGF-I. Preliminary data with the chemical inhibitors AG-480 or SB203580 suggest that neither JAK/STAT nor p38MAPK activation are required for the leptin anti-apoptotic response. Gene-array for the apoptotic pathways, showed that leptin strongly reduced the expression of two key pro-apoptotic genes; Caspase-10 and TRAIL (>2-4 fold, $p<0.001$). In conclusion, our findings demonstrate that *in vitro* leptin possesses neurotrophic/neuroprotective activities, which are achieved by modulation of cell growth and suppression of pro-apoptotic genes. It is thus likely that brain leptin, beside its metabolic functions, might also be involved in the modulation of neurotrophic/neuroprotective events.

141

DIMERIZATION OF SOX9 IS NOT NECESSARY FOR SEX DETERMINATION

Paisu Tang[1], Pascal Bernard[2], Eric Vilain[2], Vincent R. Harley

[1]Human Molecular Genetics laboratory, Prince Henry's Institute of Medical Research, Monash Medical Centre, 246 Clayton Road, Melbourne Victoria 3168 Australia, [2]UCLA Departments of Human Genetics, Pediatrics and Urology, Gonda Center, Room 6357, 695 Charles Young Drive South, Los Angeles, CA 90095-7088.

The *SRY*-related *SOX9* gene is involved in both chondrogenesis and the early steps of mammalian sex determination. Mutations in the human *SOX9* gene cause campomelic dysplasia, a severe skeletal malformation syndrome associated with male-to-female sex reversal in most, but not all, XY individuals. Here we show that SOX9 contains a dimerisation domain, and binds co-operatively as a dimer in the presence of the DNA enhancer element in genes involved in chondrocyte differentiation, such as *Col11a2*, but binds as a monomer to the regulatory region of sex-determining genes such as *SF1* (1). Frameshift *SOX9* mutations truncate its two activation domains, while all missense mutations reported to date lie in the high mobility group (HMG) DNA-binding domain. We identify a missense mutation (A76E), the first outside the HMG domain, in an XY patient presenting with campomelic dysplasia but without sex reversal. This mutation disrupts the dimerization capability of SOX9, interfering with both the DNA binding and consequent transactivation of the *Col11a2* enhancer. Consistent with the patient's phenotype, the A76E mutation does not affect DNA binding and activation of the *SF1* enhancer. DNA-dependent co-operative dimerization could represent a novel mechanism to achieve tissue-specific regulation of gene expression by a SOX transcription factor. These results establish that SOX9 co-operative dimerization is required for chondrogenesis but not for sex determination and explain why campomelic dysplasia need not be associated with XY sex reversal. This mutant is an exception to the growing list of sex-reversing clinical mutants with biochemical defects of SRY/SOX9 (2).

(1) Tang, Bernard *et al.* (2003) *Human Molecular Genetics*, (in press, July 15 issue)
(2) Harley, *et al.* (2003) *Proc. Nat'l Acad. Sci*. (USA) in press

142

ALLOSTERIC ACTIVATION OF THE CALCIUM-SENSING RECEPTOR BY L-AMINO ACIDS IN HUMAN PARATHYROID CELLS

H-C. Mun, L. Delbridge, A.D. Conigrave

School of Molecular and Microbial Biosciences, University of Sydney, NSW 2006; Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown NSW 2050 and Department of Surgery, Royal North Shore Hospital, St Leonards, NSW 2006.

We demonstrated previously that the calcium-sensing receptor (CaR) is activated allosterically by L-amino acids (1,2). As a result, in the presence of physiological concentrations of L-amino acids, the Ca^{2+} sensitivity of the receptor is markedly elevated. These observations have the potential to explain how elevated dietary protein intake influences calcium metabolism e.g., by suppressing parathyroid hormone secretion and promoting urinary calcium excretion (3).

In the current work, we have demonstrated that individual L-amino acids and physiological L-amino acid mixtures allosterically activate the CaR in parathyroid cells derived from samples of normal human parathyroid glands obtained at the time of parathyroid engraftment in patients undergoing thyroid surgery.

Parathyroid cells were prepared from samples of normal human parathyroid tissue by collagenase digestion. For studies of receptor activation, an assay of receptor-dependent intracellular Ca^{2+} mobilization was used based on cellular loading with the Ca^{2+}-sensitve fluorophore, fura-2 AM. For studies of parathyroid secretion, normal cells were loaded into a column of Sephadex G25 beads and perifused with physiological saline solutions that contained various concentrations of L-amino acids and amino acid mixtures at a constant extracellular Ca^{2+} concentration of 1.25 mM. Active individual amino acids including L-Phe, L-Trp, L-His, L-Ala and L-Glu allosterically activated the CaR as revealed by enhanced Ca^{2+} sensitivity in fura-loaded cells. In addition, active L-amino acids suppressed PTH secretion from perifused cells.

The data support the hypothesis that the parathyroid CaR is a molecular target of L-amino acids and provides a molecular explanation for the recognised effects of protein intake on calcium metabolism in humans.

(1) Conigrave, A.D., Quinn, S.J. & Brown, E.M. L-amino acid sensing by the extracellular Ca^{2+}-sensing receptor. *Proc. Natl. Acad. Sci. USA* **97**, 4814-4819 (2000).
(2) Conigrave, A.D., Franks, A.H., Brown, E.M. & Quinn, S.J. L-Amino acid sensing by the calcium-sensing receptor: a general mechanism for coupling protein and calcium metabolism? *Eur. J. Clin. Nutr.* **56**, 1072-1080 (2002).
(3) Kerstetter, J.E. *et al.* Increased circulating concentrations of parathyroid hormone in healthy, young women consuming a protein-restricted diet. *Am. J. Clin. Nutr.* **66**, 1188-1196 (1997).

143

IDENTIFICATION AND FUNCTIONAL EFFECTS ON CELL SIGNALLING PATHWAYS OF A NOVEL MUTATION (V836I) IN THE CALCIUM SENSING RECEPTOR CAUSING AUTOSOMAL DOMINANT HYPOCALCAEMIA

Bryan K. Ward[1,2], Aaron L. Magno[1,2], Alison H. Franks[3], Bronwyn G.A. Stuckey[1], Arthur D. Conigrave[3], Thomas Ratajczak[1,2]

[1]Department Of Endocrinology And Diabetes, Sir Charles Gairdner Hospital and [2]Centre For Medical Research, University Of Western Australia, Nedlands, Western Australia 6009 And [3]School Of Molecular And Microbial Sciences, University Of Sydney, Sydney, New South Wales 2006.

The calcium sensing receptor (CaR) is a cell surface-expressed G-protein coupled receptor which, when stimulated by agonist, can activate a number of cell signalling pathways including the PI-phospholipase C (PI-PLC) and MAPK pathways (1). Activating mutations of the receptor cause the inherited disorder, autosomal dominant hypocalcaemia (ADH) (2). We identified a missense mutation (G to A) at nucleotide 2506 of the CaR resulting in an amino acid change (V836I) in the proband of a family with suspected ADH and found by restriction enzyme analysis that it cosegregates with hypocalcaemia in family members. In order to determine relative expression levels, wild-type and mutant receptors, cloned into a mammalian expression

vector as Flag-tagged constructs, were transfected into HEK293 cells and examined by Western blotting using an anti-Flag antibody. For functional characterisation, receptors were transfected into HEK293 cells and assayed for activation of the PI-PLC pathway using an inositol phosphate (IP), dose-response assay and for MAPK activation using a luciferase reporter based dose-response assay. Western blotting demonstrated equivalent expression of wild-type and mutant receptors. The IP dose-response curve was left-shifted for V836I compared with wild-type receptor (EC_{50} 1.5 and 2.5 mM Ca^{++}, respectively) and the V836I receptor showed nearly double the maximal response seen with wild-type receptor. By contrast, there was no change in the EC_{50} (both 3.9 mM Ca^{++}) in the MAPK assay, and the maximal response for the V836I receptor was reduced to 70% of that for wild-type. These results confirm the activating status of this mutation with respect to the PI-PLC pathway, known to be involved in the regulation of calcium homeostasis (1). The amino acid change is a conservative one, but occurs in a region where a number of other activating mutations have been detected. We speculate that it causes conformational changes that alter G-protein coupling, differentially affecting cell signalling pathways.

(1) Brown, EM and RJ MacLeod (2001). Physiological Reviews 81: 239-297
(2) Pollak et al (1994). Nature Genetics 8: 303-307

144

REGULATION OF LIVER RECEPTOR HOMOLOGUE-1 EXPRESSION BY PROSTAGLANDIN E_2 IN HUMAN PREADIPOCYTES

Agnes Kovacic, Evan R. Simpson, Colin D. Clyne

Prince Henry's Institute of Medical Research, Clayton, VIC 3168, and Department of Biochemistry and Molecular Biology, Monash University, Clayton Victoria, 3168.

Aromatase is the enzyme responsible for estrogen production and is the product of the *CYP19* gene. This gene is under the control of many tissue-specific promoters, each of which is regulated by different cohorts of factors. In normal breast adipose tissue, relatively low levels of aromatase are expressed *via* the action of the adipose specific promoter I.4. Breast tumor-derived factors such as prostaglandin E_2 (PGE_2) strongly stimulate aromatase expression via an alternative promoter, promoter II, leading to increased estrogenic drive and tumor growth. We have previously shown that (i) PGE_2 activates PKA and PKC pathways in human preadipocytes, both of which maximally stimulate aromatase promoter II activity, and (ii) activity of promoter II is enhanced by the orphan nuclear receptor Liver Receptor Homologue-1 (LRH-1). We therefore hypothesised that the action of PGE_2 is mediated, at least in part, through activation of LRH-1. To assess the effect of PGE_2 on LRH-1 transcriptional activity, 3T3-L1 preadipocytes were co-transfected with a GAL4-LRH-1 fusion construct and a GAL4 responsive luciferase reporter. Cells were treated with PGE_2 (1 μM) or the PKA and PKC activators forskolin (25 μM) and PMA (4 nM) for 8 h, and luciferase activity measured. However, luciferase activity was not altered by any of these treatments. To examine potential effects of PGE_2 on LRH-1 expression, human preadipocytes were isolated by collagenase digestion of human subcutaneous adipose tissue and maintained in primary culture until confluent. Cells were treated with PGE_2, forskolin or PMA and LRH-1 mRNA and protein levels quantified by real time PCR and Western blotting, respectively. PGE_2 induced a dose- and time-dependent increase in LRH-1 mRNA and protein levels with maximal effect (4-fold) at 1 μM PGE_2 for 48 hours. These effects of PGE_2 were

mimicked by treatment with PMA, but not forskolin. We conclude that PGE_2 increases LRH-1 expression in human preadipocytes through activation of PKC. Since promoter II is synergistically activated by LRH and cAMP, the ability of PGE_2 to increase both LRH-1 expression (via PKC) and cAMP signalling (via PKA) greatly enhances its estrogenic effect in breast adipose. This may in part explain the recently described chemo-preventative effects of cyclooxygenase inhibitors in breast cancer development.

145

PARATHYROID HORMONE IN THE JAPANESE PUFFERFISH, *FUGU RUBRIPES*

J.A. Danks[1], P.M.W. Ho[1], A.J. Notini[2], F. Katsis[1], P. Hoffmann[1], B.E. Kemp[1], T.J. Martin[1], J.D. Zajac[2]

[1]St Vincent's Institute of Medical Research, Fitzroy and [2]Department of Medicine, The University of Melbourne, Austin Health, VIC.

A major change in calcium control between lower and tetrapod vertebrates is the evolution of the parathyroid gland. Amphibians are the first animals to have a distinct parathyroid gland. Despite the absence of a parathyroid gland, fish respond to changes in ambient calcium levels and regulate plasma calcium accordingly. Since the amino acid sequence of mammalian PTH was determined in 1970 efforts have been made to find homologs of PTH in lower vertebrates. There have been a number of reports on the presence of immunoreactive PTH-like proteins in fish detected by antisera to mammalian PTH but a fish PTH homolog has not been identified. In the course of studies of the molecular evolution of the PTH family we have isolated and sequenced the DNA which encodes a fish homolog of PTH from the pufferfish, *Fugu rubripes*. This sequence encodes a peptide which is eighty amino acids long. The N-terminal 34 residues of fugu PTH (fPTH) share 15 residues with other known PTH sequences and is most closely related to chicken PTH (1-88) (cPTH). In the amino acid sequence of fPTH after the first 34 amino acids there is no significant homology to either human PTH (hPTH) or cPTH, indicating weak evolutionary pressure to conserve the C-terminus of the PTH molecule. The potency of fPTH (1-34) in promoting cyclic adenosine monophosphate (cAMP) formation in UMR106.01 cells (ID50= 17 $\pm$ 3.6nM, n=4) is consistency less than hPTH (1-34), human parathyroid hormone-related protein (1-34) (hPTHrP) and fugu parathyroid hormone-related protein (1-34) (fPTHrP) (ID50= 1.4 $\pm$ 0.48nM, n=4). However the maximum amplitude of response was significantly greater than that achieved with the highest concentrations of hPTH or human or fugu PTHrP. The cAMP studies indicate that fPTH (1-34) acts throught the PTH/PTHrP receptor, (PTH1R). fPTH (1-34) is not recognised immunologically by a number of antisera raised to the N-terminus of hPTHrP or hPTH. Therefore fPTH is a member of the PTH family with only N-terminal homology to other family members, and with the least homology to any PTH so far sequenced.

PRESENCE OF GROWTH HORMONE SECRETAGOGUES RECEPTOR (GHS-R) AND GHRELIN IN ENDOMETRIAL ADENOCARCINOMA

N. Tawadros[1,3], L.A. Salamonsen[1,2], T. Jobling[2], C. Chen[1,3]

1. Prince Henrys Institute of Medical Research, 2. Department of Obstetrics and Gynaecology, Monash Medical Centre and 3. Department of Physiology Monash University, Clayton, Victoria.

The GHS-R and its ligand, ghrelin, are widely expressed in peripheral tissues outside the hypothalamus-pituitary axis and in neoplastic tissues including neuroendocrine, thyroid, intestinal and prostate tumour or cancer cells. Previous studies in our laboratory demonstrated the mRNA and protein expression of both ligand and receptor in the normal human endometrium and in endometrial adenocarcinoma across the pathological grades. Endometrial cancer is the most common gynaecological malignancy of the female reproductive tract. The aim of this study was to investigate the cellular localisation of the GHS-R and ghrelin in several endometrial cancer cell lines and to examine whether ghrelin has a proliferative effect on these cancer cells. Endometrial cancer cell lines were subjected to immuno-histochemistry for both GHS-R and ghrelin using specific antibodies (Merck Research Lab. USA for GHS-R antibodies and Cardiovascular Research Institute, Osaka Japan for ghrelin antibodies) and immuno-peroxidase detection methods. The intensity of immunostaining was scored semi-quantitatively from (-) negative to (++++) in relation to known positive and negative controls. Specific immunostaining for both the ligand and its receptor was observed in primary cultured cells and in ishikawa, Hec 1A and AN3CA endometrial cancer cell lines. The highest level of intensity of staining for both ligand and receptor was observed in primary cultured cancer cells derived from grade II cancer. The highest intensity of staining for both in cell lines was observed in Ishikawa cells with decreasing intensity in HEC 1A and AN3CA. Using the primary cultured cells and the Ishikawa cell line we conducted proliferation experiments. Studies showed that primary cultured cells and ishikawa cells showed an increased proliferation in vitro in response to ghrelin to levels of 141% and 137% respectively to that observed in untreated controls. This implies a potential tumour promoting role for ghrelin in this tissue. This may provide evidence that a previously unrecognised autocrine/paracrine pathway involving ghrelin to cell growth in endometrial cancer. We are currently investigating the function of the ghrelin system in the proliferation and apoptosis of tumour cells in culture.

Supported by NHMRC and Prince Henry's Institute of Medical Research.

EXPRESSION OF KAI1(CD82), A METASTASIS SUPPRESSOR, IN AN ANIMAL MODEL OF HUMAN PROSTATE CANCER

Marijana Lijovic[1], Gino Somers[2], Mark Smyth[3], Albert Frauman[1]

[1]Clinical Pharmacology & Therapeutics Unit, Department of Medicine, University of Melbourne, Austin & Repatriation Medical Centre, Heidelberg 3084, [2]Department of Anatomical Pathology, Royal Children's Hospital, Parkville 3052 and [3]Cancer Immunology-Cellular Immunity Laboratory, Peter MacCallum Cancer Institute, East Melbourne 3002.

Prostate cancer (CaP) is a major cause of morbidity and mortality in men worldwide. The 'TRansgenic Adenocarcinoma of the Mouse Prostate' (TRAMP) model (1) was used to study the KAI1(CD82) metastasis suppressor gene during CaP development. The model resembles stages of development of human CaP, including the precursor lesion 'prostatic intraepithelial neoplasia' (PIN). Previously, we showed a biphasic pattern of KAI1 mRNA (2) and protein (3) expression in primary human CaPs (overexpression in low-grade CaP & reduced expression in high-grade CaP). Results from immunohistochem-ical analysis of the TRAMP model suggest a similar pattern of KAI1 expression (Table 1). Thus, PIN and lower grade CaPs exhibit higher levels of KAI1 protein than normal tissue, although levels in higher grade CaPs approach normal tissue levels. With our human data, the TRAMP data suggest that increased KAI1 expression may be a compensatory mechanism to prevent spread of CaP, and its loss may predispose to metastases.

TABLE 1. KAI1 staining scores (median) for various prostatic tissues from TRAMP animals

	N	KAI1 staining score (density x area)*	No. specimens with KAI1 scores >95% upper limit normal prostate tissue
Normal prostate tissue	4	6.33	0/4 (0%)
Prostatic intraepithelial neoplasia	25	6.89	7/25 (28.0%)
Well differentiated CaP	14	8.62	6/14 (42.9%)
Moderate+poorly differentiated CaP	10	6.66	4/10 (40.0%)

** KAI1 protein expression scored using computer image analysis (3)*

(1) Gingrich et al. *Prostate Cancer Prostatic Dis* 1999 2:70-75.
(2) Bouras & Frauman. *J Pathol* 1999;188:382-388.
(3) Lijovic et al. *Cancer Detect Prev.* 2002;26:69-77.

HUD, A NOVEL ANDROGEN RECEPTOR MRNA-BINDING PROTEIN, IS ABERRANTLY EXPRESSED IN PROSTATE CANCER

Ricky R. Lareu, Britt Granath, Dianne J. Beveridge, Henry Furneaux[1], Dominic C. Voon, Peter J. Leedman

Laboratory for Cancer Medicine, Western Australian Institute for Medical Research and Centre for Medical Research, School of Medicine and Pharmacology, The University of Western Australia, WA. [1]Vascular Biology Program, Department of Physiology, University of Connecticut Health Centre, CT, USA.

Androgen deprivation results in the regression of most prostate cancers. However, this effect is eventually abrogated by the emergence of androgen-insensitive disease. Although multiple mutations have been described in the androgen receptor (AR) in late-stage disease, current evidence suggests that the presence of such mutations is not the major mechanism in the progression to androgen-independent disease. We have previously identified a highly conserved UC-rich element at the 3'-untranslated region (3'UTR) of the AR transcript. This region was a target for HuR, a ubiquitously expressed member of the embryonic lethal abnormal vision (ELAV)/Hu RNA-binding protein family, and poly(C)-binding protein 1 (CP1) in LNCaP cells, a human prostate cancer cell line (1).

A pilot study was conducted to detect the expression of HuD protein (an ELAV/Hu protein exclusively expressed in neuronal tissue under normal physiologic conditions) in prostate cancer. Immunohistochemistry revealed significantly higher HuD protein expression in the carcinoma tissue, compared to normal tissue in the same sections. In addition, higher levels of HuD protein were associated with increasing adenocarcinoma grade. Interestingly, LNCaP cells were found to express HuD and immunoprecipitation-RT-PCR demonstrated a close association of HuD with AR mRNA. RNA electrophoretic mobility shift assay demonstrated that recombinant HuD (rHuD) protein bound specifically to the AR UC-rich wild-type (Wt) probe. Furthermore, this RNA-protein complex could be super-shifted by HuD antibody. Specific mutations in the Wt probe abrogated the HuD complex. In addition, rHuD and recombinant CP1 proteins demonstrated cooperativity when binding the AR UC-rich Wt probe.

In summary, these data demonstrate novel ectopic expression of HuD in human prostate cancer cells. Furthermore, HuD binds avidly to the AR mRNA 3'UTR UC-rich element, and in a cooperative manner with CP1. These data suggest a new model in which HuR, HuD and CP1 play important roles in the regulation of AR mRNA expression, and ultimately in the control of AR levels in prostate cancer.

(1) Yeap *et al.*, 2002. J Biol Chem. 277(30): 27183-27192.

149

MUTATIONS OF THE RAS AND B-RAF GENES IN OVARIAN CANCER

Stacey Jamieson[1,2], Maria Alexiadis[1], Peter J. Fuller[1]

[1]Prince Henry's Institute of Medical Research, Clayton, Victoria, 3168, [2]Monash University Department of Pathology & Immunology, Prahran, Victoria, 3181.

The Ras-Raf-MEK-ERK pathway is a key signalling mechanism that regulates many cellular functions, including cell proliferation, transformation, differentiation and apoptosis. Two components of this pathway, Ras and Raf, are proto-oncogenes. The three ras genes, K-, N- and H-ras, encode four closely related, but distinct 21kDa GTPases that bind and regulate a serine/threonine-specific kinase, Raf, of which there are three isoforms, Raf-1, A-Raf and B-Raf. Although activating mutations in the K-, N- and H-ras and B-raf genes are found in many human neoplasms, an apparent association has been observed between the presence of ras and B-raf mutations in similar cancer types. Activating point mutations in ras genes predominantly arise in codons 12, 13 and 61, with those in codons 12 and 13 occurring most frequently. Similarly, a point mutation at codon 599 accounts for at least 80% of activating B-raf mutations. K-ras and B-raf mutations have been identified in ovarian epithelial tumours, however sex cord stromal tumours of the ovary, of which the most common type are granulosa cell tumours (GCT), have not been examined. The aim of this study is to determine the frequency of activating mutations in the K-, N- and H-ras and B-raf genes in ovarian GCT. Gene expression patterns of the three ras and B-raf genes will be determined in a panel of ovarian tumours, including 9 GCT, 19 epithelial tumours (serous and mucinous cystadenocarcinomas) and 10 normal premenopausal ovaries. Total RNA is used for RT-PCR analysis. The PCR products are subjected to Southern blot analysis and probed with internal gene-specific ^{32}P-labelled oligonucleotide probes for semi-quantitative analysis of gene expression. RT-PCR assays have been established for the four genes using gene specific primers. The identities of the amplicons have been confirmed by direct sequence analysis. The amplicons encompass codons 12, 13 and 61 of the ras genes and codon 599 of the B-raf gene. We anticipate that approximately 50% of the mucinous epithelial tumours will harbour mutations in the K-ras gene at codons 12 and 13, as previously reported. Identification of mutations in this signalling pathway in GCT would represent the first somatic activating mutation identified in this tumour type and may provide novel targets for tumour therapy.

150

ALTERATIONS IN CYTOSKELETAL DYNAMICS IN BREAST CANCER CELLS DUE TO PROGESTERONE TREATMENT

Hazel D. Hill[1], J. Dinny Graham[1], Melissa L. Yager[1], Geraldine M. O'Neill[2], Christine L. Clarke[1]

[1]Westmead Institute for Cancer Research, University of Sydney at the Westmead Millennium Institute, Westmead Hospital, Westmead. [2]Oncology Research Unit, The Children's Hospital at Westmead, New South Wales.

Progesterone plays a major role in the development of the normal breast and uterus and is implicated in the development of hormone-dependent cancers. Recent findings provide evidence

for the involvement of the cytoskeleton in the malignant transformation of cells, and research in our laboratory, showing that progestins regulate actin microfilaments, suggest that endocrine signalling may be implicated in cytoskeletal regulation. Cofilin or actin depolymerising factor (ADF) is an actin-monomer sequestering, F-actin depolymerizing protein that is involved in regulating actin assembly and disassembly. The phosphorylation of this protein makes it inactive and thus unable to regulate organization of the cytoskeleton. The aim of this study was to firstly, characterise the effects of progestins on actin microfilaments and secondly, to investigate their effects on the levels of both forms of cofilin. T-47D breast cancer cells were treated with ORG2058 (10nM) or vehicle for 72hr. Cells on coverslips were stained with phalloidin-FITC to visualise actin filaments and cell extracts and soluble and insoluble cell fractions were probed for total cofilin and phospho-ADF on immunoblots. The cofilin antibody detected only cofilin, while pADF detected both pcofilin and pADF, allowing the comparison of phosphorylated and total cofilin levels. Wild-type cells displayed thick, long microfilaments across regions of cells as well as much shorter and thinner fibres throughout the cytoplasm. ORG2058 treated cells showed only the shorter and thinner fibres throughout their cytoplasm, which is consistent with a more motile and invasive phenotype. Cofilin was expressed in cell extracts and soluble fractions with a MW of 18.6kDa. Treatment of cells with ORG2058 produced an increase in levels of pADF but no change in cofilin levels. This increase was also seen in the soluble fractions. Progestin augmentation of cofilin/ADF phosphorylation has been demonstrated, consistent with a progestin-mediated increase in the inactive form of cofilin/ADF. This would result in a reduction in the regulation of the actin microfilaments and re-organization of the cytoskeleton. These results provide new evidence for a role for progesterone in regulation of the actin filament assembly and provide some insight into the mechanism through which it may be exerting its proliferative effects.

151

MMPS ARE REGULATED BY STEROID HORMONES AND STROMAL FACTORS IN ENDOMETRIAL CANCER

Lisa A. Di Nezza[1,2], Tom Jobling[2], Lois A. Salamonsen[1]

[1]Prince Henry's Institute of Medical Research and [2]Department of Obstetrics and Gynaecology, Monash Medical Centre, Clayton, Victoria 3168.

Endometrial cancer (EC) is a common tumour of the female genital tract which predominantly affects postmenopausal women. Matrix metalloproteinases (MMPs) are a large family of proteases which participate in the degradation of collagens and other extracellular matrix macromolecules and are implicated in tissue destruction and remodelling. Previously we have shown a correlation between the increasing expression of MMP-2, -9 and MT1-MMP and EC progression[1]. Thus, it is important to elucidate mechanisms of MMP regulation. The endometrium is responsive to steroid hormones. In addition, tumour-stromal interactions are important in tumourigenesis and MMP production has been shown to be influenced by such interactions. Aims of the current study were a) to study the effects of estradiol (E_2) and medroxyprogesterone acetate (MPA) on MMP production in primary EC cells and cell lines *in vitro* and b) to examine MMP production in co-cultures utilising primary EC and normal endometrial cells. MMPs were assessed by zymography of culture medium. Overall, MPA treatment decreased MMP secretion in primary EC cells and cancer associated stromal cells (CSCs) from three patient biopsies as well as in EC cell lines[2]. Enhanced levels of proMMP-9,

MMP-2 and proMMP-7 were observed in co-cultures of normal epithelial cells and CSCs ($p<0.05$). The induction of proMMP-7 in these co-cultures was more pronounced when cellular contact was allowed to occur. These data suggest that CSCs contain and/or secrete factors which increase MMP secretion from epithelial cells. These MMPs have also been identified in uterine washings from EC patients. In conclusion, MMPs produced by EC are responsive to progesterone. These data may provide a rationale for the use of progestin therapy in the treatment of EC. Epithelial-stromal interactions are also important in MMP regulation. Further studies are in progress to confirm the nature of these interactions.

(1) Di Nezza et al. Cancer. 2002
(2) Di Nezza et al. Gynecol Oncol. 2003

152

CHARACTERISATION OF THE 3' UTR OF C-*ERB*B-2 *(HER-2)* IN HUMAN BREAST AND GASTRIC CANCER CELL LINES

Michael Epis, Andrew Thomson, Peter J. Leedman

Laboratory for Cancer Medicine and School of Medicine and Pharmacology, Western Australian Institute for Medical Research, Centre for Medical Research, Royal Perth Hospital, The University of Western Australia, WA, 6000.

Understanding the mechanisms that underly the expression of growth promoting tyrosine-kinase receptors, such as the EGF-receptor (EGF-R), in breast cancer is a major goal. The *HER-2* oncogene is a member of the EGFR family and is over-expressed in 30-50% of human breast cancers. While the rate of mRNA transcription is important in many cell systems, there are numerous examples of genes, including the EGF-R, that are regulated primarily at the post-transcriptional (mRNA decay) level. We have recently characterised novel *cis*-acting elements and *trans*-acting factors that play a major role in regulating EGFR mRNA decay. However, the mechanisms regulating *HER-2* mRNA turnover are yet to be determined. Here we aimed to identify and characterise novel *cis*-acting mRNA stability modifying elements within *HER-2* mRNA. We transfected multiple cancer cell lines, each with varying levels of *HER-2* protein expression (MDA-468, MDA-453, SkBr3 and N87), with a variety of different *HER-2* 3' UTR luciferase reporter constructs. Our data identified a novel GU-rich *cis*-acting element residing within the 3' UTR of *HER-2* mRNA. This element stabilises the heterologous reporter compared to other regions in the 3' UTR. *HER-2* mRNA half-life studies are underway to explore differences across low (MDA-468) and over-expressing *HER-2* breast (MDA-453 and SkBr3) and gastric (N87) cancer cell lines. Interestingly, this *HER-2* mRNA stabilising element is bound by breast cancer cytoplasmic proteins in RNA gel-shift assays. In summary, these studies suggest that the 3' UTR of *HER-2* mRNA contains a novel *cis*-acting stabilising element that is the target for *trans*-acting breast cancer RNA binding proteins. Further studies are in progress to elucidate the functional role of this region in regulating *HER-2* mRNA decay, and characterise the *HER-2* mRNA-binding proteins in breast cancer cells.

153

GRAFTING OF OVARIAN TISSUE IN A NON-HUMAN PRIMATE MODEL

M. Pangestu[1], M. Diamente[3], D. Pawitri[2], J Shaw[1], A. Trounson[1]

Monash Institute of Reproduction and Development Monash University,Clayton, Vic 3168, [2]Monash IVF, Clayton Vic 3168. [3] Centre Primate Research Centre. Bogor Agricultural University, Bogor Indonesia

Ovarian tissue can contain a large number of immature oocytes. In species such as the mouse and sheep these remain functional and can give rise to live young following frozen storage and grafting. Frozen storage of ovarian tissue has therefore been suggested as a strategy for cryobanking female germ cells for rare or endangered species and for young women who are at high risk of premature menopause. Trials on a small number of women and non-human primates show that grafts of frozen thawed ovarian tissue can restore menses, but have not ascertained how long the grafts remain functional or whether normal, fertilizable, oocytes can be collected from these grafts. In this study frozen thawed ovarian tissue were auto grafted to eleven previously ovariectomised female macaques (M. fascicularis) aged between 4 and 15 years at the start of the study. The females were studied for at least 6 months following graft replacement to a subcutaneous site on the abdomen to ascertain: the delay until menses were restored, the regularity and length of the cycles and whether cycles ceased. Oocyte retrievals were performed in a hormonally stimulated cycle to assess the number, quality and fertilizability of oocytes from the grafts. Results. Menstrual cycles were restored to all females usually within 3 months of graft replacement, but only 6 of the females developed regular cycles. Oocytes (including MII oocytes) could be recovered from some, but not all grafts, and some, but not all, females. There was evidence that graft function (regularity of cycles) declined with time. The number of oocytes recovered from the equivalent of one grafted ovary was significantly lower than the number recovered from an intact single ovary. We conclude that ovarian autografting to a subcutaneous site does permit maturation of oocytes and oocyte retrieval for assisted reproductive techniques, the efficiency is however low.

154

STEREOLOGICAL ASSESSMENT OF GONADOTROPIN EFFECTS ON OVARIAN FOLLICLE DEVELOPMENT IN MOUSE OVARY

Yuan Wang, Jenny A. Spaliviero, Charles M. Allan, David J. Handelsman, Peter J. Illingworth

Department of Reproductive Medicine, Westmead Hospital, University of Sydney, Westmead NSW 2145 Australia; ANZAC Research Institute, Sydney. NSW 2139, Australia.

While ovulation requires concerted action of gonadotrophins, the specific effects of FSH on ovarian follicle populations remains difficult to isolate from LH effects in vivo. We therefore aimed to study the effects of FSH and LH in the gonadotrophin deficient *hpg* mouse using unbiased stereology to study gonadotropin effect on ovary. Female *hpg* mice were treated for 20 days (days 21-41 days of age) with daily ip injections of hFSH (10 IU/day) or hCG (1 IU/day)

alone or combined. Ovaries fixed in paraformaldehyde, embedded in glycol methacrylate, thick sections (25 μm) stained with PAS were subjected to stereological counting of follicle number (primordial, primary, secondary, antral, preovulatory) using the oocyte nucleolus as index particle. Sections and frames for counting were selected by random uniform sampling and the particle count obtained through the middle 18μm of the section (CAST-grid system, Olympus). Follicle number per ovary was obtained by combining particle density by optical disector with ovarian volume by Cavalieri's principle. The number of primordial follicles was higher in untreated *hpg* compared with phenotypically normal littermates (2771±415 vs 1800±216) and was reduced by FSH treatment alone (1793 ± 186) or with hCG (1147 ± 273) but not by hCG alone (2715±600). By contrast, primary follicle numbers were increased by hCG treatment alone (994±62), or with FSH (889.5±104.0) compared with untreated (325±52), FSH-treated *hpg* mice (426±79) and normals (403±27.09). Antral follicles were absent in the untreated *hpg* and hCG alone treated animals, but appeared following treatment with FSH alone (101 ± 20) or with hCG (187 ± 34) and in normals (116±21.42). Preovulatory follicles were only seen in the wild type (5±3.58) and combined treatment groups (10±6.86). We conclude that, using unbiased quantitative methods, that gonadotrophins have distinct effects on early follicle recruitment with prolonged FSH reducing primordial follicle number whereas hCG had no effect, while the reverse effects were evident in primary follicle numbers. Nevertheless antral, pre-ovulatory and ovulated require joint action of both LH and FSH. Further studies are required to investigate the mechanisms involved.

Supported by NHMRC.

155

CROSSTALK BETWEEN PROGESTERONE AND INTERLEUKIN 11 SIGNAL TRANSDUCTION PATHWAYS IN HUMAN ENDOMETRIAL STROMAL CELLS DURING DECIDUALIZATION.

E Dimitriadis and L A Salamonsen

Prince Henry's Institute of Medical Research, Clayton, 3168, Vic.

Decidualization of endometrial stromal cells is critical for embryo implantation and establishment of pregnancy. IL-11 is one of the few molecules known to be obligatory for decidualization and implantation in the mouse and enhances progesterone (P)-induced human endometrial stromal cell (HESC) decidualization 1,2. IL-11 signals via a heterodimeric complex composed of an IL-11 receptor alpha (IL-11Rα) chain and gp130 and signal transduction occurs via the Janus kinase/signal transducer and activator of transcription (STAT) pathway. This study examined the regulation of STAT3 in HESC during P-induced decidualization, and the effect of IL-11 on activation of STAT3 in HESC. The decidualization of HESC was assessed using an in vitro model in which P was administered for 10 days to cells cultured in serum-free conditions with added estrogen (E). Medium was changed every 48 hours for measurement of prolactin (PRL) as a decidual marker, and cellular protein was extracted at each medium change for Western analysis. HESC were also cultured in serum free conditions for 30 min with added IL-11 and cellular protein extracted at 5 min intervals for Western analysis. Treatment of HESC with P increased the abundance of STAT3 protein from day 6 of culture coinciding with an increase in PRL secretion. Co-treatment of HESC with antiprogestin (onapristone) after decidualization was in process reduced the abundance of STAT3, but had no influence on STAT3 phosphorylation. Addition of IL-11 to HESC resulted in the phosphorylation of STAT3

from 5min. Phosphorylation was abolished following co-treatment with IL-11 neutralising antibody while STAT3 levels remained stable. Our observations demonstrate that P regulates STAT3 protein expression in HESC possibly via P receptor and IL-11 phosphorylates STAT3 in HESC. The data provides evidence of synergy between the P and IL-11 signal transduction pathways during decidualization of HESC. This knowledge is important in understanding the formation of decidua and a functional placenta, and regulation of fertility.

1. Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG (1998) Nat Med 3:303-308
2. Dimitriadis E, Robb L, Salamonsen LA (2002) Mol Hum Reprod 8:636-643

156

NOVEL ERα LIGANDS, PPT & R,R-THC, PROMOTE ANGIOGENESIS IN HUMAN MYOMETRIAL MICROVASCULAR ENDOTHELIAL CELLS

Marina Zaitseva, Peter AW Rogers, Caroline E Gargett

Centre for Women's Health Research, Monash University Dept. of Obstetrics & Gynaecology, Clayton, Vic. 3168

Angiogenesis is the growth of new blood vessels from pre-existing vessels and involves proliferation of microvascular endothelial cells (MEC). VEGF is a major promoter of angiogenesis and mediates angiogenic effects through interaction with VEGF receptor-2 (VEGF-R2). We have demonstrated that MEC derived from human myometrium (MMEC) constitutively express estrogen receptor-β (ERβ), while ERα varies between subjects and is only expressed in approximately 60% of MEC isolates[1]. 17β-estradiol (E) upregulates VEGF-R2 and promotes MEC proliferation in the ERα-expressing isolates, but not in ERα negative MMEC[2]. The aim of the present study was to determine whether ERα mediates upregulation of VEGF-R2 and the angiogenic effects of E in ERα-expressing adult human MEC using the novel ERα-selective ligands, PPT and R,R-THC[3]. Myometrial MEC were isolated from hysterectomy tissue obtained from ovulating women, cultured and used between passages 1-3 (purity >98% CD31+ cells)[4]. ERα and VEGF-R2 expression were measured by flow cytometry using an ERα antibody and biotin-rhVEGF$_{165}$ binding respectively[2]. MEC proliferation was determined by MTS bioassay[2]. We first tested the activity of PPT and R,R-THC on a breast tumor cell line known to express wildtype ERα (MCF-7) and demonstrated that both ligands significantly increased proliferation in a similar manner to E, an effect blocked by the nonspecific ER antagonist, ICI 182,780. Neither PPT or R,R-THC stimulated proliferation of the ERα negative cell line MDA-DB-453. In ERα+ MMEC, PPT and E increased VEGF binding in a dose-dependent manner, but had no effect on ERα negative MMEC samples. PPT, R,R-THC and E significantly augmented VEGF-induced MMEC proliferation in ERα positive MMEC ($P < 0.05$) but not ERα negative MMEC. These data confirm that the angiogenic effects of E on MMEC are due to upregulation of VEGF-R2 and are mediated by ERα rather than ERβ.

1. Gargett CE et al Molec Hum Reprod (2002) 8:770-775.
2. Gargett CE et al J Clin Endocrinol Metabol (2002) 87:4341-4349.
3. Kraichely DM et al Endocrinol. (2000) 141: 3534-3545.
4. Gargett CE et al (2000) Hum Reprod 15:293-301

157

THE AROMATASE KNOCKOUT (ARKO) MOUSE AS A MODEL TO STUDY THE OESTROGENIC ACTIONS OF TIBOLONE

Margaret E.E. Jones[1], Anne T. Reutens[1], WahChin Boon[1], Antwan G. Ederveen[2], Helenius J. Kloosterboer[2], Evan R. Simpson[1]

[1]Prince Henry's Institute of Medical Research, Melbourne; [2]NV Organon, Oss, The Netherlands.

Tibolone (ORG OD14) has oestrogenic, progestogenic &/or androgenic activity depending on the tissue. The Aromatase knockout (ArKO) mouse provides an ideal model to study the multiple properties of Tibolone given its inability to synthesise endogenous oestrogens. In this study, we examined the effect of Tibolone administration on the ovariectomised (ovx) ArKO mouse. Ovx or sham-operated ArKO & WT mice were orally administered Tibolone (2µg/g body mass), ethinyl estradiol (EE) (0.05µg/g body mass) or vehicle once daily for 6 weeks. As expected ovx increased body weight gain. Tibolone produced a precipitous decline in body mass in ovx ArKO mice. Within 3 weeks of administration, mice lost a mean of 25% of initial body mass, versus 10% for comparative wildtype (WT) mice, likely a reflection of adipose tissue loss. Control EE-induced body mass loss was not as great in ovx ArKO (11.2%). Uterine mass increase was significantly greater in Tibolone treated ArKO & WT groups compared to EE replacement (13.5- vs 7.7-fold increase Tibolone; 3.9- vs 2.4-fold increase EE, ArKO vs WT respectively, compared to ovx + vehicle). In line with these effects Tibolone showed an oestrogenic effect on bone yielding an increased bone mineral density in the distal femur. Ovx or intact vehicle-treated ArKO mammary glands were rudimentary, consistent with their oestrogen-naïve background. In contrast, EE-treated ArKO mammary glands had ducts extending from the nipple to beyond the lymph node, relatively small terminal end buds (TEBs), & a bifurcated branching pattern. Tibolone-replaced ovx ArKO mammary glands displayed extensive side branching. TEBs were prominent beyond the lymph node where the duct spread into the fat pad. Clearly Tibolone expresses both oestrogenic & progestogenic activity on breast development. Furthermore it is evident that Tibolone does not need to be aromatised to produce oestrogenic activity as observed on ArKO body-, adipose tissue-, uterine- & bone-mass. The effect is likely being mediated by the two Tibolone metabolites 3α- & 3β-hydroxytibolone. These results demonstrate that the ArKO mouse provides a useful model for assessing the composite steroidal activities of Tibolone.

158

PROGESTERONE RECEPTOR EXPRESSION IS MODULATED BY PROSTAGLANDINS IN HUMAN MYOMETRIAL CELLS

Gemma Madsen, Tamas Zakar, Roger Smith, Sam Mesiano

Mothers and Babies Research Center, University of Newcastle & John Hunter Hospital, Newcastle, NSW 2310.

Progesterone withdrawal transforms the myometrium to a highly contractile state required for parturition. In human pregnancy, progesterone withdrawal is mediated functionally by a decrease in myometrial progesterone responsiveness. This is attributed to increased expression

of progesterone receptor type A (PR-A), a repressor of progestin actions. Prostaglandins (PGs) are potent endogenous uterotonins and their administration at any stage of human pregnancy induces the full parturition cascade. We hypothesized that in human pregnancy PGs act, at least in part, by inducing functional progesterone withdrawal by modulating myometrial expression of PR-A relative to PR-B. To test this hypothesis we determined whether PGE_2 and $PGF_{2\alpha}$ influence PR-A and PR-B expression in the PHM1-31 human myometrial cell line. PHM1-31 cells were exposed to PGE_2 and $PGF_{2\alpha}$ (1pM to 10nM each) for 24h. Relative abundance (normalized to 18S rRNA) of mRNAs encoding total PR-A and PR-B were determined by real-time quantitative RT-PCR. Abundance of PR-A and PR-B mRNAs were differentially and dose dependently increased by PGE_2. PGE_2 more efficiently induced PR-A than PR-B expression. Consequently, the PR-A/PR-B expression ratio, which is thought to reflect the extent to which PR-A suppresses progesterone responsiveness, increased in response to low doses of PGE_2 (0.01 to 1nM) and returned to basal levels in response to higher PGE_2 levels (1-10nM). $PGF_{2\alpha}$ stimulated expression of PR-A but had no effect on PR-B. This increased the PR-A/PR-B expression ratio. These data show that PGE_2 and $PGF_{2\alpha}$ regulate PR-A and PR-B expression in human myometrial cells. Since progesterone responsiveness is inversely related to the PR-A/PR-B expression ratio, the increase in this ratio by PGE_2 (at low concentrations) and $PGF_{2\alpha}$ may cause functional progesterone withdrawal. These data suggest that PGs induce parturition in part by inducing functional progesterone withdrawal. Such a mechanism would explain why PGs alone can induce the full parturition cascade.

159

A ROLE FOR PLATELET-ACTIVATING FACTOR IN LUTEOLYTIC $PGF_{2\alpha}$ PRODUCTION BY THE OVINE ENDOMETRIUM

Omar Chami[1], Gareth Evans[2], Chris O'Neill[1]

[1]Human Reproduction Unit, Department of Physiology, University of Sydney, Royal North Shore Hospital. [2]Faculty of Veterinary Science, University of Sydney.

Pulsatile release of endometrial prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induces luteolysis in ruminants (1). It has long been help that this pulsatility is driven by the pulsatile release of ovarian oxytocin, and that the two mediators form a positive feedback loop (2). However, several studies have shown that pulsatility persist in the absence of either the ovary, corpus luteum or pituitary (2, 3). It was hypothesized that uterine $PGF_{2\alpha}$ pulses are generated by a uterine loop of platelet-activating factor (PAF) signaling (4). PAF induces a uterine sex-steroid dependent $PGF_{2\alpha}$ response that is augmented by oxytocin and inhibited by embryonic interferon tau (4). The aim of this study was to investigate whether the maturation of a uterine PAF signaling loop in the ovine endometrium occurs at the time that $PGF_{2\alpha}$-pulsatility is first observed. Caruncular endometrium and uterine luminal fluids were collected from ewes on days 10, 12, 14 and 16 of the estrous cycle. The PAF content in tissue and the amount released into media increased significantly ($P < 0.01$) between days 10-16. PAF-receptor mRNA increased in a steroid-dependent manner, with immunolocalisation of the PAF-R protein showing that it was present in stratum compactum stroma and glandular epithelium. By day 14 there was a marked increase in PAF:acetylhydrolase activity in the luminal fluids ($P < 0.001$) and endometrial tissue ($P < 0.05$). Plasma PAF:acetylhydrolase protein immunolocalisation showed that it was present in the lumen of the glandular epithelium. This may serve to prevent accumulation of PAF outside of endometrial

tissue. This study shows that the components of a localized loop of PAF signaling were assembled in the ovine endometrium at the time that the onset of uterine $PGF_{2\alpha}$ pulsatility was expected.

(1) McCracken, J.A., *et al*., PGF 2 identified as a luteolytic hormone in sheep. *Nature*, 1972. 238(83):29-34.
(2) Silvia, W.J. and R.E. Raw, Regulation of pulsatile secretion of PGF_{2a} from the ovine uterus by ovarian steroids. *J Reprod. Fertil*, 1993. 98(2):341-7.
(3) Denamur, R., J.et al., Pituitary control of the ovine corpus luteum. *J Reprod Fertil*, 1973. 32(2):207-20.
(4) Chami, O., et al., PAF may act as an endogenous pulse generator for sheep of luteolytic PGF_{2a} release. *Am J Physiol*, 1999. 276:E783-92

160

MACROPHAGE MIGRATION AND LUTEAL REGRESSION IN OVARIES OF LEUKOCYTE ADHESION MOLECULE-DEFICIENT (ICAM-1-/-) MICE.

Rebecca L. Robker, C. Wayne Smith*, Robert J. Norman and Darryl L. Russell

Department of Obstetrics and Gynaecology, University of Adelaide, South Australia 5005. *Section of Leukocyte Biology, Department of Pediatrics, Baylor College of Medicine, Houston TX, USA

Luteal regression is an ovarian remodeling event involving apoptosis of luteal cells, an influx of macrophages thought to phagocytose luteal cell debris, and resorption of the corpus luteum (CL). Macrophage adhesion and migration in many tissues is mediated by ICAM-1 (a cellular adhesion molecule which acts as a counter-receptor for leukocyte β2-integrins); and increased ICAM-1 expression has been associated with macrophage infiltration in regressing CL of the rat. To test whether ICAM-1 mediates macrophage infiltration during regression, CL of ICAM-1 null (ICAM-1-/-) mice were analyzed by immunohistochemistry for markers of macrophages (F4/80) and luteal cell apoptosis (caspase-3). Ovaries of adult cycling ICAM-1-/- females showed abundant macrophages in regressing CL indicating that ICAM-1 is not required for macrophage migration during luteolysis. In older mice (6 months of age), wildtype (WT) ovaries consisted primarily of follicles and CL, however, ICAM-1-/- ovaries consisted almost entirely of stroma with only a few follicles and CL. These "stromal" cells were reminiscent of luteal cells (hypertrophied and eosinophilic) suggesting that CL regression failed to resolve normally resulting in accumulation with time. To test this, WT and ICAM-1-/- mice were hormonally primed to stimulate luteinization and subsequent regression, with PMSG/ hCG for 4 or 6 days, and the regressing CL were compared. Preliminary results showed that the CL of ICAM-1-/- mice are less regressed than WT at 4d post-hCG, an effect which is most pronounced at 6d post-hCG. Immunohistochemical staining patterns for F4/80 and caspase-3 were similar in WT and ICAM-1-/- ovaries indicating that macrophages are present and luteal cell apoptosis is occurring in the "regressing" CL. Concurrently, to test whether the lack of ICAM-1 and a potential defect in luteal regression would impact fertility, female ICAM-1-/- and WT littermates were housed with WT males. After 6 months ICAM-1-/- females had produced normal numbers of litters and

pups. Thus, ICAM-1 is not essential for female fertility. That CL regression was delayed despite the presence of luteal macrophages in ICAM-1-/- ovaries indicates that during luteal regression ICAM-1 is likely to be more important for macrophage activation and/or phagocytosis than recruitment or migration.

161

withdrawn

162

EXPOSURE TO A BACTERIAL INFECTION DURING PREGNANCY ALTERS FETAL DEVELOPMENT WITH CONSEQUENCES FOR GLUCOSE METABOLISM

Nicolette A. Tape[1], **Frederick R. Walker**[1], **Julie A. Owens**[2] **& Deborah M. Hodgson**[1].

Laboratory of Neuroimmunology, School of Behavioural Sciences, University of Newcastle, and The Mothers and Babies Research Centre, John Hunter Hospital, NSW, Australia[1] and Discipline of Physiology, School of molecular and biomedical Science, University of Adelaide[2].

An adverse fetal environment has been implicated in predisposing the developing infant towards disease and dysfunction in later life. As maternal infection during pregnancy results in the transfer of excess cortisol and cytokines into fetal circulation, maternal infection could potentially result in the programming towards this predisposition. Particularly susceptible to programming effects caused by glucocorticoid and cytokine overexposure is the developing fetal hypothalamic-pituitary-adrenal (HPA) axis, a system critically involved in the modulation of both the stress response and glucose metabolism. Alterations to this system can be detrimental to health, given that exposure to altered glucose and/or cortisol concentrations can result in such pathologies as cardio-vascular disease, renal failure, and diabetes type2. This study aimed to identify whether exposure to bacterial endotoxin during pregnancy would affect the growth and development of the infant and result in a predisposition towards insulin resistance in late adulthood. Pregnant guinea pigs were administered endotoxin (salmonella enteritidis, 100ug.kg^{-1}, s.c.) or an equivolume of saline on gestation days 46, 48, 50 and 52 (term=68days). At term, a subset of guinea pigs had their pups delivered by caesarean section, and maternal and fetal blood samples were collected for cortisol analysis. The remaining guinea pigs were left to deliver normally. Guinea pig pups were weighed at birth and then weekly into adulthood and body mass index was determined. The guinea pigs were then exposed to a glucose challenge (2g.kg^{-1}) to assess insulin responsivity. Prenatal exposure to endotoxin was found to be associated with reduced weight at birth ($p<0.05$) and a trend towards an increased weight in adulthood as compared to control animals. These animals also exhibited reduced cortisol levels at birth and in adulthood ($p<0.05$) compared to control animal indicative that exposure of infants in utero to ~~i~~a bacterial challenge altered HPA axis development. Prenatal exposure to infection was also associated with altered glucose metabolic activity in late adulthood. These results indicate that fetal exposure to infection in utero has significant implications for fetal development and the efficacy of future metabolic functioning. As such, the prenatal environment is highlighted as a critical determinant of the programming towards future health conditions.

163

RESTRICTED FETAL GROWTH AND NEONATAL CATCH-UP GROWTH HAVE GENDER-SPECIFIC RELATIONSHIPS WITH INSULIN SECRETION IN YOUNG ADULT SHEEP

K.L. Gatford[1], M.J. DeBlasio[1], P.T. Thavaneswaran[1], J.S. Robinson[2], I.C. McMillen[1], J.A. Owens[1]

[1]School of Molecular & Biomedical Science and [2]Dept of Obstetrics & Gynaecology, University of Adelaide, Adelaide SA 5005.

Loss of first-phase or acute insulin secretion following a glucose challenge is an early defect in the pathogenesis of Type II diabetes. Small size at birth is associated with an increased incidence of Type II diabetes, but limited evidence of an insulin secretion defect in humans. Impaired placental growth and function is a known major cause of small size at birth. We therefore determined the effect of experimental placental restriction of fetal growth on insulin secretion in the young adult sheep (375 days). The study was approved by University of Adelaide Animal Ethics Committee. An intravenous glucose tolerance test (0.25 g glucose/kg bodyweight) was performed on 19 control and 17 placentally-restricted sheep. Insulin secretion was defined as area under the curve for insulin (AUCI), first-phase insulin secretion as AUCI from 0 to 20 minutes, and second-phase insulin secretion as AUCI from 20 to 210 minutes, after intravenous glucose. Insulin secretion was greater in males than in females in absolute terms ($p=0.014$) and relative to the glucose stimulus (AUC for glucose) ($p=0.09$). Restriction of placental growth did not alter total or first-phase insulin secretion, but tended to decrease second-phase insulin secretion in females only ($P=0.07$). In males, first-phase insulin secretion relative to glucose correlated negatively with weight or length at birth, whilst absolute total and second-phase insulin secretion correlated positively with neonatal catch-up growth of long bones (all $p<0.05$). In females, absolute second-phase insulin secretion correlated positively with length at birth and negatively with neonatal growth of soft tissues (all $p<0.05$). These outcomes were not altered by adjustment for insulin sensitivity. Therefore, restricted fetal growth and/or rapid neonatal catch-up growth are associated with decreased second-phase insulin secretion in females, but hyperinsulinaemia in males in the young adult sheep.

164

ARE FEMALE FETUSES MORE SENSITIVE TO THE EFFECTS OF INFLAMMATION THAN MALE FETUSES?

Naomi Scott, Annette Osei-Kumah, Vanessa Murphy, Vicki Clifton

Mothers & Babies Research Centre, John Hunter Hospital, Newcastle NSW

Previous studies from our lab have shown that the birthweight of female fetuses is significantly reduced in the presence of maternal asthma while male fetuses are of normal birthweight. These studies suggest that the female fetus may be more sensitive to an immune challenge than the male fetus. We hypothesised that immune cells derived from the female fetal cord blood will be more sensitive to an immune challenge than male fetal cells. Whole cord blood from male (n=4) and female (n=3) fetuses of normal, uncomplicated pregnancies were diluted 1 in 10 in saline, and treated with increasing doses of lipopolysaccharide (LPS) (1 ng/ml-10 μg/ml) for 48 hours at

37°C, 5%CO_2. Tumour necrosis factor alpha (TNF alpha) was measured in cell supernatants using ELISA. At the time of blood collection, blood smears were conducted which showed a mixed population of immune cells were present in the cord blood, including neutrophils, eosinophils, lymphocytes and monocytes. There was no basal production of TNF alpha by cord blood cells. LPS stimulated the cord blood cells to produce TNF alpha in a dose dependent manner. In female fetuses, TNF concentrations ranged from 0-125 pg/ml while in male fetuses concentrations ranged from 6-171 pg/ml in the presence of increasing concentrations of LPS. At all doses of LPS, the response of cord blood cells collected from female fetuses was significantly reduced when compared to male fetuses. Our preliminary data suggests that there is a significant difference in the response to an immune challenge between male and female fetuses however male fetuses appear to have a more enhanced response in this study.

165

FACTORS DERIVED FROM THE FETO-PLACENTAL UNIT ALTER BRONCHIAL SMOOTH MUSCLE SYNTHETIC FUNCTION

Annette Osei-Kumah[1], Roger Smith[1], Qi Ge[2], Alaina Ammit[3], Vicki Clifton[1]

[1]Mothers and Babies Research Centre, Hunter Medical Research Institute, The University of Newcastle, NSW 2308, Australia; Respiratory Research Group, [2]Department of Pharmacology, [3]Faculty of Pharmacy, University of Sydney, NSW 2006 Australia.

A number of recent studies have indicated that maternal asthma severity is increased as gestation progresses, particularly in the presence of a female fetus. Nevertheless the specific mechanisms that lead to these exacerbations are unknown. We have hypothesised that a factor (s) derived from the feto-placental unit will alter bronchial smooth muscle (BSM) synthetic function by upregulating inflammatory pathways associated with asthma. The aim of this study was to determine whether pregnancy derived factors from the feto-placental unit alter inflammatory mediators involved in asthma in the BSM. Cultured human BSM cells were exposed to either male (n = 8) or female (n = 8) fetal plasma collected from the umbilical cord after normal vaginal delivery for 1, 2, 6 and 24 hours. Nonpregnant nonasthmatic plasma (n = 3), nonpregnant asthmatic plasma (n = 3) and plasma free media were included as control treatments. Enzyme linked immunosorbent assay was used to determine the concentration of regulated upon activation, normal T-cell expressed and secreted (RANTES), interleukin (IL) -6 and eotaxin protein in the culture supernatant at the end of the 24-hour period. RNA was extracted from the BSM cells and quantitative real time RT-PCR was used to determine mRNA expression of intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). There was a significant increase in IL-6 production by cells treated with asthmatic plasma ($P < 0.05$) and fetal plasma ($P < 0.001$). Fetal plasma increased RANTES production ($P < 0.01$) and ICAM-1 mRNA expression ($P < 0.05$). The production of eotaxin and VCAM-1 mRNA expression was not statistically different between any of the treatment groups. The results of this study show that factors derived from the feto-placental circulation can alter the production of inflammatory mediators associated with asthma in the BSM and may contribute to the mechanism of asthma exacerbation during pregnancy.

THE EFFECT OF FETAL GENDER ON MATERNAL INFLAMMATION AND LUNG FUNCTION IN ASTHMATIC PREGNANCIES

V.E. Murphy[1], P.G. Gibson[2], R. Smith[1], W.B. Giles[1], V.L. Clifton[1]

Mothers and Babies Research Centre [1] & Department of Respiratory and Sleep Medicine [2], Hunter Medical Research Institute, Newcastle.

Pregnant women with asthma have an increased incidence of low birth weight neonates. Recently we demonstrated that the size of female neonates was specifically reduced in asthmatic mothers who did not use inhaled steroids. The present study investigated the effect of fetal gender on maternal and placental inflammatory pathways and maternal lung function. Pregnant women with asthma were grouped according to their use of inhaled steroids (no glucocorticoid, glucocorticoid) and compared to a non-asthmatic group (control). Circulating white blood cells were assessed in early (<20 weeks) and late (>30 weeks) pregnancy. Maternal lung function was assessed at 20 and 30 weeks. The placenta was collected following delivery to measure cytokine mRNA by RT-PCR.

The circulating monocyte count and percentage of all white blood cells that were monocytes significantly increased from early to late pregnancy only in women from the no glucocorticoid group who were pregnant with a female fetus (P=0.02, Mann Whitney test). Other white blood cells did not differ significantly between groups.

Maternal lung function (forced expiratory volume at 1 second to vital capacity ratio, FEV_1:VC) decreased significantly from 20 to 30 weeks only in asthmatic women pregnant with a female fetus, regardless of glucocorticoid use and was correlated with female birth weight in the no glucocorticoid group only (P<0.02).

There was a significant increase in placental Th2:Th1 cytokine mRNA ratios in females from the no glucocorticoid group, as assessed by measuring TNF-α (Th1) and IL-5 and IL-4 (Th2) mRNA (P<0.05, ANOVA). There was a significant inverse correlation between placental Th2:Th1 ratio and 11β-HSD2 activity in female neonates.

Our study provides evidence that the female fetus increases inflammation and reduces lung function in asthmatic mothers. Our data suggest that this alteration in maternal inflammation, when not controlled by the use of inhaled glucocorticoids, results in changes in placental 11β-HSD2 activity, alterations in placental cytokine expression and reduced female fetal growth.

167

REDUCED NUMBERS OF TYPE-1 ALVEOLAR EPITHELIAL CELLS IN THE FETAL LUNG OF GLUCOCORTICOID RECEPTOR NULL MICE

Timothy J. Cole[1], Daniel Bird[1], Nicola Solomon[2], Rosemary Van Driel[3], Rod Dilley[3], Stuart Hooper[4]

[1]Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, 3010, Victoria, [2]Murdoch Institute, Royal Children's Hospital, Parkville, 3010, Victoria, [3]Baker Heart Research Institute, Commercial Road, Prahran, 3181,Victoria, [4]Department of Physiology, Monash University, Clayton, 3168, Victoria.

Glucocorticoids regulate many physiological processes including aspects of embryonic development. They provide important signals for the maturation of the fetal lung and antenatal glucocorticoids are used to reduce the respiratory insufficiency suffered by preterm infants. To further understand the role of glucocorticoids in fetal lung maturation, we have analysed mice with a targeted null mutation for the glucocorticoid receptor (GR) gene (1, 2). In the absence of glucocorticoid signaling via GR, lung development is severely retarded. The lungs of fetal GR null mice are condensed, have hypercellularity and reduced septal thinning with increased lung weight and DNA content. However, in fetal GR null mice, expression of the surfactant protein genes in type-II alveolar cells is relatively unaffected as is production and release of pulmonary surfactant. Analysis of epithelial cell types by electron microscopy revealed that the proportion of differentiated type II cells were increased whereas the proportion of differentiated type-1 alveolar epithelial cells were markedly reduced (by ~50%). Similarly, we found a 50% reduction in mRNA levels for T1α and aquaporin-5, two type-1 alveolar epithelial cell-specific markers and a 20% reduction in aquaporin-1 mRNA levels. This demonstrates that during murine embryonic development, glucocorticoids are essential for the complete differentiation of appropriate numbers of type-I alveolar epithelial cells that allows proper gas exchange at birth, but are not obligatory for type-II cell differentiation or lung surfactant production and release.

(1) Cole T.J., Blendy J.A., Monaghan A.P., Krieglstein K., Schmid W., Aguzzi A., Fantuzzi, G., Hummler E., Unsicker K., Schütz G. (1995) Genes Dev. 9, 1608-1621.

(2) Cole T.J., Myles K., Purton J. F. Brereton P. S., Solomon N. M., Godfrey D. I., Funder J. W. (2001) Mol Cell Endocrinol 173**,** 193-202.

168

CHARACTERIZATION AND EXPRESSION OF FIBRONECTIN AND RELATED INTEGRINS IN SHEEP AMNION NEAR TERM

Fahad Hanna[1], Sarah Fraser[1], Mrinal Bhave[1],Graham Jenkin[2], Ross Young[2], Robert Fairclough[1]

[1] School of Molecular Sciences, Victoria University, Victoria 3029, [2] Department of Physiology, Monash University, Clayton, Victoria

Preterm rupture of fetal membranes is a major cause of premature birth. It has been estimated that fetal membrane rupture is associated with 30-40% of preterm deliveries. However the factors responsible for preterm rupture of fetal membranes are still uncertain. Previous studies have, however, indicated that the extracellular matrix plays an important role in maintaining fetal

membrane intregrity and function. Given that alternatively spliced isoforms of fibronectin are key constituents of the extracellular matrix together with the associated integrins, we have determined the expression of fibronectin and integrin mRNAs in the amnion of sheep during spontaneous labor. Amnion samples were collected from sheep on days 136-137 of gestation (n=4) and at term (n=4). RNA was extracted using TRIZOL reagent and used for cDNA synthesis using a oligo(dT) primer. The reverse transcription polymerase reaction (RT-PCR) was used to examine alternative splicing of fibronectin and expression of various integrin subunit mRNAs in the sheep amnion. Real-time PCR and/or Northern analysis was used to assess any changes in the level of expression of these molecules in the sheep amnion. The results showed that both EDA^+ and EDA^- forms of fibronectin were present in the sheep amnion at term. Alternative splicing of fibronectin is knowm to influence interaction with integrins. A number of different integrin subunits were found in fetal membranes before labor and at term at the mRNA level using RT-PCR including α_5, α_4, α_6, α_v and β_1, β_4. Preliminary results were also obtained indicating that there was a decline in the mRNA expression of fibronectin in sheep amnion near term. These results suggest that the interaction of fibronectin isoforms with a number of identified integrins might be important in the extracellular matrix of the sheep amnion near term and that these interactions might influence fetal membranes rupture.

169

EXPRESSION OF THE ANDROGEN RECEPTOR AND ENHANCEMENT OF FSH SIGNALLING BY DIHYDROTESTOSTERONE IN A GRANULOSA TUMOUR CELL LINE

T.E. Hickey, R.J. Norman

Reproductive Medicine Unit, Dept. Obstetrics & Gynaecology, University of Adelaide, The Queen Elizabeth Hospital, Woodville, SA, 5011.

Androgen receptors (AR) are strongly expressed in granulosa cells of developing ovarian follicles but their role in the orchestration of folliculogenesis is unclear. In part, androgens modulate FSH signalling through a post-cAMP mechanism that remains unresolved[1]. Recently a human granulosa tumour cell line, KGN, was established and shown to have functional FSH signalling which induces oestradiol (E2) synthesis[2]. We have further characterised these cells for expression of the AR and a selection of AR associated proteins (ARAs) that co-regulate AR transcriptional activity. These were similarly expressed in KGN and human granulosa lutein cells. We also investigated cellular responses to the most potent natural AR ligand, dihydrotestosterone (DHT), proposing that DHT would enhance FSH and/or cAMP- induced E2 synthesis in KGN. Cells were cultured under various treatment protocols, which included the addition of androstendione (A4) as substrate for E2. Media was analysed for E2 by RIA and cells were either counted with a haemocytometer or treated with H^3-thymidine to measure DNA synthesis. FSH had no effect on cellular proliferation and stimulated low levels of E2. Conversely, cAMP inhibited cellular proliferation 7-fold over controls and stimulated oestradiol synthesis 26-fold over that induced by FSH. The addition of DHT significantly enhanced both FSH (2-fold) and cAMP (5-fold) stimulated E2 production when cells were pre-treated for 2 days prior to A4 addition. The enhancement was reduced with shorter pre-treatment intervals. Although FSH-signalling was weaker than expected in these cells, it was enhanced by androgen. More significantly, post-cAMP events were potently affected by androgen activity, which

concurs with data from animal studies[2]. We conclude that KGN represents a good model for the study of AR-mediated activity in human granulosa cells. Such a model could lend new insights into normal ovarian function, granulosa cell cancers, and the pathogenic consequences of hyperandrogenic states that alter ovarian function and often lead to infertility.

(1) Hillier and Tetsuka. Baillieres Clin Obstet Gynaecol: 11(2), 1997
(2) Nishi Y *et al*. Endocrinology: 142(1), 2001.

170

UNEXPECTED TRANSREPRESSION OF ERβ-MEDIATED SIGNALLING IN GRANULOSA CELLS

Simon Chu, Peter J. Fuller

Prince Henry's Institute of Medical Research and Monash University Dept of Medicine, Clayton, Victoria, 3168.

Estrogen receptor β (ERβ) is the predominant estrogen receptor in granulosa cells of the ovary and is expressed at high levels in granulosa cell tumours (GCT). The functional significance of this expression is not known. Our aim is to gain insight into the function of ERβ in GCT and normal ovary, by examining 2 human GCT lines, COV434 and KGN that express ERβ mRNA. Although the cells bind estradiol (E_2), transcriptional activation is not observed. Transactivation was also not observed with transfected ERα and β in response to E_2. This transcriptional resistance is specific to steroid receptor transactivation; reporter plasmids that are activated by the transcription factors AP1and NFκB demonstrate both constitutive and inducible transactivation. Given that these transcription factors are known to cause transrepression of both ERα and GR mediated transcription, we examined the possibility that activation of these pathways was responsible for the lack of an E_2 response. COV434 and KGN cells were transfected with an estrogen responsive reporter plasmid, and treated with inhibitors of AP1 and NFκB alone or together, in the absence or presence of E_2. The AP1 inhibitors alone had no effect, whereas inhibition of NFκB signalling elicited a 2-3 fold induction in response to E_2. This response was ligand and ER dependent. Interestingly, inhibition of both AP1 and NFκB signalling pathways caused a further increase in the ER response indicating complex cross-talk between the 3 signalling pathways causing transrepression of ERβ. An interaction between ERβ and NFκB has not previously been reported, nor has a synergistic co-transrepression been previously documented. We are currently investigating whether there is a direct interaction between the p65/p50 subunits of NFκB and ERβ. The relevance of this observation to GCT *in vivo* is unclear. There is increasing evidence that suggests ERβ may function as a transcription factor to promote differentiation. The inhibition of ERβ in combination with the anti-apoptotic properties of NFκB may therefore contribute to pathogenesis of GCT.

171

CHARACTERISATION OF NOVEL SRA-PROTEIN INTERACTIONS INTRINSIC TO ESTROGEN RECEPTOR PATHWAY TRANSACTIVATION

Andrew Redfern, Dianne Beveridge, Rainer Lanz[1], Lisa Stuart, Bert O'Malley[1], Peter J. Leedman

Laboratory for Cancer Medicine, School of Medicine & Pharmacology, Centre for Medical Research, Western Australian Institute for Medical Research and the University of Western Australia, Perth, WA, Australia, [1]Division of Cell and Molecular Biology, Baylor College of Medicine, Houston, Texas, USA.

Estrogen receptor (ER)-mediated activation of target genes involves interactions between ER and a diverse range of cofactors. SRA (steroid receptor RNA coactivator) (1), the only described RNA coregulator, is involved in the transactivation process for Type 1 nuclear receptors. SRA contains a number of stable stem loops, several of which contribute to the transactivation capacity of SRA as a whole (2). These structures may act as docking motifs for co-regulator proteins. To identify such SRA stem loop–protein interactions we used SRA stem loop STR1 as bait in yeast three-hybrid screening of a human breast cancer library. A novel family of SRA-binding proteins was identified, that has a characteristic RNA-binding motif. Binding of these proteins to STR1 in RNA gel-shift assays and co-purification with full length SRA in IP-RT-PCR experiments confirms close association of family members with SRA in breast cancer cells. The two family members functionally investigated to date, SRA binding proteins (SBP) 1 and 4, demonstrate activation and repression respectively, of SRA-mediated ER co-activation in transient transfections of HeLa cells. Mutation of either RNA binding or enzymatic domain of SBP4 abrogates the repressive effect of this molecule, indicating both are required for this co-regulator function. Furthermore, initial SRA transfection experiments in MCF7 breast cancer cells, which overexpress SBP4, suggest that SRA corepresses rather than coactivates ER-driven gene activation in this cell line, possibly contributed to by SBP4 action resulting from SRA binding. In summary, we have identified a family of novel SRA-binding proteins that contain a specific structural motif and function to modulate SRA-potentiated transcription. Their role as regulators of estrogen action in human breast cancer is under investigation.

(1) Lanz RB, et al. Cell 1999, 97: 17-27.
(2) Lanz RB, et al. Proc Natl Acad Sci USA 2002, 99(25): 16081

172

VALPROATE IS AN ANDROGEN AND PROGESTERONE ANTAGONIST IN AN IN-VITRO YEAST BIOASSAY

Alison K. Death, Kristine C.Y. McGrath, David J. Handelsman

Dept of Medicine & ANZAC Research Institute, Concord Hospital, University of Sydney, Sydney NSW.

Epilepsy is associated with a higher prevalence of reproductive dysfunction including menstrual disturbance, anovulation and hyperandrogenism in women and sexual dysfunction and infertility in men compared with age-matched non-epileptics. The contributions from the convulsions, underlying neurological disease, anticonvulsant drugs and/or psychosocial concomitants to the

reproductive endocrine disorders remains difficult to disentangle. We therefore examined the widely used anticonvulsants valproate (VPA) and carbamazepine (CBZ) for steroidal bioactivity using yeast transformed with a steroid receptor-reporter system. Yeast was stably transformed with human cDNA for human androgen receptor (AR), progesterone receptor A (PR) or estrogen receptor α (ER) together with a reporter plasmid containing a β-galactosidase gene under transcriptional control of an androgen (ARE), progestin (PRE) or estrogen (ERE) reporter element. Bioassays were established by culturing transformed yeast in the absence (agonist bioassay) or presence (antagonist bioassay) of fully stimulating doses of cognate ligands (5 nmol/L testosterone for AR, 1.6 nmol/L progesterone for PR and 5 nmol estradiol for ER) for 4 (PR) or 16 (AR, ER) hours. The bioassay end-point was β-galactosidase activity measured by a standard method in yeast cell lysates. VPA showed dose-dependent inhibition of progesterone-induced PR activity (10μM, 22±7%, $p<0.001$; 3μM, 58±5%, $p<0.005$; 1μM, 81±10%, $p<0.05$; 0.5μM, 98±14%, p=NS) first significantly detectable at a VPA concentration of 1 μM. Similarly, VPA inhibited testosterone-induced AR activity (10μM, 47±10%, $p<0.005$; 3μM, 74±10%, $p<0.05$; 1μM, 98±6%, p=NS; 0.5μM, 98±9%, p=NS) first significantly detectable at 3 μM. VPA had no ER antagonist bioactivity nor any AR, PR or ER agonist bioactivity. CBZ had no significant agonist or antagonist AR, PR or ER bioactivity. We conclude that VPA is a non-steroidal antagonist for human AR and PR but not ER whereas CBZ has no detectable agonist or antagonist steroidal bioactivity. VPA's androgen and progesterone antagonism at concentrations two order of magnitude lower than therapeutic blood levels (350-700 μM) seems likely to contribute to the frequency of reproductive endocrine disturbances among patients treated with VPA.

173

THE NOVEL METALLOPEPTIDASE, ANGIOTENSIN-CONVERTING ENZYME 2, IS SELECTIVELY EXPRESSED IN MATURE LEYDIG CELLS

Gabrielle C. Douglas[1], Moira K. O'Bryan[2], Mark P. Hedger[2], Rebecca A. Lew[1], A. Ian Smith[1]

[1]Baker Heart Research Institute, Melbourne, [2]Monash Institute of Reproduction and Development, Monash University, Melbourne.

The metallopeptidase angiotensin-converting enzyme (ACE) plays a pivotal role in the cardiovascular system by generating the vasoconstrictor peptide angiotensin II (Ang II). A splice variant of ACE is expressed in the germ cells of the testis, where it is essential for fertility. A homologue of ACE, termed ACE2, has recently been cloned from human heart and shows an expression pattern restricted to coronary and renal endothelial cells and to testis, implicating the enzyme in both cardiovascular and reproductive function. *In vitro* studies suggest that ACE2 is strictly a carboxypeptidase with a limited substrate specificity, which is insensitive to inhibitors of its homologue ACE. Indeed, the most likely physiological substrate is Ang II itself, which is cleaved to form Ang 1-7, a putative vasodilator. To date, no study has examined the cellular localisation of ACE2 in the testis. Using specific antisera raised against synthetic peptide sequences from the cloned sequence of ACE2, we have localised ACE2 to Leydig cells within the rat testis and to both Leydig and Sertoli cells in the human. Immunoreactive ACE2 is also present in foetal rat Leydig cells up to three days post-partum. As the foetal cells differentiate

into immature Leydig cells, ACE2 staining disappears and does not reappear until the cells have matured and are capable of secreting testosterone. Destruction of adult Leydig cells with the selective toxin ethane dimethane sulfonate ablated ACE2 staining in the testis. ACE2 catalytic activity assayed using a specific quenched fluorescent substrate, was detected in both testis and Leydig cell membranes; this activity was substantially reduced by an ACE2-specific inhibitor. Western blot analysis was employed in parallel to assess both the expression level and molecular size of ACE2, as confirmed by expressed recombinant human ACE2. These results suggest a role for ACE2 in the control of testicular function in the rat, possibly in the degradation of Ang II, which is believed to modulate testosterone synthesis and germ cell development.

174

MORPHOLOGY OF THE HUMAN TESTIS FOLLOWINGVASECTOMY

R.I. McLachlan[1], D.A. Raleigh[1], L. O'Donnell[1], G. Southwick, D.M. de Kretser[2]

[1]Prince Henry's Institute of Medical Research, and [2] Monash Institute Reprod. & Development. Clayton, 3168.

Introduction: Vasectomy reversal (VR) surgery only restores fertility in ~50% of men. Adverse effects on the testis and epididymis may contribute to infertility after VR. We performed a quantitative study of the effect of vasectomy on the testis focusing on changes in germ cell populations and interstitial fibrosis. Methods: Bouins-fixed, paraffin-embedded testicular biopsies were obtained from 34 men at VR, after an obstructive interval of 1-20 years. A portion of each biopsy was reprocessed into methacrylate resin to obtain 25µm H&E-stained sections for counting of germ cell populations using the optical disector stereological approach and germ cell data were expressed per Sertoli cell (N/SC). Two µm paraffin wax sections were stained using the Masson's Trichrome method and the percentage of testicular fibrosis quantified by point counting. Germ cell numbers and testicular fibrosis content were compared to values from biopsies obtained from 10 age-matched normal men. Results: Compared to control men, vasectomy did not change Sertoli cell numbers (N/g testis). Spermatogonial & pachytene spermatocyte numbers were unchanged but round and elongated spermatids were decreased ($p<0.001$) in vasectomised subjects (by 31.7 % & 37.8%, respectively). Both spermatid populations were related to the obstructive interval ($p<0.05$). A 2.7 fold increase ($p<0.001$) in testicular fibrosis was observed with vasectomy. Fibrosis worsened with an increasing obstructive interval ($p<0.001$) and showed a negative relationship with decreased spermatid numbers ($p<0.05$). Serum sperm antibody levels were not associated with effects on germ cell numbers. Neither spermatid number nor the extent of fibrosis were predictive of sperm counts 3-6 months after VR. Conclusion: Vasectomy causes a decline in spermiogenic efficiency and an increase in fibrosis, with these changes worsening with increasing time since vasectomy. The impact of vasectomy on the testis should be taken discussed when advising the procedure and when considering the basis for unsuccessful VR.

175

INHIBITION OF OXYTOCIN RECEPTORS AND ESTROGEN RECEPTOR ALPHA IN RELAXIN GENE KNOCKOUT MICE

Andrew L. Siebel, Laura J. Parry

Department of Zoology & Howard Florey Institute, University of Melbourne, Victoria 3010.

The oxytocin receptor (OTR) is an important contractile-associated protein highly up-regulated in the myometrium at term. Activation of this receptor stimulates myometrial contractions and leads to delivery of the young. In rodents, the increase in OTRs in the myometrium is primarily influenced by estrogen (1). However, regulation of OTRs may also involve an interaction with the ovarian peptide hormone relaxin (RLX). Relaxin decreases oxytocin-stimulated rat myometrial contractions through inhibition of phosphatidylinositolphosphate turnover (2). Recent evidence suggests that the uterotrophic effects of RLX are mediated by activation of estrogen receptors (ERs) (3). Thus, an alternative regulatory effect of RLX on uterine OTRs may be stimulatory via activation of the ERα. This study used a RLX gene knockout mouse ($Rlx^{-/-}$) to investigate the effects of RLX on myometrial OTR and ERα expression in late pregnancy. Tissues were obtained from time-mated C57/Blk6J $Rlx^{+/+}$ mice (Howard Florey Institute) at 5 stages of gestation (days 7.5, 10.5, 14.5, 17.5, 18.5 post coitum, *pc*) and $Rlx^{-/-}$ littermates on days 14.5 and 18.5 *pc*. Quantitative analysis of OTR and ERα mRNA was performed using real-time PCR. OTR mRNA expression in the myometrium of $Rlx^{+/+}$ mice was significantly ($p<0.025$) higher on day 18.5 *pc* compared to all other stages of gestation. In contrast, myometrial OTR mRNA concentrations were significantly ($p<0.02$) lower in $Rlx^{-/-}$ mice on both days 14.5 and 18.5 *pc* compared to $Rlx^{+/+}$ mice. Myometrial ERα gene expression increased significantly ($p<0.05$) on day 14.5 *pc* in $Rlx^{+/+}$ mice and remained high throughout gestation. However, there was no increase in ERα gene expression in the myometrium of $Rlx^{-/-}$ mice on days 14.5 or 18.5 *pc*, with significantly ($p<0.02$) lower concentrations compared to $Rlx^{+/+}$ mice. This study demonstrated that the up-regulation in OTR and ERα expression observed in the myometrium of late pregnant $Rlx^{+/+}$ mice did not occur in $Rlx^{-/-}$ mice. We suggest that the effects of RLX on OTRs are mediated via activation of ERαs and that RLX potentially regulates both OTRs and ERαs in the myometrium.

(1) Fang X *et al.* (1996) Endocrinology 137:3213-3219
(2) Sanborn *et al.* (1995) Progress in Relaxin Research p289
(3) Pillai SB *et al.* (1999) Endocrinology 140:2426-2429

176

EXPRESSION OF THE RELAXIN RECEPTOR (LGR7) AND RELATED GENES DURING THE PHASES OF THE MENSTRUAL CYCLE

Courtney P. Bond[1], Laura J. Parry[2], Chrishan S. Samuel[2], Helen M. Gehring[2], Peter A.W. Rogers[1], Roger J. Summers[1]

[1]Monash University, Victoria 3800, [2]Howard Florey Institute, Victoria 3010.

Relaxin (Rlx) is a 6kD insulin-like peptide that acts at its own receptors and is produced by the ovaries to cause connective tissue remodeling of the rodent and pig reproductive tract to facilitate parturition[1]. In humans, plasma relaxin levels increase in the 1st trimester of pregnancy in

contrast to rodents and pigs where relaxin levels increase towards the end of pregnancy[1]. Recent studies indicate that relaxin is secreted from glandular epithelial and stromal cells[2]. Here we examined the binding of [^{33}P]-H2Rlx, the expression of 2 relaxin peptides, the LGR7 receptor (the relaxin receptor[3]) and VEGF in samples from human uterus at different stages of the menstrual cycle. Quantitative autoradiography identified a 3.5 fold increase in relaxin receptors in the uterine endometrium in sections obtained from the secretory phases compared to the proliferative phases. Receptor binding was greater in the glandular epithelial cells than stromal cells. Competition binding on human uterus sections showed that H2Rlx (pK_i 8.0 ± 0.06) had a greater affinity for [^{33}P]-H2Rlx binding than PRlx (pK_i 7.4 ± 0.21). RT/PCR carried out on RNA samples isolated at different stages of the menstrual cycle showed an up regulation of LGR7 receptor expression during the secretory phases that paralleled the [^{33}P]-H2Rlx binding in the autoradiographic study. RT/PCR performed on RNA extracted from isolated cells showed greater LGR7 receptor expression in glandular epithelial cells than in stromal cells. H1Rlx was not expressed by the uterus while H2Rlx expression was low and not altered by the stage of the menstrual cycle. VEGF expression increased during the late-secretory phase. The studies show that the expression of the relaxin receptor (LGR7) changes markedly in glandular epithelial cells with the phases of the menstrual cycle suggesting a specific role for the hormone in the physiology of the uterus.

(1) Sherwood, O.D. (1994) *The physiology of reproduction*, Raven Press Ltd, NY. 861-1009.
(2) Palejwala, S., Tseng, L., Wojtczuk, A., Weiss, G. & Goldsmith, L.T. (2002) *Biol of Reprod*., **66**, 1743.
(3) Hsu, S.Y., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., Sherwood, O.D. & Hsueh, A.J.W. (2002) *Science*, **295**, 671.

177

AUSTRALIAN AND NEW ZEALAND ACROMEGALY REGISTRY (ANZAR): DEVELOPMENT AND AIMS

Ross C. Cuneo[1], Ken Ho[2], Mark McLean[3], Warrick Inder[4], Ian Holdaway[5], Steven Boyages[3], Stephen Judd[6], R. Leigh Atkinson[7]

[1]Metabolic Research Unit, University of Queensland and Department of Diabetes and Endocrinology, and [7]Department of Neurosurgery Princess Alexandra Hospital, Brisbane; [2]Garvan Institute for Medical Research, Darlinghurst, Sydney, [3]Department of Endocrinology, Westmead Hospital, Sydney; [4]Department of Endocrinology, St. Vincent's Hospital, Melbourne; [5]Department of Endocrinology, Auckland General Hospital, Auckland; [6]Department of Medicine, Flinders Medical Centre, Adelaide.

Acromegaly is an uncommon disorder characterised by excessive secretion of Growth Hormone (GH) usually from a pituitary adenoma. Treatment options include neurosurgical resection, radiotherapy or radiosurgery, and medical options directed at reducing secretion (with dopamine agonists and somatostatin analogues) or blocking GH-receptor activation (with a genetically engineered GH receptor antagonist). A number of controversies exist in the timely diagnosis and selection and optimisation of treatments. Since the disorder is uncommon, with international incidence of 1-3 new cases per million per year, few centres generate extensive experience. Delayed diagnosis implies a larger adenoma, with an attendant reduction in the efficacy of most treatment options. The relative cost-effectiveness of various treatment options are poorly described. The data regarding Australian incidence, prevalence, treatments offered and outcomes

have not been adequately reported. The New Zealand experience has received wide recognition (1).

A Steering Committee has been formed to explore these and related issues through the development of a regional tumour registry for Australia and New Zealand. The initial aims are to describe the demographics of patients with acromegaly seen in tertiary referral centres between 1980 and the present, to assess whether therapies have changed with time, and to describe the efficacy and adverse effects of those treatments. Progress will be reported on the identification of the ethical issues involved in such data collection, in developing a reporting and analysis system, and in securing sponsorship. Endocrinologists, neurosurgeons, radiotherapists and other interested parties are invited to participate in this on-going project. Future developments will aim to answer more specific questions regarding the diagnosis and management of acromegaly, and later to expand to include other pituitary tumour types.

(1) Determinants of clinical outcome and survival in acromegaly. Rajasoorya, C, Holdaway IM, Wrightson P, Scott DJ and Ibbertson HK. Clin-Endocrinol 1994; 41(1): 95-102.

178

COMBINED THYROXINE/LIOTHYRONINE TREATMENT DOES NOT IMPROVE WELL-BEING, QUALITY OF LIFE OR COGNITIVE FUNCTION COMPARED TO THYROXINE ALONE: A RANDOMIZED CONTROLLED TRIAL IN PATIENTS WITH PRIMARY HYPOTHYROIDISM

John P. Walsh[1], Lauren Shiels[2], Ee Mun Lim[3], Chotoo I. Bhagat[3], Lynley C. Ward[1], Bronwyn G.A. Stuckey[1], Satvinder S. Dhaliwal[1], Gerard T. Chew[1], Minoti C. Bhagat[1], Andrea J. Cussons[1]

[1]Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands WA; [2]Department of Psychiatry and Behavioural Science, Sir Charles Gairdner Hospital; [3]Western Australian Centre for Pathology and Medical Research (PathCentre),Nedlands, WA.

Thyroxine is standard treatment for hypothyroidism. A recent study reported that combined thyroxine/liothyronine (T_3) treatment improved well-being and cognitive function compared to thyroxine alone. We conducted a double blind, randomized, controlled trial with a crossover design in 110 patients (101 completers) with primary hypothyroidism in which liothyronine 10 μg was substituted for 50 μg of the patients' usual thyroxine dose. No significant (P<0.05) difference between thyroxine and combined thyroxine/T_3 treatment was demonstrated on cognitive function, quality of life scores (SF-36), Thyroid Symptom Questionnaire scores, subjective satisfaction with treatment or 8 of 10 visual analog scales assessing symptoms. For the General Health Questionnaire-28 and visual analog scales assessing anxiety and nausea, scores were significantly (P<0.05) worse for combined treatment than for thyroxine alone. Serum TSH was lower during thyroxine treatment than during combined thyroxine/T_3 treatment (mean ± SEM 1.5 ± 0.2 *vs.* 3.1 ± 0.2 mU/L, P<0.001), a potentially confounding factor; however, subgroup analysis of subjects with comparable serum TSH concentrations during each treatment showed no benefit from combined treatment compared to thyroxine alone. We conclude that, in the doses used in this study, combined thyroxine/T_3 treatment does not improve well-being, cognitive function or quality of life compared to thyroxine alone.

CHALLENGES IN THE MANAGEMENT OF THYROID CANCER

Georgia Soldatos, Helena Teede

Endocrinology Unit, Monash Medical Centre, Clayton, Victoria 3169.

We report 2 cases of thyroid carcinoma presenting with the unusual clinical manifestation of spinal cord compression (SCC) secondary to spinal metastases.

A 61 year old man presented in October 2002 with progressive bilateral leg weakness and clinical signs of SCC. Initial MRI confirmed the presence of cord compromise at T7 secondary to a probable bony metastasis. Urgent decompressive laminectomy of T6/7/8 revealed follicular carcinoma and was followed by spinal irradiation. Total thyroidectomy identified bilateral, well differentiated follicular carcinoma. Although he remained asymptomatic during 6 months of follow up, T3 toxicosis developed. A WBI scan demonstrated iodine avid disease in the neck, thorax and pelvis and he proceeded to 30mCi of I^{131} under steroid cover to avoid cord compression and bone marrow toxicity given mild leucopenia pre treatment. The plan is for further, small doses of I^{131} depending on disease activity and response.

This case is a reminder of this rare presentation of thyroid carcinoma and its even rarer association with thyrotoxicosis. The latter is important when deciding on an appropriate dose of RAI, as its conversion to radiothyroxine by functioning carcinoma increases the potential for systemic toxicity (1).

The second case is a 24 year old who presented with progressive mid - thoracic pain and clinical signs of SCC, on the background of a right struma ovarii containing benign thyroid tissue, approximately 1 year earlier. A destructive bony lesion at T8 was confirmed on imaging and she proceeded to decompressive laminectomy followed later by spinal stabilisation. The histopathology was consistent with follicular carcinoma of the thyroid. A total thyroidectomy was performed but no focus of tumor was identified, so the source of the vertebral lesion was presumed to be malignant struma ovarii, despite the "benign" initial pathology. Clinically the patient made a good recovery and has been treated with 3 doses of RAI of 100, 200 and 100mCi respectively, 5, 11 and 23 months post thyroidectomy. She has also completed a course of spinal irradiation.

The ongoing question in this lady is the value of further RAI for an isolated, relatively asymptomatic, low iodine avid (1%) vertebral lesion in the setting of a raised, but stable thyroglobulin.

(1) Sisson et al, 2001. J Nucl Med 42:975 - 983

180

THE CLINICAL EFFECTS OF THYROID STUNNING AFTER DIAGNOSTIC WHOLE BODY SCANNING WITH 5 MCI ^{131}I

William Lees[1], Robert Mansberg[1], John Roberts[1], Jocelyn Towson[1], Elizabeth L. Chua[2], John R. Turtle[2, 3]

[1]Department of PET and Nuclear Medicine and [2]Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, NSW 2050, and [3]Department of Medicine, University of Sydney, NSW 2006.

'Thyroid stunning' from diagnostic ^{131}I imaging prior to ablative therapy with ^{131}I for well-differentiated thyroid carcinoma has been well reported, but documentation of effect on clinical outcome is sparse. The purpose of this retrospective study was to investigate the clinical effects of stunning. The outcome of ^{131}I ablative therapy in a group of patients (n=36) who had diagnostic scans using 185 MBq (5 mCi) of ^{131}I was compared to that in a group (n=36) who had diagnostic scans using 740 MBq (20 mCi) of ^{123}I. Patients were imaged at least 4 weeks after near-total thyroidectomy, prior to their first ^{131}I ablative therapy. Follow-up imaging was performed every 3-6 months, and further ^{131}I treatment administered when indicated. A group of patients (n=36) who proceeded directly to their first therapy dose without a diagnostic scan and were followed up with ^{123}I was compared to the group who did have a ^{123}I diagnostic scan prior to the first ablative therapy. The efficacy of therapy was evaluated using ablation of the thyroid, evidenced by the absence of uptake in the thyroid bed on the diagnostic scan, as endpoint. Only 47% of patients in the ^{131}I diagnostic group had the thyroid gland ablated after a single administration of therapy ^{131}I, compared to 86% in the ^{123}I diagnostic group ($p < 0.005$). Patients who had ^{131}I diagnostic scans required higher total ^{131}I therapeutic activity (6.7 GBq or 180 mCi) to ablate the thyroid gland than those in the ^{123}I diagnostic group (4.4 GBq or 119 mCi). There was no difference in outcome between the group who did and the group who did not have a diagnostic study with ^{123}I prior to their first ablative therapy. The difference in outcome between the ^{131}I and the ^{123}I diagnostic groups demonstrates that the efficacy of ^{131}I therapy is reduced subsequent to the use of 185 MBq (5 mCi) of ^{131}I for diagnostic imaging. This indicates that the phenomenon of stunning is clinically significant and affects the outcome of therapy.

181

THE IMPORTANCE OF SUBTLE THYROGLOBULIN CHANGES IN FOLLOW UP OF THYROID CANCER

Carol Tong, Peter Colman

Department of Diabetes and Endocrinology, Royal Melbourne Hospital.

A 41 yo lady, with no significant past history, presented in 1993 with a neck lump, which was papillary carcinoma on biopsy. Histopathology following total thyroidectomy revealed multifocal papillary thyroid cancer and five metastatic lymph nodes. Following surgery, an iodine-131 whole body scan (WBS) showed residual thyroid tissue and cervical lymph node metastases. Ablation with 80 mCi iodine-131 followed. The post-ablation whole body scan showed uptake in the left lobe of the thyroid only. She was commenced on thyroxine 150 mcg daily. From 1993 to 2002 thyroxine dose was titrated to achieve TSH suppression. Between 1995-1998 TSH was greater than 0.10 mU/L on 4/9 occasions. Iodine-131 WBS in 1994 and 1996 showed no residual

thyroid disease or metastases. An unstimulated thyroglobulin in 1996 was < 2.5 pmol/L. Thyroglobulin remained < 1 pmol/L until February 2002, when the thyroglobulin increased to 3.3 pmol/L (2.1 ng/ml). The laboratory report noted that following a change of assay in 2001, results were 1.5 times those of the previous assay. As a result the change was not considered significant. She presented with cough in November 2002. TSH was 1.27 mU/L and thyroglobulin 20 pmol/L. CT chest and PET scan showed pulmonary and neck metastases, which were resected and confirmed as metastatic papillary carcinoma. An iodine-131 WBS in April 2003 showed no uptake in the neck or chest, despite a stimulated thyroglobulin of 7.3 pmol/L. Neck ultrasound and CT chest and abdomen were negative.

This case illustrates the importance of thyroglobulin in surveillance of thyroid cancer. A recent consensus report (Mazzaferri et al, JCEM 88(4);1433-1441) suggests a TSH-stimulated thyroglobulin cutoff of 2 ng/mL (3.1 pmol/L) as a marker of recurrence. Additionally it highlights the importance of a reproducible and robust thyroglobulin assay. In this case, the change in assay may have confused identification of a true and significant rise in thyroglobulin.

182

PERSONAL EXPERIENCE OF THE DIAGNOSIS AND MANAGEMENT OF TSHOMA

Marianne Elston, Louise Wolmarans, John V. Conaglen

Department of Endocrinology, Waikato Hospital, Hamilton, New Zealand.

Thyrotropin-producing pituitary adenoma (TSHoma) is a rare condition with an estimated prevalence of 0.05 to 2% of pituitary adenomas. Patients are often misdiagnosed as having primary hyperthyroidism and may receive inappropriate treatment. As a result the adenoma is often large at diagnosis and cure rates are low.

We present the clinical characteristics of seven patients who presented between 1990 and 2003 with clinical thyrotoxicosis and inappropriate secretion of TSH (thyroid-stimulating hormone) and were diagnosed as having a TSH-secreting pituitary adenoma. Four of the patients were female and the mean age of the group at presentation was 41 years (range 20 to 58 years). Five of the seven patients had macroadenomas on imaging and four were inappropriately treated for primary hyperthyroidism before the diagnosis of TSHoma was made.

Data on the presentation, clinical findings, results of investigations, treatment, and outcome after a mean follow up of five years (range 0.5 to 12 years) will be presented. With increased awareness and earlier diagnosis, management can be appropriately directed towards the pituitary.

CLINICAL BEHAVIOUR OF THYROID CANCER IN NEW CALEDONIA

A. Omari, E.L. Chua, J.R. Turtle

Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown 2050, NSW, Australia.

Background. New Caledonia, located between Australia and Fiji, has the highest prevalence of thyroid cancer in the world, at 35/100000 population in 1992. A profile of thyroid carcinoma in New Caledonia has been evaluated over a 7 year period. **Methods**. Between 1/1/1995 and 31/12/2001, 227 patients with thyroid cancer were managed in one unit at Royal Prince Alfred Hospital. Demographic data, histology and extrathyroidal metastases at diagnosis (clinical, radionuclide, computerised tomographic scanning) were examined. Tumour size was available in 97 patients. Statistical analysis utilised Chi-squared and t-tests.

	Total	Female	Male	Papillary	Follicular
Number, n (%)	227	202 (89%)	25 (11%)	207 (91%)	18 (8%)
Age, mean (range)	47.2 (21-77)	46.6 (21-77)	52.3 (32-73)	47.0 (23-77)	49.1 (21-69)
Metastases, n (%)	53 (23%)	43 (21% of F)	10 (40% of M)	46 (22% of PTC)	7 (39% of FTC)

Results. The mean age at diagnosis was 47.2 years, with males on average 6 years older than females ($p<0.05$). Papillary thyroid cancer (PTC) was the most frequent type accounting for over 90% of cancers. The average size of thyroid cancers at surgery was 15 mm with 56% of the PTC tumours greater than 10mm. At diagnosis, 53 patients (23%) had extrathyroidal spread with a significantly higher proportion in the FTC group ($p<0.02$). 19 patients had markedly elevated serum thyroglobulin in excess of 1000 μg/L (RR:0-30). **Conclusions**. Thyroid cancer in New Caledonia is a major health problem when compared to the rest of the world. A higher female to male ratio of 8:1 was noted, compared to published data elsewhere suggesting a ratio of 3:1. Most cases are PTC, reflecting a trend reported in other countries with up to 80% comprising PTC. Unlike other studies with an incidence of metastases of <10%, metastatic spread (23%) was more common, representing an aggressive form of the disease present in New Caledonia. The reasons for these differences are not completely understood, but both genetic and environmental factors may be involved.

PREVALENCE OF ABNORMAL TSH LEVELS AND UNDIAGNOSED THYROID DISEASE IN A RANDOMLY SAMPLED AUSTRALIAN FEMALE POPULATION

L. Rivera-Woll, S.L. Davison, R. Bell, S.R. Davis

***The Jean Hailes Foundation Research Unit, 173 Carinish Rd, Clayton, Victoria, 3168. phone 61 3 95439612 facsimile 61 3 95439609*. email research@jeanhailes.org.au**

Objective: To establish the prevalence of abnormal TSH in a random sample of Victorian women and to determine the prevalence of undiagnosed thyroid disease and inadequately treated thyroid disease.

Design: Population-based cross-sectional study.

Participants: 1234 women (aged 18–75 years) randomly recruited across Victoria via electoral rolls.

Measurements: All women completed a health questionnaire in which they reported the presence of any thyroid disease and any treatment for this. TSH was measured in a single fasting blood sample using an immunometric assay. A TSH level between 0.5-4.0mIU/L was defined as normal.

Results: 9.9% of women had an abnormal TSH and 7.7% had undiagnosed thyroid disease. Of the latter 64% had a TSH>4.0mIU/L and 36% had a TSH<0.5mIU/L. 6.2% of women reported pre-existing thyroid disease and 51% of these were on thyroxine. 22% of women with known thyroid disease had a TSH>4.0mIU/L, 59% of these were on thyroxine and thereby having inadequate replacement therapy. 13% had a TSH<0.5mIU/L, 90% of these women were on thyroxine and hence being overtreated. Refer to table.

TABLE 1. Number of women with abnormal TSH

Thyroid disease	known		no known	total
	thyroxine	no thyroxine		
TSH>4.0mIU/L	10	7	61	78
TSH<0.5mIU/L	9	1	35	45
TSH normal	21	29	1061	1111
total	40	37	1157	1234

Conclusions: There is a high prevalence of undiagnosed thyroid disease amongst Victorian women, in particular hypothyroidism. Given the association with common illnesses that affect the community screening for thyroid disease in women may be appropriate but further studies into the cost effectiveness of this are required.

This study was supported by a philanthropic by Sue Ismiel and daughters. No conflicts of interest.

DISSECTING THE BIOLOGY OF FOLLISTATIN ISOFORMS USING TRANSGENIC MICE

Shyr-Yeu Lin, David M. de Kretser, John R. Morrison

Monash Institute of Reproduction and Development, Monash University, Melbourne.

The importance of follistatin in regulating reproductive processes and development is well recognised. Follistatin is widely expressed as two separate isoforms: FS-288 and FS-315. The biology of each isoform is poorly understood although FS-315 is the likely circulating form while FS-288 appears to be bound to heparin-sulphate proteoglycans. FS-315 is expressed at approximately 20 times the level of FS-288. The follistatin knockout mice die soon after birth due to breathing difficulties. To further address the role of follistatin, several transgenic mouse lines were generated expressing specific follistatin isoforms using human genomic constructs that were 95Kb in length and were then crossed onto the follistatin knockout background to generate "rescued mice". Three transgenic lines were made: i) FS-tg expresses both FS-288 and FS-315 isoforms; ii) FS-288tg expressing only FS-288 isoform; iii) FS-315tg expresses only FS-315 isoform.

FS-tg (control) transgenic lines, failed to rescue the knockout phenotype. These mice were able to breathe normally but were unable to feed for reasons that are not entirely clear. The transgene expression levels indicated that the human gene was expressed at levels well below that of the endogenous gene. Detailed histological examination of all major tissues did not identify major developmental defects that led to the death of FS-tg rescue mice. The phenotype of the FS-288tg rescue mice appeared to be identical to that of the FS-tg rescue mice. In contrast the FS-315tg did rescue many aspects of the knockout phenotype. The FS-315tg rescued mice displayed some external abnormal appearances including whisker disorientation, microphthalmia, ear patterning defects and angiogenesis abnormalities in the tail. The males mated but appeared infertile. The FS-315tg rescue mice are smaller than their littermates and while they initially appeared healthy, the males died at approximately 2 months of age.

The observation that FS-315 isoform is expressed at approximately the same levels in both the FS-315tg and FS-tg models presents a conundrum. Why do the FS-tg rescued mice die? I hypothesize that the FS-288 is acting to neutralize the biological actions of the FS-315. To test this, I am currently crossing both transgenic lines onto the FS knockout background to generate doubly rescued mice with the prediction that these mice will also die.

REGIONAL DISTRIBUTION OF ACTIVIN -βA, AND βC SUBUNIT PROTEINS AND FOLLISTATIN WITHIN ADULT MOUSE PROTATE DUCTS

Elspeth Gold[1], Hong Wang[1], Sally Mellor[1], Mark Cranfield[2], Nigel Groome[2], Gail Risbridger[1]

[1]Monash Institute of Reproduction and Development, Monash University, Melbourne Australia, School of Biological and [2]Molecular Sciences, Oxford Brookes University, Headington, Oxford, United Kingdom.

The overall growth of the prostate is a co-ordinated balance between positive and negative regulators in epithelium and stroma. As well, the glands exhibit regional variation in proliferative activity, being highest in distal tips and lowest in the proximal region. Conversely the highest apoptotic activity is present proximally and lowest in distal tips (1). Stromal-epithelial interactions mediated by growth factors may be responsible for this regional variation in proliferative and apoptotic activity (2). Activin inhibits cellular proliferation in the prostate and this effect is negated by follistatin (3,4). The aim of this study was to determine if there is a correlation between expression of activin A and follistatin regionally within prostate ducts. In addition the expression of the related activin-βC subunit (a putative antagonist of activin A via intracellular dimerisation) was examined. Adult anterior and ventral prostate lobes were collected, ductal spreads performed and tissues orientated proximal to distal under a dissecting microscope. Tissue was Bouins fixed, paraffin embedded and prepared for histology. Serial sections were used for immunolocalisation of activin-βA and βC subunit proteins, follistatin, basal epithelial cells and smooth muscle. Activin-βA subunit and follistatin were localised to secretory epithelial cells in the intermediate and distal regions. There was marked activin-βC subunit protein within the proximal epithelium. Activin-βC subunit immunoreactivity was also evident within prostate smooth muscle in all regions. Abundant smooth muscle proximally displayed intense activin-βC-activin subunit immunoreactivity. We conclude that co-localisation of activin-βA subunit protein and follistatin implies in vivo interplay in prostate secretory epithelial cells in regions with high proliferative activity. Activin-βC subunit proteins were present in stroma where they cannot dimerise with the activin-βA subunit, suggesting activin-βC ligands function independently of activin-βA within prostate stroma. Regional variations in stromal and epithelial activins may be involved in prostate tissue homeostasis.

(1) Lee C et al. Biol Reprod 43: 1990;
(2) Tenniswood M. Prostate 9: 1986;
(3) McPherson SJ et al. J Endocrinol 154: 1997;
(4) Wang QF et al. Endocrinology 137: 1996.

187

INHIBIN ALPHA SUBUNIT - REGULATION IN PROSTATE CANCER CELL LINES

Preetika Balanathan[1], Emma M.A. Ball[1], Sarah E. Harris[2], Andrew N. Shelling[2], Gail P. Risbridger[1]

[1]Centre for Urological Research, Monash Institute of Reproduction and Development, Monash University, Melbourne, Australia. [2]Department of Obstetrics and Gynaecology National Women's Hospital, Auckland, New Zealand.

Inhibin and activin are members of the TGFβ superfamily of growth and differentiation factors. They were first identified as gonadal-derived regulators of pituitary FSH and were subsequently assigned multiple actions in a wide range of tissues. The tumour suppressive role of the inhibin alpha subunit (INHα) in the gonads and adrenal glands was initially identified and characterised by using INHα knockout mice. Prostate cancer cell lines derived from human prostate carcinomas do not express INHα, whereas normal human prostate is positive for INHα expression. The aim of this study is to determine the cause of this inactivation of INHα gene expression in prostate cancer cell lines and determine the role INHα plays in prostate tumourigenesis.

Methylation of the regulatory region of INHα gene in LNCaP, DU145 and PC3 prostate cancer cell lines was revealed by bisulphite genomic sequencing. The pattern of methylation observed in the cell lines demonstrates that INHα gene is silenced since methylation of the CpG sites in the regulatory region of tumour suppressor genes is a common aberration in human cancers. Re-activation of INHα gene transcription was observed after treatment of the cell lines with reagents that inhibit DNA methylation and histone deacetylation. Down-regulation of gene expression was also demonstrated by transient transfection of a luciferase reporter gene driven by a methylated INHα promoter in a mouse adrenal cell line suggesting that the mechanism of methylation alone is sufficient to down-regulate transcription from the INHα promoter.

The data above demonstrate that promoter methylation is associated with the regulation of the INHα gene in prostate cancer cell lines. The results are consistent with a tumour suppressive role of INHα in prostate carcinomas and further support the characterisation of INHα as a tumour suppressor.

188

USE OF REAL-TIME PCR TO MEASURE PERI-OVULATORY CHANGES IN INHIBIN SUBUNIT MRNA EXPRESSION

Khiem Hua, Peter Illingworth

Department of Reproductive Medicine, Westmead Hospital, University of Sydney. NSW 2145.

INTRODUCTION: It was previously found (Newton *et al.*, Hum Reprod, 17: 38-43, 2002) that ovulation is associated with changes in the relative concentrations of inhibin A and activin A proteins. In order to study the molecular control of these protein changes, the mRNA expression of inhibin alpha, betaA, and betaB were studied relative to ovulation. The effects of a COX-2 inhibitor (NS-398) were also studied to investigate the role of prostaglandins.

METHODS: Murine preantral follicles from 21-day-old BALB/c mice were mechanically isolated and cultured in a non-spherical environment. After 8 days of culture, the follicles were studied for 24 hours ± hCG (2.5 IU/ml), ± NS-398 (0.1, 1, 10μM) with medium collected at 6-hour intervals. Quantitative changes in the expression of inhibin subunit mRNAs were measured using real-time reverse transcription PCR (RT-PCR). To improve the specificity for mRNA detection, intron-spanning primers were designed for inhibin alpha, betaA, and betaB. Beta-actin was used as internal standard for normalisation. Results are presented as the mean ± SEM ratio (n =5-7 follicles) of target gene and beta-actin.

RESULTS: In control follicles, without hCG, the expression of the different genes were inhibin alpha =2.23 ± 0.42, inhibin betaA =18.34 ± 5.63, inhibin betaB =15.90 ± 3.53. Following administration, of hCG the corresponding level of gene expression was significantly lower (than that observed without hCG) for all three genes (alpha, 0.18 ± 0.03; $p < 0.01$: betaA, 1.47 ± 0.31; $p < 0.01$: betaB, 1.33 ± 0.34; $p< 0.001$). NS-398 at 10μm caused a reduction in the number of follicles ovulating after hCG (54.4%) compared to hCG alone (77.9%). Progesterone secretion was not affected by NS-398. The treatment of follicles with NS-398 alone resulted in a 2-2.7 fold increase in the expression of the inhibin/activin subunits mRNA, but no influence on the effect of hCG.

CONCLUSIONS: This study demonstrates that the previously described increase in pre-ovulatory activin A secretion is not a result of increased betaA gene expression. These data raise the possibility that the rate of activin A secretion may be regulated by falls in inhibin alpha subunit synthesis.

189

REGULATION OF ACTIVIN/INHIBIN SUBUNIT AND STEM CELL FACTOR MRNA BY INTERLEUKIN-1 AND FOLLICLE-STIMULATING HORMONE IN RAT SERTOLI CELLS

Kazutaka Saito, Julie Muir, Anne O'Connor, David de Kretser, Mark Hedger

Monash Institute of Reproduction and Development, Monash University.

Spermatogenesis is under complex regulation involving the hormones, testosterone, follicle-stimulating hormone (FSH) and inhibin, and locally-produced cytokines, including interleukin-1α (IL-1α), activin A and stem cell factor (SCF). In a previous study, we have shown that FSH and IL-1α exert reciprocal effects on production of the activin A and inhibin B proteins in rat Sertoli cell cultures. The present study investigates this regulation, as well as that of SCF, at the mRNA level. Sertoli cells from 20 day old rats were cultured (48h, 37°C) with human IL-1α and/or human FSH or dibutyryl cAMP (dbcAMP). Activin A was measured in the culture medium by specific ELISA. The activin/inhibin subunits (α, β_A, β_B) and SCF mRNA were measured in cell extracts by real-time PCR using the LightCycler®. In a time course study, activin A secretion progressively accumulated in the culture medium over 48 hours in both basal and IL-1α-stimulated cultures. Increased secretion of activin A in the IL-1α-stimulated cultures was associated with a 4-fold increase of β_A mRNA peaking around 4-8h of culture. IL-1α caused a decline in β_B and α-subunit mRNA expression with maximum inhibition at 24h of culture, but had little or no effect on SCF mRNA expression over this time-period. In contrast, FSH (and dbcAMP) inhibited β_A mRNA expression, and stimulated β_B and SCF mRNA expression in the

cultures. These effects of FSH were also partially blocked by co-incubation with IL-1α. These data clearly demonstrate that the reciprocal regulation of activin A and inhibin B by IL-1α and FSH involves regulation at the transcriptional level. These data also suggest that hormonal control of spermatogenesis, by FSH in particular, is mediated through interactions between IL-1α, activin A and SCF produced by the Sertoli cells in the adult testis.

190

SUPPRESSING BETAGLYCAN EXPRESSION BY RNA INTERFERENCE (RNAI) REDUCES INHIBIN ACTIVITY IN LβT2 GONADOTROPES

Ruth Escalona, Paul Farnworth, Jock K. Findlay, Guck T. Ooi

Prince Henry's Institute of Medical Research, Clayton, Victoria 3168, Australia.

Inhibin, a gonadal peptide, is a key endocrine hormone that provides negative feedback control to the pituitary gonadotropes to regulate the expression and release of follicle-stimulating hormone (FSH). In gonadotropes, activin stimulates the expression of FSHβ-subunit as well as gonadotropin releasing hormone receptor (GnRHR), and both effects are antagonized by inhibin. Although gonadotropes possess a number of inhibin-specific binding proteins, the molecular mechanisms underlying inhibin regulation of FSH and GnRHR expression are not well understood. Betaglycan, a membrane proteoglycan, was recently shown to bind inhibin with high affinity in a complex with activin type II receptor, thereby antagonising activin signalling. Cells non-responsive to inhibin become inhibin-responsive when engineered to overexpress betaglycan, but the consequences of removing betaglycan were not known. We now demonstrate that reducing endogenous betaglycan expression in gonadotropes can relieve the antagonism of activin-A stimulated transcription of target genes. Two activin-responsive promoter constructs were used; 5.5 kb of the ovine FSHβ promoter (oFSHβ-lux), and three copies of the activin-responsive sequence of the GnRHR promoter (3XpGRAS-PRL-lux). These constructs were transfected into LβT2 mouse gonadotrope cells either alone or in the presence of a mixture of small (21bp) duplex RNAs corresponding to the betaglycan gene. These transfected siRNAs initiate RNA interference, causing the 'knock-down' of expression of genes whose sequence is homologous to the duplex RNA. To control for non-specific effects, a similar but unrelated siRNA corresponding to the BF-1 forkhead-like protein was used. Activin (1 nM) increased the activity of both promoters by 5-8 fold compared to untreated cells. Inhibin (0.5 nM) was able to suppress this activity by 51 $\pm$ 7% (mean $\pm$ SD, n=2) for 3XpGRAS-PRL-lux and 52 $\pm$ 11% for oFSHβ-lux. Similar suppression by inhibin was also seen for cells co-transfected with the control BF-1 siRNA. In contrast, inhibin's ability to suppress activin-stimulated activity was reproducibly and significantly reduced (24 $\pm$ 4%, $p<0.045$ for 3XpGRAS-PRL-lux, and 33 $\pm$ 3%, $p<0.005$ for oFSHβ-lux) in cells co-transfected with betaglycan siRNA, but not those receiving the control siRNA. These results indicate that expression of betaglycan in pituitary gonadotropes is essential for the endocrine effects of inhibin in the negative feedback control of FSH. Supported by the NHMRC of Australia (RegKey 241000)

REGULATION OF INHIBIN BINDING AND BETAGLYCAN EXPRESSION BY TGF-β SUPERFAMILY MEMBERS IN MOUSE ADRENAL AC CELLS

Yao Wang, Ruth Escalona, Jock K. Findlay, Guck T. Ooi, Paul Farnworth
Prince Henry's Institute of Medical Research, Clayton, Victoria 3168.

Inhibin A, a member of the transforming growth factor (TGF)-β superfamily, binds to adrenocortical cells with high affinity via at least eight membrane proteins, including the TGF-β co-receptor, betaglycan, but the adrenal function and signalling pathway(s) of inhibins are not clear. In engineered systems, inhibin A dominantly inhibits the actions of activin and BMP by sequestering the type II receptors for each agonist in high affinity complexes with betaglycan. We have found that inhibins A and B differentially antagonize activin and BMP action in the mouse adrenocortical (AC) cell line. In the present studies, we have examined competition for binding of radiolabelled ligands at the AC cell surface as the basis of the observed interactions, and investigated whether betaglycan expression and subsequent inhibin binding in AC cells are regulated by TGF-β superfamily members .

Inhibin A competed for binding of [^{125}I]activin A to AC cells, whereas activin A competition for [^{125}I]inhibin A binding was negligible. A 15 amino acid residue sequence in a putative loop of the inhibin α-subunit shows >80% similarity to BMPs, and a portion of specific [^{125}I]inhibin A binding to AC cells was competed by BMP-2 (IC50 of 0.35 nM), and BMP-6 and –7 (each IC50 »1 nM). Affinity labelled protein species of deduced size >170 kDa, consistent in size with glycosylated betaglycan, were the primary target for competition from BMPs. Overnight pre-treatment of AC cell cultures with activin A or B did not affect subsequent binding of [^{125}I]inhibin A to AC cells, whereas BMP-2, BMP-6 and BMP-7 pre-treatments each reduced such binding by around 30%. Betaglycan mRNA levels in AC cells measured using real-time RT-PCR were 34±5, 30±16 and 37±8 % lower (mean±SE, *n*=4, 3 and 5, respectively) after treatment of AC cells with BMP-2, BMP-6 or BMP-7, respectively, for the preceding 24 h, whereas inhibins and activins had little effect.

In summary, inhibin blocks activin from binding to its receptors on the adrenocortical cells, but resists competition from activin for its own binding, consistent with the dominant antagonism of activin action by inhibin. On the other hand, BMP competes with inhibin for binding to the adrenal cell membrane proteins, and suppresses the expression of betaglycan. According to the current model for inhibin action, these BMP effects would reduce the effectiveness of inhibin to sequester type II receptors for BMP. In conclusion, the adrenal effects of inhibin will be determined in part by the relative levels of extracellular BMP and activin, and the expression of betaglycan.

Funded by the NH&MRC of Australia (RegKey 241000)

REGULATION OF ADRENAL STEROIDOGENESIS BY ACTIVIN AND BMP, AND THEIR DIFFERENTIAL ANTAGONISM BY INHIBIN

Paul Farnworth, Yao Wang, Ruth Escalona, Peter Stanton, Jock Findlay, Guck T. Ooi
Prince Henry's Institute of Medical Research, Clayton, Victoria 3168.

Cytochrome P450 17α-hydroxylase 17,20-lyase (*Cyp17*) controls glucocorticoid and sex hormone biosynthesis from substrates such as progesterone (P) in steroidogenic tissues, including the adrenal cortex. Although inhibin A, a member of the transforming growth factor (TGF)-β superfamily, binds to adrenal cells with high and low affinity via multiple membrane proteins, including betaglycan, the adrenal function of inhibins is not clear. Current models limit inhibin action to the antagonism of agonists (activin, BMP) via sequestration of their type II receptors in complexes with betaglycan. We have used the mouse adrenocortical AC cell line to determine whether inhibins act alone or in combination with other TGF-β superfamily members to alter *Cyp17* expression and P metabolism.

The level of *Cyp17* mRNA in AC cells, measured using real-time RT-PCR, was inhibited by activins A and B, and BMP-2, -6 and -7, whereas BMP-3, a BMP antagonist in other systems, was a partial agonist relative to the other BMPs. These inhibitory effects occurred at the level of transcription since the luciferase activity of a human *Cyp17* promoter-reporter construct was similarly suppressed by each of these ligands. The *Cyp17* mRNA level was unaffected by either inhibin A or inhibin B acting alone. Nevertheless, both inhibins (2 nM) abolished the suppression of *Cyp17* expression by activin A or B, and relieved the suppression of *Cyp17* expression by BMP-7, but only inhibin A partly reversed the inhibitory effects of BMP-2 and -6. Consistent with the *Cyp17* mRNA results, the conversion of [^{3}H]P to 17α-hydroxyprogesterone and androstenedione was little affected by overnight treatment with inhibin A (2 nM), but was inhibited by BMP-2 (4 nM). However, inhibin A did not relieve the inhibition caused by BMP-2.

In summary, activins and BMPs suppress adrenal *Cyp17* expression by transcriptional mechanisms, with consequent inhibition of the 17α-hydroxylation of P, at least in the case of BMP-2. Inhibins A and B differentially antagonize the suppression of *Cyp17* expression by activins and BMPs, but neither inhibin alters *Cyp17* expression when acting alone, providing no support for an agonist-independent local action of inhibin in the adrenal. These interactions of inhibins suggest one of their roles in the adrenal cortex is to integrate the inputs from multiple members of the TGF-β superfamily to control androgen precursor synthesis.

Funded by the NH&MRC of Australia (RegKey 241000).

THE USE OF HORMONE TREATMENT TO REDUCE THE ADULT HEIGHT OF TALL GIRLS: LONG-TERM EFFECTS ON FERTILITY

Alison Venn[1], Fiona Bruinsma[2], Penelope Jones[2], Judith Lumley[2], Priscilla Pyett[2], Jo Rayner[2], George Werther[3]

[1]Menzies Centre for Population Health Research, University of Tasmania; [2]Centre for the Study of Mothers' and Children's Health, La Trobe University; [3]Centre for Hormone Research, Royal Children's Hospital, Melbourne.

Introduction: Although uncommon in recent years, treatment with high doses of oestrogen to reduce the adult height of tall girls has been available since the 1950s. Little is known about long-term outcomes.

Methods: This retrospective cohort study identified eligible subjects from the medical records of Australian paediatricians who assessed or treated tall girls from 1959 to 1992, and from self-referrals. Eligible subjects were those who had sought a medical opinion about their tall stature and had radiological assessment of their skeletal age: they included girls who received oestrogen treatment (diethylstilbestrol or ethinyl estradiol) in adolescence (treated group) and those who did not (untreated group). Women were traced and invited to complete a postal questionnaire and computer assisted telephone interview (CATI). Data on reproductive outcomes included history of infertility, pregnancies and births. Age adjusted relative risks (adj RR) are presented with 95% confidence intervals.

Results: 1,436 eligible subjects were identified: 1,221 from medical records and 215 from self-referrals. 1240 women (86.4%) were traced and of these, 79.5% (n=395) of treated and 62.1% (n=450) of untreated women agreed to participate. The mean age was 39.8 years (range 20-55) in the treated group and 37.7 years (range 23-54) in the untreated group. Women who were treated were more likely to have ever tried to become pregnant without success for a period of 12 months or more (35.2%) than untreated women (18.3%) (adj RR=1.9, 95% CI 1.5-2.4). Treated women were more likely to have seen a doctor because they were having difficulty getting pregnant (33.3%) than untreated women (17.9%) (adj RR=1.9, 95% CI 1.4-2.4) and to have ever taken fertility drugs (14.4% of treated, 6.1% of untreated women) (adj RR=2.3, 95% CI 1.4-3.6). The proportion of women in each group that had ever been pregnant was similar (77.6% treated, 77.1% untreated) (adj RR=1.0, 95% CI 0.9-1.1). Exclusion of women who self-referred to the study had little effect on the risk estimates.

Conclusions: Treatment with high dose oestrogens to reduce the adult height of tall girls appears to have long-term effects on fertility.

USING HORMONE TREATMENT TO REDUCE THE ADULT HEIGHT OF TALL GIRLS: ARE WOMEN SATISFIED WITH THE DECISION IN LATER YEARS?

Priscilla Pyett,[1] Jo Rayner,[1] Alison Venn,[2] Judith Lumley,[1] George Werther,[3] Fiona Bruinsma,[1] Penelope Jones [1]

[1]Centre for the Study of Mothers' and Children's Health, La Trobe University; [2]Menzies Centre for Population Health Research, University of Tasmania, [3]Centre for Hormone Research, Royal Children's Hospital, Melbourne.

Introduction: Treatment with synthetic oestrogens to reduce adult height has been available for tall girls in Australia since the 1950s. Treatment aims to reduce psychosocial problems of concern to tall girls and their families but little is known about the long-term outcomes.

Methods: This retrospective cohort study identified eligible women from the medical records of Australian paediatricians who assessed or treated tall girls from 1959 to 1992, and from self-referrals. Eligible women were those who had sought a medical opinion about their tall stature and had radiological assessment of their skeletal age: they included girls who received oestrogen treatment in adolescence (treated group) and those who did not (untreated group). Women were invited to complete a postal questionnaire and to comment on a range of issues including their satisfaction with their current height, the assessment and/or treatment procedures, and the decision about whether or not to have treatment.

Results: 1,436 eligible women were identified, 1,221 from medical records and 215 from self-referrals. 1240 women (86.4%) were traced and of these, 79.5% (n=395) of treated and 62.1% (n=450) of untreated women agreed to participate. Women were allocated to three groups according to their current self-reported height: less than 177cm (n=317), 177.1-182.9cm (n=401), and 183cm or more (n=88). 98.6% of untreated women in the shortest group were satisfied with the treatment decision compared to 49.5% of treated women of the same height. In the middle group, 99.4% of untreated women and 61.4% of the treated women were satisfied. 100% of the tallest untreated women were satisfied with the decision compared to 59.5% of treated women. Dissatisfaction was associated with a range of feelings about the decision, with concerns about side effects already experienced, or with a perception that treatment resulted in little or no reduction in height.

Conclusion: In the long-term, many women who were treated as tall girls were dissatisfied with the decision to have treatment, irrespective of their final adult height. In marked contrast, very few untreated women were dissatisfied with the decision.

195

VARIABILITY IN CORTICOSTEROID-BINDING GLOBULIN (CBG) LEVELS WITH WEIGHT LOSS IN HUMANS

E. Mitchell[1], C. Bagley[1], P. Clifton[2], M. Noakes[2], J. Lewis[3], D. Torpy[4]

[1]Hanson Institute, Frome Road, Adelaide SA, [2]CSIRO Health Sciences and Nutrition, Kintore Avenue, Adelaide, SA, [3]Canterbury Health Laboratories, Christchurch, NZ, [4]Univ. of Adelaide Dept. of Medicine, Royal Adelaide Hospital, Adelaide, SA.

Plasma levels of the cortisol transport glycoprotein, corticosteroid-binding globulin (CBG) are lower in insulin-resistant subjects[1]. The mechanism may involve insulin or fat-derived cytokines, both of which can reduce CBG synthesis *in vitro*. A rise in CBG levels with weight loss may reduce free cortisol and help prevent weight gain, in the setting of ongoing central HPA axis stimulation by fat-derived cytokines. Hence, we hypothesized that diet-induced weight loss may lead to an increase in plasma CBG, and that this CBG elevation may correlate with falls in plasma insulin or ultra-sensitive CRP (US-CRP), a marker of pro-inflammatory cytokine secretion. We measured plasma CBG by a novel monoclonal ELISA assay, (developed by JL), before and after a 16-week intensive weight loss program in 86 subjects (58 idiopathic obesity, 28 with PCOS). Subjects achieved a mean weight loss of 8.0 ± 0.3% (95.9 to 88.2 kg, $P<0.0001$). Mean fasting serum insulin levels fell by 28 ± 3% (19 ± 1.2 to 13 ± 0.9 mU/L, $P< 0.0001$). Plasma US-CRP levels decreased by 15.1 ± 4.4% (7.3 ± 0.83 to 5.3 ± 0.47 mg/L, $P=0.016$) Mean plasma CBG levels increased by 5.2 ± 2.6% (484 ± 14.6 to 499 ± 15.5 nmol/L, $P=0.2$). There was a significant correlation between the rise in CBG and degree of weight loss ($r= -0.25$, $P=0.03$), however the change in CBG was not related to changes in insulin, US-CRP or free fatty acids, which can alter cortisol-CBG binding. Although CBG rose in proportion to weight loss, individual responses varied widely. Only 52% of subjects exhibited a rise in CBG (to a maximum of 92%) and 48% of subjects actually had a decrease in plasma CBG (max. 47.5%). This pilot study suggests that there is a wide variation in responses in plasma CBG to weight loss – correlations with other HPA parameters and the tendency for regain of weight after dieting may allow differentiation of subjects in whom cortisol plays an important role in obesity.

(1) Fernandez-Real J-M et al. J Clin Endocrinol Metab 2002;87:4686-4690.

196

THE WAKING CORTISOL RESPONSE IN OBSTRUCTIVE SLEEP APNOEA

F.D. Mackenzie, R.V. Jackson, A.J. Finnimore, J.E. Grice, D.J. Torpy

University of Queensland Department of Medicine,Greenslopes Private Hospital and University of Adelaide Department of Medicine,Royal Adelaide Hospital.

Backgrond: Salivary and plasma cortisol levels rise considerably by 20-40 mins after waking compared to levels immediately after waking (0 min). This phenomenon is light dependent and may represent an alternative non-invasive test of central hypothalamic-pituitary-adrenal (HPA) axis integrity.

Aim: To test the hypothesis that the normal waking cortisol response is not affected by obstructive sleep apnoea.

Methods: Twelve subjects with suspected sleep apnoea were recruited. Subjects on medication that interfere with HPA axis function were excluded. The sleep laboratory was modified to provide 800 Lux of light intensity on waking. Commercial salivettes (Sarstedt,Germany) were used-participants collected saliva at five time points:bedtime, waking (0 min) and 20, 40 and 60 min after waking. Timers and prompting from the sleep technician were used to ensure accurate sampling time.

Results: Diagnosis in the 12 subjects included:OSA (mean RDI 11.5) – 8 subjects ,and no OSA (RDI< 5)- 4 subjects.The mean rise (0 min to peak) in salivary cortisol was: 154% in subjects without OSA; 130% in subjects with OSA .Two patients (1 with mild OSA and 1 with moderate OSA) showed a paradoxical reduction in the normal waking cortisol response.

Conclusion: These preliminary results suggest that the waking cortisol response in OSA, although generally preserved may be deficient in some individuals.

(1) Scheer FAJL,Buijs.RM.J Clin Endocrinol Metab 1999;84:3395-3398.

197

RELATIONSHIP BETWEEN TESTOSTERONE, SEX HORMONE BINDING GLOBULIN AND PLASMA AMYLOID BETA PEPTIDE 40 IN OLDER MEN WITH SUBJECTIVE MEMORY LOSS OR DEMENTIA

M.J. Gillett[1], R.N. Martins[2], R.M. Clarnette[3], P. Chubb[4], D.G. Bruce[3,5], B.B. Yeap[1,5]

[1]Department of Endocrinology and Diabetes, Fremantle Hospital, [2]Sir James McCusker Alzheimer's Disease Research Unit, Hollywood Private Hospital and School of Psychiatry and Clinical Neurosciences, University of Western Australia, [3]Department of Community and Geriatric Medicine Fremantle Hospital, [4]Biochemistry Department, Fremantle Hospital and [5]School of Medicine and Pharmacology, University of Western Australia.

Introduction: Alzheimer's disease is the most frequent cause of dementia in Western societies. The prevalence of dementia increases with increasing age, whilst in men total and free testosterone levels decline by 0.8-2%/year. Higher testosterone levels predict better performance in tests of cognitive function, and men with Alzheimer's disease have lower testosterone levels than controls. We sought to examine the effect of androgen status on plasma expression of the Alzheimer's disease-related peptide amyloid beta 40 (Aβ40).

Methods: 28 community dwelling older men with subjective memory loss or dementia had serum testosterone, oestradiol, sex hormone binding globulin (SHBG), luteinizing hormone (LH), dehydroepiandrostenedione sulphate (DHEAS) and plasma Aβ40 measured. Free testosterone was derived from total testosterone and SHBG.

Results: The age of men in our cohort was 75.4±6.9 years (mean±SD). The mean MMSE score was 22.6±5.6, and the mean total testosterone was 12.2±4.4 nmol/L. Using linear regression analysis, plasma Aβ40 showed significant inverse correlations with total and free testosterone (r=-0.5, *P*=0.01, r=-0.4, *P*=0.03) respectively. SHBG was also significantly negatively correlated with Aβ40 (r=-0.4, *P*=0.04) and there was a non-significant negative trend for DHEAS and Aβ40 (r=-0.4, *P*=0.06). Neither LH nor estradiol correlated with Aβ40.

Conclusions: These data demonstrate that lower androgen levels are associated with increased plasma Aβ40 in older men with memory loss or dementia, suggesting that subclinical androgen

deficiency enhances the expression of Alzheimer's disease-related peptides *in vivo*. An inverse correlation exists between SHBG and Aβ40, warranting further investigation.

198

D_2 DOPAMINE RECEPTOR A1 ALLELE FREQUENCIES IN TYPE 2 DIABETES

J. Grice[1], B. Lawford[2], A. Bachmann[1], S. Kannan[1], R. Jackson[3]

School of Medicine, [1]Greenslopes Private Hospital & [3]Logan Hospital and [2]Dept. of Psychiatry, The University of Queensland.

The presence of the less common *Taq*I A1 allele of the D_2 dopamine receptor (DRD2) is strongly associated with addictive behaviours (alcohol, opioid dependence) and (less strongly) with obesity. In view of the increased risk of type 2 diabetes in obesity and the metabolic syndrome, we hypothesized that the DRD2 A1 allele frequency might be increased in patients with type 2 diabetes, particularly those who are obese, or with concomitant features of the metabolic syndrome.

We studied 60 patients (53 M, 7 F; aged 52-73 yrs; mean BMI = 31.4 ± 4.9). None were on cholesterol-lowering medication or alcohol dependent. DNA was extracted from whole blood (Nucleon BACC2) and genotyping was done by PCR, digestion with *Taq*I and agarose gel electrophoresis.

The table shows genotypes and A1 allele frequencies [c.f. previously reported (1) non-alcoholic control groups (0.185)]. Comparing $A1^+$ and $A1^-$ patients, we found no significant effect on cardiovascular risk factors (BMI, WHR in males, BP, glucose, cholesterol, triglycerides, HDL), HbA1c, liver enzymes or alcohol consumption (MANOVA). A1 frequencies were slightly increased in sub-groups with elevated SBP, BMI and WHR and in drinkers (NS compared to the total group - χ^2 test), but not in those with a positive family history of diabetes or those selected from the upper ranges of the above parameters.

	A1/A1	*A1/A2*	*A2/A2*	*A1 Freq.*
All (n=60)	2	19	39	0.192
SBP ≥ 150 (n=21)	1	8	12	0.238
BMI ≥ 30.00 (n=37)	2	9	17	0.230
Positive FH (n=26)	0	10	16	0.192
≥ 7 drinks/wk (n=19)	1	8	10	0.263

In conclusion, we found no evidence that the DRD2 A1 allele frequency is increased in this diabetic group, despite their significant obesity. A NS trend, seen in some sub-groups, warrants investigation in greater numbers of subjects.

(1) Lawford, BR et al. 1997 Biol Psychiatry 41:386-393.

199

ENDOCRINE DISCRIMINATION OF CLINICAL GROUPS USING HPLC DRIVEN BY SUPRAMOLECULAR CHEMISTRY AND PCA STATISTICS

P.K. Zarzycki[1], V. Clifton, A. Bisits, K.M. Kulhanek, R. Smith

Mothers and Babies Research Centre, The University of Newcastle and JHH, Newcastle, NSW 2310, [1]The Medical University of Gdansk, Faculty of Pharmacy, Hallera 107, 80-416 Gdansk, Poland.

The great diversity of steroid hormones present many difficulties for the simultaneous analysis of steroids, present in plasma, when using radioimmunoassay (RIA) or classical separation techniques (GC, CE, TLC, HPLC). In our previous studies we reported a simple strategy for the optimization of steroid separation using temperature as the critical parameter for selectivity when the liquid phase was modified with β-cyclodextrin (1,2). The aim of this work was to apply the above mentioned method for quantification of free steroids that are present in human plasma, as well as characterisation of individual subjects based on chromatographic profiles and multivariate statistics: principal component analysis (PCA). Biological samples were collected from pregnancies with male (n=12) and female (n=12) fetuses that were delivered by elective Caesarean section with no labour (females n=4, males n=6) or spontaneous vaginal delivery (females n=8, males n=6). Samples were pre-purified and concentrated by reversed-phase solid-phase extraction (SPE) and afterwards separated and quantified using a HPLC system equipped with a UV-VIS diode-array detector. Each chromatogram composed of at least 27 individual peaks including the steroids: estetrol, estriol, 17β-estradiol, estrone, cortisol, cortisone, 17αOHprogesterone, 20αOHprogesterone and progesterone. PCA was used to reduce the dimensions of the initial data matrix (27 peaks versus 24 objects) to fewer uncorrelated variables. Preliminary results indicate that in the three dimensional space that was formed by PCA 1,2 and 3 the investigated subjects can form clear associations in relation to fetal gender or type of delivery. The appropriate selectivity of chromatographic systems can be achieved by modifying the liquid mobile phase with an inclusion agent.

(1) P K. Zarzycki, R. Smith, J. Chromatogr. A., (2001) 912:45-52.
(2) P.K. Zarzycki, K. M. Kulhanek, R Smith, J. Chromatogr. A. (2002) 955:71-78

200

COMPLEX REGULATORY INTERACTIONS CONTROL HYPOTHALAMIC CRH GENE EXPRESSION

Richard C. Nicholson, Bruce R. King, Roger Smith

Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, NSW 2310, Australia.

Corticotropin-releasing hormone (CRH), a 41 amino acid neuropeptide, has a key role in integrating hormonal, autonomic and behavioral responses to stress. CRH is synthesized in the paraventricular nucleus (PVN) of the hypothalamus from where it is released into the hypophyseal portal circulation to orchestrate the pituitary-adrenal stress response, leading to production and release of glucocorticoids. Glucocorticoids in turn inhibit hypothalamic PVN

production of CRH and pituitary production of ACTH, hence ensuring that serum glucocorticoid levels are appropriate to the stress experienced and that the system can be regulated by negative feedback. In the brain CRH has also been found in the posterior pituitary, thalamus, cerebral cortex, cerebellum, pons, medulla oblongata and spinal cord where it acts as a neurotransmitter. In peripheral tissues CRH is produced in the adrenal medulla, ovary, testis, heart, lung, liver, stomach, duodenum, pancreas, T-lymphocytes and placenta. In tissues outside the PVN the effect of glucocorticoids on CRH gene expression is different. For example, glucocorticoids inhibit CRH production in the hypothalamus but stimulate production from the placenta. The mechanism by which glucocorticoids inhibit CRH gene expression in the PVN but stimulate CRH gene expression in other tissues is a major focus of our research. To identify key elements regulating the CRH gene, mouse pituitary tumor-derived cells (AtT20 cells) were used as a hypothalamic model in an analysis of the CRH promoter. Two cAMP responsive elements were identified: (I) a consensus cAMP response element (CRE) and (II) a previously unrecognized caudal-type homeobox response element (CDXRE). Glucocorticoids inhibit only the component of cAMP-stimulation occurring via the CRE through an action involving a negative glucocorticoid response element (nGRE). We also identified two regions that, in the absence of the nGRE, can be stimulated by glucocorticoids: (I) the CRE and (II) a region between -213 to -99bps. Electrophoretic mobility shift assays identified binding of the transcription factors CREB and Fos at the CRE in AtT20 cells, whereas CREB and cJun were detected in placental cells. A model is presented whereby CRH gene regulation is mediated via tissue specific expression of transcription factors.

201

CHARACTERISATION OF THE MEN1 PROMOTER

A. Sweet[1], J. Prins[1], N. Hayward[2], J. Cardinal[1]

[1]Department of Diabetes and Endocrinology, Princess Alexandra Hospital, Woolloongabba 4102 [2]Human Genetics, Queensland Institute of Medical Research, Herston 4006.

Menin, a nuclear protein encoded by the tumour suppressor gene MEN1 appears to function as a regulator of cell cycle (1,2). Mutations in the MEN1 gene have also been shown to cause a variety of endocrine cancers (3). While the majority of recent studies focus on menin function in terms of its protein binding partners, this study will instead focus on transcriptional regulation; the agents that stimulate or inhibit expression from MEN1. In order to identify transcription factors, along with the upstream signalling pathways to which MEN1 is responsive, a variety of pEGFP-MEN1 promoter constructs were generated and transfected into CCL-228 with the resultant expression measured by FACS analysis. A region of 1.5kb containing a previously suggested 542bp promoter was identified as the MEN1 promoter. The 542bp region showed significantly reduced activity, only 43% of that seen from the 1.5kb region. It does however; contain a variety of putative transcription factor binding sites over its entire length. Significantly, the deletion of the proximal 22bp of the 542bp region, which contains a putative p300 binding site, saw promoter activity drop by a further 33%. There is a putative E2F binding site immediately adjacent to this p300 site. It is known that p300 is a co-activator for several isoforms of E2F (4) and it is therefore postulated that these two factors co-operate to regulate expression from MEN1. These results provide initial information on the location and size of the MEN1 promoter and evidence of possible transcription factors binding sites for further investigation.

(1) Sukhodolets KE, Hickman AB, Agarwal SK, Sukhodolets MV, Obungu VH, Novotny EA, Crabtree JS, Chandrasekharappa SC, Collins FS, Spiegel AM, Burns AL, Marx SJ. (1997). The 32-kilodalton subunit of replication protein A interacts with menin, the product of the MEN1 tumour suppressor gene. *Molecular and Cellular Biology* 23(2):493-509
(2) Ikeo Y, Sakurai A, Suzuki R, Zhang MX, Koizumi S, Takeuchi Y, Yumita W, Nakayama J, Hashizume K. (2000) Proliferation-associated expression of the MEN1 gene as revealed by in-situ hybridisation: possible role of menin as a negative regulator of cell proliferation under DNA damage. *Laboratory Investigation* 80(6):797-804
(3) Guo SS and Sawicki MP. (2001). Molecular and genetic mechanisms of tumorigenesis in multiple endocrine neoplasia type-1. *Molecular Endocrinology* 15(10):1653-64
(4) MarzioG, Wagener C, Gutierrez M, Cartwright P, Helin K, Giacca M. (2000) E2F family members are differentially regulated by reversible acetylation. *Journal of Biological Chemistry* 275(15):10887-92

202

THE MRNA FOR PROSTAGLANDIN E RECEPTOR 3 (EP3) AND P450SCC ARE REGULATED BY ACUTE PGE_2 INFUSION IN THE OVINE FETAL ADRENAL GLAND

P.J. Simmonds, R.M. Escalona[1], G.T. Ooi[1], I.R. Young, B.J. Canny

Department of Physiology, Monash University, VIC, 3800 & [1]Prince Henry's Institute of Medical Research, Clayton, VIC, 3168.

Previous *in vitro* and *in vivo* studies in our laboratory have demonstrated that PGE_2 can stimulate secretion of cortisol directly at the level of the fetal adrenal gland. The current study was undertaken to examine if the mRNA for PGE_2 receptors and other genes critical in the cortisol synthesis were affected by a 24-hour infusion of PGE_2. Intact fetuses underwent surgery at 125 d gestational age to implant catheters. Saline (1 ml/h) was infused from this time to maintain catheter patency. At 140 d GA fetuses received either a 24 hour (h) infusion of saline (1 ml/h, n=5) or PGE_2 (1 μg/kg/min, n=5). This infusion of PGE_2 was shown to be effective as it led to a significant ($P<0.05$) increase in the plasma concentrations of PGE_2, ACTH and cortisol. At the end of the infusion the animals were humanely killed and the adrenals collected. Total RNA was extracted and a fixed amount of RNA was reverse transcribed by RT-PCR. The cDNA was then used to perform real-time PCR using specific primers that encoded the Prostaglandin E Receptors (EP1, EP2, EP3 and EP4), the ACTH receptor, P450scc, 3βHSD and P450c17 steroidogenic enzymes. mRNA for each subtype of the Prostaglandin E receptor (EP1-4) was found in the ovine fetal adrenal. The relative abundance of the mRNA for EP3, but not EP1, 2 and 4, was increased ($P= 0.06$) by the infusion of PGE_2 when compared to the saline-infused controls. Similarly, the PGE_2 infusion increased ($P= 0.06$) the mRNA for P450scc, but had no effect on the mRNA encoding ACTH receptor or 3βHSD and P450c17. This study demonstrates that the EP receptors are present in the ovine fetal adrenal, suggesting that PGE_2 can act directly at this gland. Furthermore, it appears that the relative expression of the mRNA encoding two critical genes involved in PGE_2 signalling and steroidogenesis may be up-regulated by an acute infusion of PGE_2. At this stage it is not clear if the effects of PGE_2 are mediated directly at the adrenal gland or *via* a central pathway.

THE CREAPS: A FAMILY OF CRE BINDING PROTEINS

Kristy L. Shipman[1], Phil J. Robinson[2], Bruce R. King[1], Roger Smith[1], Richard C. Nicholson[1]

[1]Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, NSW, [2]Children's Medical Research Institute, University of Sydney, NSW.

The cAMP regulatory element (CRE) is one of the most important regulatory elements determining up regulation of corticotropin releasing hormone (CRH) in the placenta and hypothalamus. In order to identify the proteins involved in CRH gene regulation, a yeast one-hybrid system was used to screen human placental cDNA library for transcription factors capable of functionally binding the CRE. This resulted in the discovery of a human cDNA encoding a protein with a distinctive combination of modular domains. The protein contains two leucine-zipper-like domains typical of bZIP transcription factors, a zinc finger-like domain with potential DNA binding properties, a zinc finger-like domain typical of RNA binding proteins, two coiled-coil domains typically found in transcription factors and an SR-rich domain characteristic of proteins involved in RNA splicing. This new protein has been named CREAP1, for CRE Associated Protein.

Protein from yeast transformed with a CREAP clone has been shown to specifically bind to the CRE of the CRH promoter using an electrophoretic mobility shift assay. A multiple tissue expression array has shown that CREAP is present in a wide variety of human tissues, with high expression seen in the fetal brain, heart, kidney, thymus and lung and in adult cerebellum, putamen, pituitary and thymus. Little to no expression was found in the adult substantia nigra, heart, esophagus, colon and ovary.

The CREAP peptide sequence was compared to the protein databases and three highly related proteins of unknown function were found. They are 95% similar to each other over the N-terminal two-thirds and are all very similar (60%) to CREAP1. All four proteins share the coiled-coil, zinc finger, leucine zipper and SR domains. These protein sequence and domain similarities suggest that a new family of human proteins uniquely capable of binding to the CRE and to function in RNA splicing has been identified.

ELUCIDATION OF A REGION MEDIATING CHAPERONE ACTIVITY IN CYCLOPHILIN 40 AND ANALYSIS OF RESIDUES IN AN HSP90 MICRODOMAIN CRITICAL TO CHAPERONE FUNCTION AND DIMERISATION

Danny Mok, Aaron L. Magno, Rudi K. Allan, Bryan K. Ward, Thomas Ratajczak

Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, WA, Western Australia Institute for Medical Research, University of Western Australia, Nedlands, WA.

The function of steroid receptors are thought to be modulated by the immunophilins cyclophilin 40 (CyP40), FKBP51 and FKBP52 by participating in receptor folding, stabilisation and transport to their target genes. These co-chaperones exist in a steroid receptor complex in association with Hsp90, a molecular chaperone that is essential for promoting steroid hormone signalling. Immunophilins interact with a common acceptor site found at the extreme C-terminus of Hsp90 through highly conserved tetratricopeptide repeat (TPR) domains. A hydrophobic α-helical microdomain in Hsp90, located between residues 649-673, is involved in dimerisation and may also provide a second binding site for TPR-containing immunophilins. The aims of this study were to map the region in CyP40 that mediates chaperone activity, and to examine the hydrophobic microdomain in Hsp90 to identify residues or regions that may influence chaperone function and Hsp90 dimerisation, respectively.

Chaperone activity was assessed by a rhodanese aggregation assay using GST-fusion proteins of either CyP40 or Hsp90. For CyP40, plasmid constructs were prepared to express full-length protein (1-370), and the CyP40 deletion mutants 1-185, 1-213, 91-370, 185-370 and 185-370Δ243-256 containing a disrupted TPR domain. Our preliminary results suggest a region between residues 91-213 in CyP40, corresponding to a cleft between the N-terminal cyclophilin-like domain and the C-terminal TPR domain, to mediate chaperone activity. Lysine and large hydrophobic residues were substituted for alanine by site-directed mutagenesis in the central Hsp90β hydrophobic microdomain. Chaperone activity was shown to be diminished with modification of residues K649, K652, L654, L663, L664 and F668, while no change or an increase in activity was observed for residues L657, F659 and L670. The role of individual amino acid residues within the Hsp90 hydrophobic segment in dimerisation and oligomerisation is currently being examined in an assay involving chemical cross-linking. The finding of a chaperone domain in CyP40 provides further evidence of its fine-tuning role in modulating steroid receptor function. Our results provide further evidence that the C-terminal domain of Hsp90 represents a multi-functional region with critical roles in TPR immunophilin binding, chaperone activity and dimerisation.

205

IDENTIFICATION OF HADHB, HUR AND CP1 AS HUMAN RENIN MRNA BINDING PROTEINS

Dianne J. Beveridge[1], David J. Adams[2], Louise van der Weyden[2], Peter J. Leedman[1], Brian J. Morris[2]

[1]Laboratory for Cancer Medicine and School of Medicine and Pharmacology, Western Australian Institute for Medical Research, Centre for Medical Research, Royal Perth Hospital, The University of Western Australia, WA, 6000 and [2]Basic & Clinical Genomics Laboratory, Department of Physiology, School of Medical Sciences and Institute for Biomedical Research, The University of Sydney, NSW, 2006.

Renin is the rate limiting enzyme in the generation of the potent cardiovascular effector hormone angiotensin II. Production of renin is critically dependent on the modulation of renin (*REN*) mRNA stability. However, the mechanisms involved, particularly the *cis*-acting mRNA stability elements and the *trans*-acting factors that target *REN* mRNA are yet to be elucidated. Transfections of the renin-expressing cell line Calu-6, with various luciferase-reporter gene constructs, identified a distal 34 nt *cis*-element which was shown to be an important contributor to basal *REN* mRNA instability. Using the yeast three-hybrid system with the *REN* 3'-UTR as bait we isolated HADHB. REMSA and UV cross-linking assays (UVXL) with Calu-6 cytoplasmic extracts and recombinant HADHB showed specific binding of HADHB with *REN* mRNA. A band observed in Calu-6 extracts via UVXL was of similar size to HuR, a member of the family of embryonic lethal abnormal vision (ELAV) RNA-binding proteins that stabilizes mRNAs. Another band was of similar size to poly-C binding protein-1 (CP1), an RNA binding protein previously implicated in the control of mRNA turnover. REMSA with recombinant HuR and CP1 confirmed binding to *REN* mRNA. Immunoprecipitation studies with Calu-6 cells confirmed that HADHB, HuR and CP1 associate with *REN* mRNA *in vivo*. Use of siRNA directed at HuR markedly reduced HuR as well as renin protein. In contrast HADHB siRNA increased renin protein. In conclusion, these data suggest that HADHB and HuR are novel renin mRNA-binding proteins that target a destabilizing *cis*-element in the 3'UTR of *REN* and have destabilizing and stabilizing effect, respectively, on renin production.

206

THE COUMARIN ANTIBIOTIC NOVOBIOCIN FUNCTIONS AS A PROBE FOR THE DIFFERENTIAL RECOGNITION OF STEROID RECEPTOR-ASSOCIATED TPR-IMMUNOPHILINS BY HEAT SHOCK PROTEIN 90

Rudi K. Allan [1,2] , Bryan K. Ward [1,2] , Danny Mok [1,2] , Malcolm Walkinshaw[3], Thomas Ratajczak [1,2]

[1] Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, WA 6009; [2] Centre for Medical Research, University of Western Australia; [3] Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, Scotland EH9 3JR.

Mature steroid receptor complexes capable of binding hormone are comprised of the receptor, heat shock protein 90 (hsp90), p23 and one of a number of immunophilins: CyP40, FKBP51,

FKBP52 or PP5. These immunophilins are thought to modulate receptor function and they compete for a common binding site in the C-terminal domain of Hsp90 via their conserved tetratricopeptide repeat (TPR) domains. Essential for this interaction is the strongly conserved MEEVD peptide at the extreme C-terminus of Hsp90 which is accommodated within a binding groove assembled from the TPR domain of CyP40, comprising three units of the TPR motif. Evidence suggests that a leucine-rich microdomain N-terminal to this motif, comprising residues 649-670, may also contribute to Hsp90-TPR protein interaction (1). A recent study has shown that the coumarin antibiotic novobiocin binds within a region spanning residues 657-677 and was able to disrupt the chaperone activity of Hsp90 (2). Thus novobiocin is presented as a potential inhibitor of TPR-protein binding to Hsp90. The aim of the present study was to investigate the effects of novobiocin on the binding of CyP40 and other TPR-proteins to Hsp90.

CyP40 (185-370) and PP5 were bacterially expressed as GST fusion proteins and assayed for binding to His-tagged C-terminal human Hsp90β (527-724) in the presence of novobiocin using an enzyme-linked immunoabsorbent assay. Results show novobiocin to be a potent inhibitor of both CyP40 and PP5 binding to Hsp90, with IC50's of ~3mM and ~1.6mM respectively. This difference in sensitivity to novobiocin suggests that Hsp90 recognition of TPR immunophilins may not be identical. We are currently using novobiocin to probe the Hsp90 interactions with FKBP52 and Cdc37, a co-chaperone of Hsp90 involved in modulating Hsp90 control over kinase signalling pathways.

(1) Chen et al (1998), Cell Stress & Chaperones, 3, 115-129.
(2) Marcu et al (2000), Journal of Biological Chemistry, 275, 37181-37186.

207

SEX STEROID TREATMENT AFFECTS ACUTE ACTH AND CORTISOL (F) RESPONSES TO A SELECTIVE SEROTONIN REUPTAKE INHIBITOR (SSRI) IN MALE AND FEMALE SHEEP

J.H. Broadbear, T. Nguyen, I.J. Clarke[1], B.J. Canny

Department of Physiology, Monash University, VIC, 3800 & [1]Prince Henry's Institute of Medical Research, Clayton, VIC, 3168.

We have previously demonstrated that sertraline (SERT), an SSRI, induces an acute increase in ACTH and F secretion in sheep (1). As the therapeutic effectiveness of SSRIs may be enhanced in women when compared to men (2), we have examined whether the effects of SERT on ACTH and F secretion vary according to the sex and sex steroid status. Gonadectomised male and female sheep (n=6/group) were treated with vehicle and 5 and 10 mg/kg SERT by *sc* injection in the presence and absence of sex steroids (testosterone (T) implants 400 mg for males, estrogen (E, 1 cm implant) & progesterone (P, CIDR) for females) using a cross-over design. Blood samples were collected every 10 mins around the time of injection, and the concentrations of ACTH and F in plasma were determined by RIA. Basal concentrations of ACTH and F were not different between male and female sheep, nor were they affected by sex steroid treatment. In female sheep, SERT treatment resulted in dose-dependent increases ($P<0.05$) in both ACTH and F. Sex steroid (E+P) treatment had no effect on the responses to SERT in female sheep (P=NS). In contrast, T had profound effects on the ACTH and F responses to SERT in male sheep. In the absence of T, 10, but not 5, mg/kg SERT caused an increase in both ACTH and F ($P<0.05$). In the presence of T, no dose of SERT was effective. These results suggest that the HPA axis of the

female sheep is more responsive than that of male sheep to the stimulatory effects of SERT, and indicate that T has an inhibitory effect on any responses seen. It is not clear, however, if the differences observed reflect sex differences in serotonergic pathways, or more generally within the HPA axis. These results underline the importance of reproductive status responses of the the HPA axis, and may have implications for understanding depression.

This work was supported by the NHMRC; SERT was a generous gift of Pfizer

(1) Proc ESA 45 309 (2002),
(2) Am. J Psych 158:1531 (2000)

POSTER ABSTRACTS

301

RECOMBINANT HUMAN TSH STIMULATED RADIOIODINE TREATMENT OF THYROID CANCER IN A YOUNG WOMAN

S. Hocking[1], R. Mansberg[2], B. Crawford[1, 3]

[1]Department of Endocrinology and [2]Department of PET and Nuclear Medicine, Royal Prince Alfred Hospital, and [3]University of Sydney, NSW.

Stimulation by recombinant human TSH (rhTSH) has been demonstrated to be an effective alternative to thyroid hormone withdrawal (THW) in the diagnostic follow-up of patients with thyroid cancer (TC). We present the case of a 17 yr old woman diagnosed with papillary thyroid carcinoma (PTC) following biopsy of a thyroid nodule. Her risk factors for TC included radiotherapy (4860cGy) at 5 yrs of age for a Ewing's sarcoma of the scapula and a first degree relative with PTC. Total thyroidectomy was performed during Year 11 at high school. Over the next 12 months she had three iodine-131 (I_{131}) treatments (total 361mCi) following THW for persisting disease in the neck. In 2001, her final year of high school, she had further neck surgery removing 13 cervical and mediastinal lymph nodes with metastatic PTC. Post-operatively, 200mCi I_{131} was administered following THW. Symptoms of hypothyroidism following THW became worse with each treatment, especially in winter, with fatigue, cold intolerance and cognitive decline impairing her studies and her ability to continue her sporting, community and social activities. As further I_{131} doses were planned every 3 months during 2002, her enrollment in university was deferred. Due to the debilitating effects of repeated THW, I_{131} treatment was administered following rhTSH stimulation. rhTSH 0.9mg was given intramuscularly on 3 consecutive days and 207mCi I_{131} was administered on day 5. No side-effects were experienced, she continued her normal activities, and was maintained on thyroxine 200 μg daily. Following rhTSH, thyroglobulin (Tg) level rose from 4.3 μg/L to 12.7μg/L on day 5. Post therapy scan on day 8 revealed sites of uptake comparable with the previous post-therapy scan. Based on the marked improvement in functional capacity with rhTSH stimulation compared with THW, and evidence of effective uptake, she has commenced a full-time university course this year, resumed competitive sports, and is having further rhTSH-stimulated radioablative therapy.

302

UNILATERAL ADRENAL HYPERPLASIA RESULTING IN HYPERALDOSTERONISM AND HYPERCORTISOLISM

Nirusha Arnold, David Chipps

Department of Diabetes & Endocrinology, Westmead Hospital, NSW.

A 53 year old man with uncontrolled hypertension, was found to have normokalemia, but a raised aldosterone to renin ratio, an elevated 24-hour urinary aldosterone and a nodular left adrenal gland on abdominal CT scan. Blood pressure did not improve with spironolactone. Intravenous and oral salt loading failed to suppress urinary and plasma aldosterone. Adrenal venous sampling (AVS) demonstrated high levels of aldosterone in the left adrenal vein with a raised aldosterone to cortisol ratio of 8 and a significant left to right gradient of 24 indicating a unilateral source of aldosterone secretion. Cortisol levels in the right adrenal vein however were

low suggesting suppression of cortisol secretion from the right adrenal gland by contralateral cortisol overproduction. Urinary free cortisol was elevated, plasma ACTH was suppressed and serum cortisol did not suppress overnight with Dexamethasone (1mg). We concluded he had aldosterone and cortisol overproduction by the left adrenal gland.

The patient had a left laparascopic adrenalectomy. Histopathology showed hyperplastic micronodules composed of eosinophilic and clear cells resembling the zona glomerulosa and zona fasciculata respectively. Blood pressure post-operatively was well controlled on spironolactone. Renin, aldosterone, the aldosterone to renin ratio, response to salt loading and urinary free cortisol all normalized and blood pressure has remained well controlled eighteen months post-operatively.

Unilateral Adrenal Hyperplasia (UAH), a rare cause of hyperaldosteronism, can only be diagnosed histologically , after the detection of unilateral hypersecretion of aldosterone by AVS and the apparent cure of hyperaldosteronism by unilateral adrenalectomy. Excessive co-secretion of aldosterone and cortisol in UAH has not previously been described.

(1) Haenal LC , Hermayer LC A Case of Unilateral Adrenal Hyperplasia: The Diagnostic Dilemma of Hyperaldosteronism. Endocrine Practice 2000;6(2):152-158

303

TEMPORAL TRENDS AND REGIONAL PATTERNS IN ANDROGEN PRESCRIBING IN AUSTRALIA

Paula Anderson, David J. Handelsman

ANZAC Research Institute& Dept of Andrology, Concord Hospital, Univ of Sydney, Sydney NSW 2139.

The availability of a national Pharmaceutical Benefits Scheme (PBS) scheme in Australia, which subsidizes prescriptions of essential medications allows an evaluation of national androgen prescribing patterns, which have not been reported. As testosterone (T) has been used clinically for over 65 years for its main indication, treatment of classical androgen deficiency (AD) due to well-known hypothalamo-pituitary or testicular pathologies, which have not varied, a stable and uniform pattern of androgen prescribing might be expected. Yet, international androgen prescription sales have increased 3-fold over the last 5 years of the 1990's for reasons including (a) under-diagnosed of classical AD, (b) the epidemic of androgen abuse originating in elite sports during the Cold War leading to endemic androgen abuse for recreational, cosmetic and occupational motivations for androgen-induced body-building and (c) commercial pressures from franchised clinics and pharmaceutical companies to promote testosterone as an anti-ageing tonic. We therefore obtained monthly records of prescriptions filled for PBS-approved androgen products from 1991 to 2002. These were grouped into injectable T ester (TE), oral T undecanoate (TU) and implantable T pellets (TP), and by State taking into account to take into account changing product costs and state populations. Over the decade, the standard treatment, TE prescriptions and sales increased gradually by ~50% over 10 years. By contrast, TU demonstrated a steep 7-fold increase between 1991 to 1994 with a sharp fall (~50%) immediately following with introduction of an authority requirement for all androgens followed by a gradual rise which was again reduced with the introduction of specific PBS restrictions modeled on the ESA Prescribing Guidelines. Analysis according to State demonstrated marked systematic differences, which were mostly stable over the decade except for a steep rise reaching 4-fold

above national average in prescribing of TU and TP (but not TE) in Western Australia. This followed the opening, and corresponded to the habitual prescribing patterns, of a franchised "Andropause" clinic. These data suggest that androgen prescribing is gradually increasing for both legitimate and inappropriate indications. There is also evidence that androgen prescribing differs regionally and is sensitive to both communal and professional pressures both for and against misuse of androgen prescribing.
Funded by Andrology Australia.

304

CYTOMEGALOVIRUS INFECTION: A CAUSE OF SEVERE GLUCOCORTICOID RESPONSIVE THYROIDITIS?

James J. Gome, Duncan J. Topliss, Jim Stockigt

Dept of Endocrinology &Diabetes, Alfred Hospital, Melbourne, Vic, 3004.

Cytomegalovirus (CMV) has been demonstrated in the thyroid of immunocompromised patients but there are no reports of clearly associated thyroid dysfunction. CMV infection has not been implicated in typical subacute thyroiditis. We present a case of severe transient thyrotoxicosis due to painless glucocorticoid-responsive thyroiditis in an immunocompromised patient with disseminated CMV.

A thirty four year old CMV negative, euthyroid man received a cardiac transplant for congenital aortic valvular heart disease from a CMV positive donor. Amiodarone, used for sixteen months, was ceased at the time of transplantation and was not given subsequently. Fourteen months after transplantation he developed severe thyrotoxicosis (see table) with marked weight loss, lethargy, severe proximal myopathy, diarrhoea, sweats and low grade fevers. Acute CMV infection was confirmed by positive blood PCR with a viral load of 98800 copies/ml. Thyroidal technetium uptake was negligible and ESR was 88 mm/h. Material from needle biopsy of the thyroid revealed follicular epithelial cells with no viral inclusions. PCR was negative for CMV on the biopsy material.

Weeks from diagnosis	FT4 (pmol/L)	FT3 (pmol/L)	TSH (mU/L)	Treatment changes	Wt (kg)
- 60	21.4		2.47		88
0	>75	33.6	<0.02		77
3	>75	>46.0	<0.02	15mg CBZ tds	69
4	>75	32.3	0.02	20mg PNL bd	
5	54.4	6.3	<0.02		73
6	24.7	3.5	<0.02	10mg PNL od *	
12	35.7	8.0	0.02	CBZ ceased	
17	17.0	4.0	<0.02		

He was treated initially with carbimazole (CBZ); prednisolone (PNL) was added one week later [* PNL decreased in setting of perforated duodenal ulcer]. He remained severely ill and emergency thyroidectomy was considered. However, serum fT3 decreased abruptly within a week of commencement of PNL, followed by gradual clinical recovery. While the role of past amiodarone in the pathogenesis of the thyrotoxicosis cannot be absolutely ruled out and no viral

material was found in the thyroid, this sequence suggests that severe thyrotoxicosis due to CMV thyroiditis may be a distinct clinical entity that is responsive to glucocorticoid treatment.

305

DISTRIBUTION AND CO-LOCALISATION OF CORTICOTROPHIN-RELEASING HORMONE (CRH), ARGININE VASOPRESSIN (AVP) AND ENKEPHALIN (ENK) IN THE PARAVENTRICULAR NUCLEUS (PVN) OF THE EWE

E.T.A. Rivalland[1], J. Iqbal[2], I.J. Clarke[2], A.I. Turner[1], A.J. Tilbrook[1]

[1]Department of Physiology, Monash University, Clayton, 3800; [2]Prince Henry's Institute of Medical Research, Clayton, 3168.

The PVN is central to the activation of the hypothalamo-pituitary-adrenal (HPA) axis. At the hypothalamic level the axis consists of CRH and AVP neurones found in the PVN. The ENK neurones of the PVN are also activated by various stressors. Although the distribution of these neurones in the rat has been well documented there is a paucity of information in the sheep. Our aim was to map the distribution of CRH, AVP and ENK immunoreactive cells (ir) and to determine the co-localisation of CRH, AVP and ENK–ir in the PVN of non-stressed ewes. Six gonadectomised Corriedale ewes were killed by humane means, their brains perfused and the hypothalami collected. Immunohistochemistry was performed for CRH, AVP and ENK-ir using 30 µm sections at 180µm intervals. Maps of the rostral-caudal distribution of these three peptides were generated in two ewes. The distribution of AVP and ENK-ir cells was seen to start rostrally, at the level of the periventricular nucleus (PeV) and to extend throughout the PVN forming a continuum between the two nuclei. In contrast, the distribution of CRH was localised to the medial–caudal PVN. Using anatomical landmarks, sections representing the rostral, medial and caudal extent of the PVN were chosen from four animals. Double-immunofluorescence was then carried out for CRH/AVP, CRH/ENK and AVP/ENK, to determine the extent of peptide co-localisation. The number of cells immunoreactive for each hormone was counted and the percentage of co-localization calculated. On average 6.8% of CRH cells contained AVP and 26.5% contained ENK. Two % of AVP cells contained CRH and 3.1 % contained ENK. Apart from the CRH cells that also produce ENK, the co-localisation of these peptides in the PeV and PVN is low. The distribution of these peptides is different than that seen in the rat and these results may underlie the species differences observed in responses to stress.

306

A CRITICAL REGION FOR ALDOSTERONE BINDING TO THE MINERALOCORTICOID RECEPTOR

Fraser M. Rogerson, Yi-zhou Yao, Peter J. Fuller

Prince Henry's Institute of Medical Research, Clayton, Victoria 3168.

The steroid hormone aldosterone is a critical mediator of blood pressure, via its effects on sodium reabsorption in the kidney and colon, and has hypertrophic and pro-inflammatory effects in the heart. Aldosterone acts by binding to the mineralocorticoid receptor (MR). The MR is closely

related, both structurally and functionally, to the glucocorticoid receptor (GR). The MR and GR both bind the glucocorticoids, cortisol and corticosterone, with high affinity, whereas the MR alone binds aldosterone with high affinity. The aim of our work is to determine the amino acids in the MR that confer aldosterone binding specificity. Previously, using a panel of chimeras created between the MR and GR ligand-binding domains (LBDs), we discovered that amino acids 804-874 are critical for aldosterone binding (1). In the present study we created a second panel of MR:GR chimeras concentrating on this region. The chimeras were initially analysed by a transactivation assay performed in transfected CV-1 cells. Analysis of the second panel of chimeras revealed a critical importance for amino acids 820-844 of the MR. The EC_{50} of aldosterone activation of chimeras containing this region is equal to that of the full-length MR LBD. Chimeras that did not contain this region of the MR were not activated by aldosterone. To confirm the transactivation results, Scatchard analysis of [^{3}H]-aldosterone binding was performed in transfected COS-1 cells. The K_d value of [^{3}H]-aldosterone binding to the MR(820-844)-GR chimera was very similar to the full-length MR LBD, and two-orders of magnitude greater than that to the GR LBD. In a model of the MR LBD, based on the GR LBD crystal structure, amino acids 820-844 lie on the surface of the protein and do not form part of the ligand-binding pocket. Our results strongly suggest that aldosterone binding specificity in the MR is conferred by amino acids that do not directly contact the ligand. The corresponding regions of the GR, androgen and progesterone receptors have also been identified as important mediators of ligand binding specificity, suggesting a common underlying mechanism.

(1) Rogerson FM et al (1999) J.Biol.Chem. 274: 36305.

307

PRENATAL EXPOSURE TO LPS ALTERS DEVELOPMENT OF THE ENDOCANNABINOID SYSTEM IN THE RODENT

A.R. Bromley[1], T.K. Roberts[2], R.H. Dunstan[2], T. Rothkirch[2], H. Kiernan[1], D.M. Hodgson[1]

Laboratory of Neuroimmunology, School of Behavioural Sciences [1], School of Biological and Chemical Sciences [2], University of Newcastle, NSW, Australia.

The increased focus on the role of endocannabinoid (EC) receptors and ligands has led to the investigation of the development of this system. This has resulted in the emerging role of ECs (ie., anandamide (AEA)) in various reproductive functions and their influence on the development of other critical systems. ECs are fatty acids that interact with the cannabinoid receptor in the brain (CB-1) and peripheral tissues (CB-2). They have regulatory roles in many systems activated in response to infection. Dysregulation of the EC system may impair immune responses, predisposing to infection and chronic illness. Functional maturation of the EC ligand system continues during the early post natal period. As such, we questioned whether exposure to bacteria during this time could influence sensitivity of the EC system. Initial in vitro studies demonstrated that exposure of rat brain tissue to bacterial exotoxin significantly ($P<0.05$) increased AEA levels. The aim of this study was to determine whether prenatal exposure to bacteria in Fischer 344 rats could alter adult host responses to AEA *in vivo.* Pregnant rats were administered endotoxin (salmonella enteritidis, 100ug.kg^{-1}, s.c.) or an equivolume of saline on gestation days 16, 18 and 21. At term, a subset of dams had their pups delivered by caesarean section, and brain and foetal tissue was removed for Gas Chromatography-Mass Spectrometry (GC/MS) analysis of endocannabinoid levels. The remaining dams were left to deliver normally. On post natal day 80, the response of offspring to AEA (10mg/kg iv) was assessed in terms of

locomotor activity, catalepsy, nociception, temperature. Prenatal exposure to endotoxin was found to be associated with reduced weight at birth ($p<0.05$) and a trend towards an increased weight in adulthood as compared to control animals. These animals also exhibited reduced cortisol levels at birth ($p<0.05$). Brain tissue from endotoxin-treated dams demonstrated altered levels of AEA when compared to controls. Exposure to prenatal endotoxin was also found to significantly alter the tetrad of symptoms induced by AEA administration ($p<0.05$). The results of this study will help elucidate the link between prenatal exposure to bacterial infections and the development of the endocannabinoid system.

308

OESTROGEN AND SELECTIVE OESTROGEN RECEPTOR MODULATORS (SERMS) EXERT DIVERGENT EFFECTS ON GH SIGNALLING THROUGH DIFFERENT MECHANISMS

K.C. Leung, N. Doyle, G.M. Leong, K. Sjogren, K.K.Y. Ho

Pituitary Research Unit, Garvan Institute of Medical Research, St Vincent's Hospital, Sydney, NSW.

Oestrogen inhibits GH activation of JAK2/STAT5 signalling by suppressing JAK2 phosphorylation, an action mediated by SOCS-2[1]. To determine whether SERMs exert similar effects on GH signalling, we have compared the effects of 17ß-estradiol (E_2), 4-hydroxytamoxifen (4HT) and raloxifene (Ral) on the GH/JAK2/STAT5 cascade in HEK293 cells stably expressing human GHR. The cells were transiently transfected with an expression plasmid for human ER-alpha and a luciferase reporter containing the STAT5 binding element, and treated with GH (500ng/ml) and E_2 or SERMs (1, 10, 100nM) for 18h before measuring transcriptional activity. JAK2 phosphorylation was assessed by immunoprecipitation of JAK2 and Western blotting for tyrosine-phosphorylated proteins.

GH alone increased the reporter activity by 3.6±0.7 fold (mean±SE; $P<0.02$). Co-treatment with E_2 resulted in a dose-dependent reduction of the activity, to a maximum of 62±4% ($P<0.02$) at 100nM. In contrast, 4HT and Ral increased the activity maximally by 58±5% and 43±5% ($P<0.02$), respectively. GH-induced JAK2 phosphorylation was reduced by E_2 to 57±4% of control, but increased by 4HT and Ral to 178±15% and 184±11%, respectively ($P<0.01$). We next examined whether SOCS-2 or phosphotyrosine phosphatases play a role in mediating the enhancing action of SERMs on GH signalling. Quantitative RT-PCR study revealed that the SOCS-2 mRNA level was increased by E_2 to 156±12% of control ($P<0.05$), but unaffected by 4HT and Ral. Co-treatment with a phosphatase inhibitor (vanadate) did not alter the inhibitory effect of E_2, but completely abrogated the enhancing effects of 4HT and Ral on GH-induced reporter activity. In summary, SERMs enhanced the transcriptional activity of GH by promoting JAK2 phosphorylation, an effect likely involving phosphatases. We conclude that estrogen and SERMs exert opposite effects on GH signalling through different mechanisms.

Supported by NHMRC and Eli Lilly Australia.

(1) Leung *et al.* (2003) PNAS 100:1016-1021

309

STRUCTURAL DETERMINANTS OF SPIRONOLACTONE BINDING TO THE MINERALOCORTICOID RECEPTOR

Yi-zhou Yao, Peter J. Fuller, Fraser M. Rogerson

Prince Henry's Institute of Medical Research, Clayton, Victoria 3168.

Aldosterone increases sodium reabsorption in the kidney and colon to increase blood pressure, and has hypertrophic and pro-inflammatory effects in the heart. Spironolactone antagonises aldosterone action by competing for its binding to the mineralocorticoid receptor (MR). Recent multi-centre clinical trials show that MR antagonists, such as spironolactone, dramatically increase the chances of surivival of patients with heart failure. The clinical usefulness of spironolactone is limited by its "cross-reactivity" with the androgen receptor. The design of specific, high-affinity MR antagonists would be facilitated by a better understanding of ligand binding to the MR. Spironolactone binds with very low affinity to the glucocorticoid receptor (GR). The difference in binding affinities of spironolactone to the MR and the GR has allowed us to use chimeras, created between the receptor ligand-binding domains (LBDs), to determine the region of the MR critical for spironolactone binding. We examined spironolactone binding to four chimeras in which a region of the GR was replaced with the corresponding MR sequence. Spironolactone binding was assessed by its ability to compete for [^{3}H]-dexamethasone binding to the chimera. Whole-cell ligand binding assays were performed in transiently-transfected COS-1 cells. The concentration of [^{3}H]-dexamethasone used varied with the affinity (K_d) of the radioligand for the chimera. Spironolactone was used in 1-, 5-, 10- and 50-fold molar excesses of the concentration of [^{3}H]-dexamethasone. Using the initial panel of chimeras, it was found that amino acids 804-874 were required for spironolactone binding. Examination of a second panel of MR:GR LBD chimeras, created within this region, narrowed the critical region to amino acids 820-844. Intriguingly, this same region of the MR is critical for aldosterone binding. In a model of the MR LBD, based on the GR LBD crystal structure, amino acids 820-844 lie on the surface of the protein and do not form part of the ligand-binding pocket. Our results strongly suggest that ligand-binding specificity (agonist and antagonist) in the MR is conferred by amino acids that do not directly contact the ligand.

310

NO EFFECT OF LEPTIN IMMUNISATION ON BODY WEIGHT OF GROWING LAMBS

Cheryl Schelbach, Kate G. Kauter, Tim O'Shea, James R. McFarlane

Physiology, University of New England, Armidale, NSW 2350.

Leptin is produced by a variety of tissues in the body but primarily by adipose tissue, with a close relationship existing in humans between leptin in cord blood and body weight at birth (1,2). Leptin also has a role in the regulation of food intake and energy expenditure (1,3). This study sought to manipulate circulating leptin via immunisation with bovine leptin and describes the effect of immunisation on growth rate in Merino lambs and the onset of behavioural oestrus in immunised ewes. Female (n=9) and castrate male (n=6) Merino lambs were immunised with recombinant bovine leptin (75 ug/animal) at 5 weeks of age. Booster immunisations (37.5ug/animal) were administered at 4 and 8 weeks following primary immunisation. Control

female (n=12) and castrate male (singleton n=5) Merino lambs were grazed on the same pasture as the same-sex immunised animals. Body weights of these groups were monitored and at 51 weeks post primary immunisation, yearling ewes (control and immunised) were grazed on open pasture with vasectomised rams, fitted with harness and marking crayons to indicated the onset of behavioural oestrus in this group of ewes. In this study there was no effect on growth weight following leptin immunisation in either female or castrate male animals. The was also no change in the onset of behavioural oestrus between leptin immunised and control yearling ewes. Leptin immunisation of growing lambs did not affect either body weight or onset of behavioural oestrus when compared to control lambs grazed on the same pasture, even though antibody titres were significantly elevated for the 12 month experimental period. This data suggests that plasma leptin concentrations have little effect on growth rate or on the onset of puberty in ewes.

(1) Zhang *et al* 1994. *Nature* 372 425-432
(2) Jaquet *et al* 1998 *JCE&M* 83 1243-1246
(3) Campfield *et al* 1995. *Science* 269 546-549

311

GENOMIC RESPONSE TO GLUCOPENIA IN VITRO

K. Kobayashi, S. Metaxas, Y. Xin, G.A. Werther, V.C. Russo

Centre for Hormone Research, Murdoch Childrens Research Institute, Department of Paediarics, University of Melbourne, Royal Children's Hospital, Parkville 3052, Victoria.

Intensive insulin therapy in IDDM patients as well as inadequate nutrition in premature infants potentially leads to hypoglycaemia. Glucose is the major source of energy for neuronal cells and therefore its reduced supply to the brain may affect neuronal viability/survival with consequent impairment of various brain functions. We recently demonstrated, *in vitro*, that cell growth/survival as well as glucose transport is dramatically altered in neuronal cells exposed to low glucose. These effects are ameliorated by the potent anti-apoptotic factor IGF-I via stimulation of cell growth, suppression of a number of pro-apoptotic genes and potentiation of glucose transport/transporters. These data thus suggest that glucopenic stress in neuronal cells leads to a complex modulation of genes involved in cell growth/survival and metabolic functions.

We therefore we aimed to identify low glucose-regulated genes and performed differential gene expression analysis.

Human neuroblastoma cells, SK-N-SH-SY5Y were used in this study as an in vitro model for neuronal cells. SY5Y were exposed to optimal (25mM) and low (2.5mM) glucose for 24 hours, total RNA extracted and ~10μg of poly-A RNA were purified from each treatment. Differentially expressed genes were identified by PCR-Select subtraction(Clonotech). Subtractive hybridization enabled us to compare two populations of mRNA (cells cultured in low or optimal glucose) and to obtain clones of genes that are exclusively expressed in only one population. This starting material was used to generate cDNA fragments for subsequent adaptor ligation, hybridisation and PCR. Two pools of cDNA (480 clones) were thus obtained, a control pool and one enriched for sequences expressed in response to glucose starvation. Of the 480 isolated clones only 43 were specifically regulated in low glucose. These 43 cDNA clones (~0.3-1.5 kb) are currently being sequenced and BLAST analysed. Of interest one of the determined sequences showed 96% homology with the transcription factor E2F 1 (neuronal apoptosis) and another sequence matched 94% of the cytochrome C gene (depolarization mitochondrial membrane). Sequences of several

unknown genes were also determined. Differentially expressed genes will be further confirmed by Northern analysis and RT-PCR.

In conclusion we have investigated, in neuronal cells, the genomic response to glucopenic stress and determined that this response involves the regulation of a number of genes which control mitochondrial activity, cell cycle/proliferation and apoptosis.

312

EXPRESSION OF OREXIN RECEPTORS IN THE BRAIN AND PERIPHERAL TISSUES OF RAMS

Song Zhang, Dominique Blache, Margaret A. Blackberry, Philip E. Vercoe, Graeme B. Martin

School of Animal Biology, Faculty of Natural & Agricultural Sciences, University of Western Australia, Crawley, WA 6009.

The neuropeptides, orexin-A and –B. are involved in the regulation of several functions such as food intake, sleep-wake cycle, and the activity of the endocrine, cardiovascular and autonomic nervous systems. Orexins exert their effects mainly through two specific receptors (OXR1 and OXR2) that have been found mainly in the brain (1) but also in peripheral tissues (2) of rats and humans. Both orexins are found in the peripheral blood supporting the existence of both peripheral and neurophysiological actions. Here we present the localisation of the expression of mRNA encoding for ovine OXR1 and OXR2 in central and peripheral tissues of sheep. Specific primers for ovine OXR1 and OXR2 were designed according to the published sequences of rat and human orexin receptors. PCR products for OXR1 and OXR2 were 216 bp and 229 bp. Using quantitative real time RT-PCR, we found high levels of expression of OXR1 mRNA in the hypothalamus and lower levels in the preoptic area, pineal gland, pineal recess, pituitary gland, testis and kidney. Only very small amounts of OXR1 mRNA were found in the adrenal gland. High levels of OXR2 mRNA were present in the hypothalamus and preoptic area, and there were lower levels in the pineal recess and pituitary gland. Neither OXR1 nor OXR2 were detected in liver or adipose tissue. The expression of mRNA for orexin receptors in peripheral tissues of sheep indicates the existence of direct actions of orexins at the peripheral levels in this species.

(1) Lu, X.Y., Bagnol, D., Burke, S., Akil, H., Watson, S.J. 2000. Hormones and Behavior 37, 335-344.

(2) Johren, O., Neidert, S.J., Kummer, M., Dendorfer, A., Dominiak, P. 2001. Endocrinology 142, 3324-3331.

313

LOCALISATION OF ADHESION AND STRUCTURAL PROTEINS DURING SPERMIATION

A. Beardsley[1,2], L. O'Donnell[1]

[1] Prince Henry's Institute of Medical Research, Monash Medical Centre, Clayton, [2] Dept of Anatomy and Cell Biology, Monash University, Clayton, Victoria, Australia, 3168.

Spermiation is the process by which mature spermatids are released from Sertoli cells. When FSH and testosterone (T) are suppressed, spermiation fails and spermatids are retained by Sertoli cells. The molecular mechanisms involved in spermiation and spermiation failure after T&FSH suppression are largely unknown. We showed previously that the ectoplasmic specialisation (ES), which is an adhesion junction between Sertoli cells and spermatids, is removed ~30hrs prior to spermatid release, and that an unknown adhesion complex containing β_1-integrin persists until spermatid disengagement. Since our previous studies suggest that defects in this unknown adhesion complex at disengagement are likely to underlie spermiation failure during hormone suppression, the aim of this study was to further characterise this adhesion complex by investigating the presence of α_6-integrin and microtubules (MT). Immunohistochemistry was performed in testes from normal rats and from rats with spermiation failure induced by T&FSH suppression. Immunolocalisation of α_6-integrin was consistent with that of β_1-integrin during spermiation and spermiation failure; α_6-integrin persisted around spermatids after ES removal and was localised around spermatids until their release and on retained spermatids after T&FSH suppression. This suggests that the $\alpha_6\beta_1$-integrin dimer is likely involved in spermatid disengagement. MT were abundant within the Sertoli cells during spermiation, and co-localised with ES junctions before their removal. MT in Sertoli cell cytoplasm associated with spermatids after ES removal, but were not associated with spermatids at disengagement. Moreover MT did not associate with retained spermatids. We conclude that 1) Sertoli cell MT are not involved in the disengagement or retention of spermatids during spermiation and 2) $\alpha_6\beta_1$-integrin is a likely meditator of spermatid release, and spermiation failure may be caused by functional changes in integrin-containing adhesion complexes between spermatids and Sertoli cells.

314

GENERATION OF AN ANDROGEN RECEPTOR (AR) LOXP MOUSE LINE FOR TISSUE-SPECIFIC GENE KNOCKOUT

A.J. Notini, R.A. Davey, J.F. McManus, K. Bate, J.D. Zajac

Department of Medicine, The University of Melbourne, Austin Health, VIC

Androgens mediate their effects in target cells via an interaction with the androgen receptor (AR) which directly modulates target gene expression. AR protein has been detected in a wide variety of genital and non-genital mammalian tissues including testis, prostate, brain and bone. Conditional gene targeting, using the Cre/loxP site-specific recombination system combined with standard techniques of homologous recombination, may be used to restrict AR gene disruption either spatially or temporally thus allowing investigation into the tissue-specific role of androgens. We have cloned and characterised exon 3 of the AR from a 129SV/J mouse genomic library. DNA from this clone was used in the design and generation of an AR-loxP targeting

construct. The construct contains exon 3 of the mouse AR gene flanked by loxP sites and a neomycin resistance cassette. We believe that deletion of exon 3 will result in the permanent inactivataion of the AR as this exon is highly conserved among species and it encodes the second zinc finger of the DNA binding domain. This zinc finger plays a critical role in stabilising receptor-DNA interaction and receptor dimerisation. Further evidence which supports the vital role of this region comes from a study conducted on a pair of siblings with complete androgen insensitivity syndrome (cAIS) due to an in-frame, natural deletion of the third exon of the AR gene. The AR-lox targeting construct was introduced into 129SV/J mouse embryonic stem cells via homologous recombination. Neomycin resistant ES cell colonies were selected and screened by PCR analysis. Sixteen positive ES cell clones were identified and expanded for microinjection into C57Bl6 mouse blastocysts. Blastocysts were transferred to the uteruses of pseudopregnant females. Male chimeras derived from blastocysts injections were backcrossed to female C57Bl6 mice and resulting female agouti mice were genotyped by PCR to confirm germline transmission. Thus we have successfully generated an AR-loxP mouse line which will be bred with Cre recombinase expressing mice to generate tissue specific knockout lines.

315

STEREOLOGICAL COMPARISON OF MOUSE SPERMATOGENESIS IN GENETIC MODELS WITH SELECTIVE FSH ACTIVITY

Charles M. Allan, Alvaro Garcia, Jenny Spaliviero, Fu-Ping Zhang, David J. Handelsman

ANZAC Research Institute, Concord Hospital, University of Sydney, Sydney, NSW 2139 & Institute of Biomedicine/Physiology, University of Helsinki, Fin-00014 Helsinki, Finland.

Identifying the spermatogenic effects of FSH distinct from those of testosterone remains difficult. We compared Sertoli and germ cell development in 3 recently created mouse models of selective FSH activity in the absence of LH effects. Two transgenic (tg) models used LH-deficient *hpg* mice to selectively study *i)* FSH independent of the hypothalamic-pituitary-gonadal axis (Allan CM *et al* Endocrinology *142*: 2213-20, 2001), or *ii)* a mutated ligand-independent activated FSH receptor ($FSHR^+$)(Haywood M *et al* Mol Endocrinol *16*: 2582-91, 2002). Another model examined mouse FSH effects in the presence of a permanently disrupted LH receptor (LHR) (Zhang F *et al* Mol Endocrinol *15*: 172-183, 2001). Stereological evaluation revealed increased total Sertoli cell numbers/testis in both tg-*hpg* models, relative to undeveloped *hpg* testes. Serum tg-FSH dose-dependently restored *hpg* Sertoli cells to non-*hpg* wildtype (*wt*) levels, and LHR(-/-) testes had normal Sertoli numbers, confirming the central role of FSH in Sertoli cell proliferation. Spermatogonia numbers were increased in both tg-*hpg* models, and were 45% of *wt* in LHR(-/-) testes despite elevated serum mouse FSH. Tg-FSH dose-dependently restored *hpg* spermatogonia to *wt* levels and increased meiotic spermatocytes in *hpg* testes to levels (34% of normal) found in LHR(-/-) testes, demonstrating the advantage of the tg-*hpg* paradigm where it is possible to vary circulating FSH levels. Tg-FSH and -$FSHR^+$ *hpg* testes exhibited elongated spermatid production, which was absent in LHR(-/-) males and may reflect higher testicular testosterone levels in *hpg* relative to LHR(-/-) testes. Tg-FSH increased post-meiotic round and elongated spermatid numbers in *hpg* testes to 16% and 6% of *wt*, without altering intratesticular testosterone levels, demonstrating limited FSH-dependent post-meiotic maturation. These findings show that postnatal Sertoli and mitotic/meiotic germ cell development can be stimulated by FSH activity without LH requirement, but LH-dependent Leydig cell testosterone production remains a critical determinant of initiating complete spermatogenesis.

316

SEX DIFFERENCES IN THE DISTRIBUTION AND ABUNDANCE OF ANDROGEN RECEPTOR (AR) MRNA-CONTAINING CELLS IN THE PREOPTIC AREA AND HYPOTHALAMUS OF THE SHEEP

Christopher J. Scott[1, 3], Iain J. Clarke[2] Alix Rao[2], Alan J. Tilbrook[1]

[1] Department of Physiology, Monash University, Vic, [2] Prince Henry's Institute of Medical Research, Clayton, Vic, [3] Present address: School of Biomedical Science, Charles Sturt University, Wagga Wagga, NSW.

Rams and ewes show a negative feedback response to peripheral treatment with testosterone, with both sexes having a similar degree of suppression in LH secretion during the breeding season (1). At least part of the action of testosterone to suppress GnRH/LH secretion is exerted via interaction with an AR (2). The distribution of AR-containing cells in the hypothalamus has been described for the ram (3) but the distribution and levels of AR in the hypothalamus of the ewe are unknown. In this study, we have tested the hypothesis that there is no difference in level of AR mRNA expression in the preoptic area and hypothalamus between rams and ewes. Perfusion-fixed brain tissue was obtained from adult Romney Marsh ewes (luteal phase) and rams during the breeding season (n=4/sex). AR mRNA expression was quantified in hypothalamic sections by *in situ* hybridisation using an ^{35}S-labelled riboprobe and image analysis. AR mRNA-containing cells were found in the medial preoptic area, bed nucleus of the stria terminalis, anterior hypothalamic area, ventromedial nucleus, arcuate nucleus and premamillary nucleus. The levels of AR mRNA expression were higher ($P<0.05$ in each case) in rams than ewes in the rostral preoptic area, caudal preoptic area and rostral portion of the bed nucleus of the stria terminalis. In all other regions there was no sex difference in the level of AR mRNA expression. The preoptic area and bed nucleus of the stria terminalis are important for reproductive behaviour and the sex differences in AR mRNA expression may reflect sex differences in the behavioural response to testosterone treatment. The lack of sex difference in the basal hypothalamus is consistent with this region's role in the regulation of gonadotrophin secretion.

(1) Lubbers & Jackson (1993) Biol Reprod 49:1369-1379.
(1) Hileman et al. (1994) Biol Reprod 50:1244-1250. (3) Herbison et al. (1996) Neuroendocrinology 63:120-131.

317

EFFECTS OF THE INSECTICIDE AMITRAZ ON HUMAN GRANULOSA CELLS

Fiona Young, Margaret Menadue[1], [§]Tina Lavranos[2]

Medical Biotechnology, Flinders University; [1]Endocrinology Unit, Flinders Medical Centre, [2]Cell Biol & Anatomy, Louisiana State University Health Sciences Center, USA.

The insecticide Amitraz is commonly used to control ticks and mites on cattle, sheep, pigs (125mg/ml), dogs (0.25mg/ml), and heliothis larvae on cotton (200mg/ml). Amitraz is a reproductive toxin to rats (12mg/kg/day, US EPA) and mice, but its effects on human fertility are

unknown. Granulosa cells were collected from women who had undergone a superovulation regime. Follicular aspirates were centrifuged to collect granulosa cells, which were washed and counted before $1.6x10^4$ cells per well were cultured in 96 well plates with 100ul of DMEM/F12 + 10% FCS with or without Amitraz (1, 10, 100ug/ml) for 24, 48 or 72 hours. Media were collected and replenished at 24hr periods, and estradiol and progesterone measured by RIA. Cell numbers at the end of exposure periods were measured by MTT assay. One-way ANOVA was conducted on raw data, and results presented as the mean percentage of control ±sem, $p<0.05$ [a], $p<0.01$ [b], $p< 0.001$ [c].

Hrs of Exp	Dose of Amitraz (ug / ml)								
	1 (n=3 women)			10 (n=6 women)			100 (n=3 women)		
	Cells	E_2	P_4	Cells	E_2	P_4	Cells	E_2	P_4
24	91±3	87±1	72±1	89±7	94±5	58±8[b]	51±14[b]	82±8[a]	43±6[a]
48	92±5	87±6	79±6	114±1	98±1	58±4[a]	26±17[a]	56±9[c]	42±2[b]
72	89 n=1	90±1	75±2	71±32	83±1	69±10	29 n=1	58±4[b]	27±1[a]

1ug/ml amitraz had no significant effect on luteinized human granulosa cells, but 10ug/ml amitraz significantly reduced progesterone production without affecting cell number or estrogen production. Exposure to 100ug/ml amitraz significantly reduced cell numbers and caused a corresponding decrease in steroid hormone production. Further work is required to identify the mechanism of action, and to determine the extent of the risk amitraz may pose to human fertility.

318

VALIDATION OF TIME RESOLVED IMMUNOFLUOROMETRIC ASSAY (IFMA) FOR MOUSE FSH

Jennifer A. Spaliviero, Mark Jimenez, Charles Allan, Jos A. Verhagen, Arijan J. Grootenhuis, David J. Handelsman

ANZAC Research Institute, Concord Hospital, University of Sydney, Sydney NSW 2139 & Department of Pharmacology (Steroids), NV Organon, The Netherlands.

IFMA has many advantages over RIA for measurement of FSH including higher sensitivity to permit smaller sample size, higher specificity and a nonradioactive format. Using monoclonal/polyclonal reagents developed for IFMAs of rat LH and FSH (Van Casteren JI et al Biol Reprod, *62:* 886-894, 2000), we aimed to validate an IFMA for mouse FSH (mFSH) and to compare its performance with the existing standard NIH mFSH radioimmunoassay (RIA) for studies of hormonal regulation of spermatogenesis involving expression of a human FSH transgene in the mouse. IFMA had much higher (~34 fold) sensitivity with a working detection limit of ~5 pg/tube compared with 170 ± 11 pg/tube (n=7 assays) for RIA so that all normal intact male mice (n= 97) had detectable serum FSH using a sample size of 25 μL (vs 100 μL for RIA) in duplicate. Serial dilutions of castrate mouse serum (n=8) and rat FSH (NIH RP2) were parallel to mFSH standard (AFP 5308D). Human FSH standard (hFSH, WHO 78/549) was not

detectable in the IFMA but displayed strong and non-parallel crossreactivity in the RIA. Neither IFMA nor RIA had any crossreactivity with mouse (NIH AFPn 5306A) or human (WHO 80/552) LH. Sera from FSH deficient mice with genetic inactivation of the FSH beta subunit were undetectable in both the IFMA (<0.2 pg/mL, n= 5) and RIA (<2ng/mL, n=3). In both assays, mFSH was increased by orchidectomy and suppressed by T treatment and was also reduced in GnRH analog (Zoladex) treated normal intact mice. The presence of human transgenes expressing either dimeric hFSH or Sertoli cell targeted activating mutant FSH receptor did not significantly influence mFSH immunoreactivity in the IFMA. In gonadotropin-deficient *hpg* mice, mFSH was undetectable in RIA before or after T or DHT treatment but was just detectable by the IFMA assay after T but not DHT treatment. We conclude that the mFSH IFMA is valid for measurement of mouse FSH, is accurate and has much higher sensitivity and specificity than the conventional NIH mouse FSH RIA. This makes it useful for measurements in mice where serum volume is limited and especially in mice co-expressing a human FSH transgene.

319

CRH IN MARMOSET PREGNANCIES

Maria Bowman, Suzette Tardif[1], Donna Layne[1], Roger Smith

Mothers and Babies Research Centre, Hunter Medical Research Institute, John Hunter Hospital, Newcastle, NSW. [1]Southwest National Primate Research Center, San Antonio, USA.

Maternal plasma Corticotropin Releasing Hormone (CRH) is only elevated in primate pregnancies. In humans, gorillas and chimpanzees, CRH rises as pregnancy progresses, peaking at labour. Baboons (Old World monkey) and humans have similar CRH concentrations at the end of pregnancy, however CRH in the baboon peaks during the first trimester, followed by a gradual decline reaching a plateau in the third trimester. Humans, apes and old world monkeys usually give birth to a single fetus, unlike the marmoset (New World monkey), which tends to give birth to twins. CRH has previously been measured in pregnant marmosets, however the pattern has not been clear, as longitudinal data was only available on two animals. In this study, CRH was measured using radioimmunoassay (RIA) in 5 pregnant common marmosets (*Callithrix jacchus)* from day 60 to the end of pregnancy. Samples were transported from the USA on dry ice and kept frozen at -20^{o}C. Plasma was extracted using methanol, dried and reconstituted in assay buffer. Marmoset plasma CRH diluted parallel to human CRH standard. The pattern of CRH appears similar to that observed in the baboon, peaking in early pregnancy. Interestingly, unlike the baboon, the marmoset appears to possess a CRH-BP. CRH concentrations were highest in a triplet pregnancy, followed by 2 twin pregnancies and a singleton. CRH was lowest in a fetus that was later aborted. Twin human pregnancies have also been shown to have higher levels of CRH than singletons. The biological significance of these different patterns of placental CRH secretion remains unclear however a correlation between CRH and rate of fetal adrenal zone growth is noted. We have previously demonstrated the presence of CRH receptors in fetal adrenal zone tissue further suggesting a physiological interaction.

320

LEPTIN CLEARANCE RATES DURING THE EARLY FOLLICULAR PHASE ARE HIGHER THAN DURING THE LUTEAL PHASE OF THE ESTRIUS CYCLE IN EWES

Melanie L. Hunt[1], Theresa Kokta[2], Timothy O'Shea[1], Rod A. Hill [2], James R. McFarlane[1]
[1]Physiology, University of New England, Armidale NSW Australia 2351and [2]Animal and Veterinary Science, University of Idaho, Moscow Idaho USA.

Leptin is a 16kDa peptide made primarily by adipose tissue and involved in energy homeostasis, but there is increasing evidence that it is also involved in many other systems including reproduction. Plasma concentrations of leptin has been reported to vary across the oestrus cycle in a number of species with the lowest levels occurring in the late luteal / early follicular phase (1). In this study we examined the hypothesis that the changes in leptin concentrations may be due to changes in clearance rates. Merino ewes at day 7 (n=4) and day 14 (n=4) of estrus received a single intravenous dose of ^{125}I ovine leptin. Blood samples were taken via a catheter in the jugular vein at regular intervals for 240 minutes. The blood was centrifuged and the TCA precipitable radioactivity in the plasma determined by a gamma counter. Samples from 60 minutes post-leptin tracer administration were fractionated by gel permeation HPLC and 0.5 ml fractions collected. Leptin pharmacokinetics data were fitted using a two pool exponential decay model $Y=Ae^{-at} + Be^{-bt}$ (2). The disappearance of radioactive leptin from the plasma was significantly faster (1.61 ml/min/kg) at day 7 than at day 14 (0.26 mi/min/kg). This supports the hypothesis that changes in clearance rate at the different stages of the cycle may influence leptin concentrations and provides further evidence that leptin has a role in reproduction. HPLC analysis of samples from the day 7 and day 14 groups revealed that the radioactive leptin eluted in a complex with a molecular size of approximately 66kda. The exact nature of the complex remains to be found but it suggests that the major binding protein in ewe plasma is not likely to be ObRe, the soluble form of the receptor.

(1) Garcia *et al.*, 2002. Serum leptin and its adipose gene expression during pubertal development, the estrous cycle , and different seasons in cattle. J Anim. Sci 80:215-2167.
(2) Hill et al 1998. Leptin: its pharmokinetics and tissue distribution. Int J Obes 22:765-770.

321

ESTROGEN REPLACEMENT RESCUES THE DYSFUNCTION OF PITUITARY SOMATOTROPES OF OVARIECTOMISED AROMATASE KNOCKOUT(ARKO) MOUSE

Ming Yan, Margaret E.E. Jones, Maria Hernandez, Evan R. Simpson, Chen Chen
Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Victoria 3168. <ming.yan@med.monash.edu.au>

Available data on the influence of estradiol (E_2) on growth hormone (GH) levels remain controversial. A factor contributing to this uncertainty is lack of knowledge of long-term E_2 action on pituitary somatotropes. The molecular mechanism of E_2 action on somatotropes is poorly understood due to difficulties in strictly controlling E_2 levels. In the present study, we investigated the influence of E_2 on pituitary GH expression and secretion, as well as the

expression of mRNAs encoding for GH-secretagogues receptor (GHS-R), GH-releasing hormone receptor (GHRH-R), pituitary-specific transcription factor (Pit-1) and somatostatin receptors (sst1-5). Female ovariectomised (Ovx) aromatase knockout (ArKO) mice were used this study in which the disruption of the *cyp19* gene encoding aromatase leads to a block in the production of estrogen. The Ovx procedure also prevented accumulation of androgen occurred in ArKO female mice. The concentration of serum GH was measured by radioimmunoassay. GH, Pit-1, GHS-R, GHRH-R, sst1-5 mRNA levels were measured using semi-quantitative RT-PCR methods. Ovariectomised wild-type mice (Ovx/WT) for 5 weeks decreased the levels of pituitary GHS-R and increased sst1, sst-3 mRNA expression compared to WT mice. Ovx with ArKO mice significantly reduced the levels of GH, GHS-R, GHRH-R, Pit-1 and increased sst1, sst-3 mRNA levels comparison with WT mice. The levels of serum GH in Ovx/WT and Ovx/ArKO mice were significantly lower than that in WT mice. Replacement of E_2 (1μg/day/mouse) for 3 days in OVX mice increased the levels of GH mRNA to control levels and reduced sst-4,-5 to 64%, 80% of control levels in WT mice, leading to an increase of serum GH concentration. Moreover, exposure of Ovx/ArKO mice to E_2 for 21 days (0.05mg 21 day slow release pellet) elevated the levels of mRNA encoding for GH and GHRH-R, and augmented serum GH concentration to control levels obtained in WT mice. The results indicate that E_2 replacement rescues the dysfunction of pituitary somatotropes from long-term E_2 deficient conditions in Ovx/ArKO mice. Supported by NHMRC.

322

IDENTIFICATION OF TESTOSTERONE-REGULATED GENES IN THE ADULT RAT TESTIS

P. Sluka[1,2], L. O'Donnell[1], P.G. Stanton[1]

[1]Prince Henry's Institute of Medical Research, Monash Medical Centre, Clayton, Victoria, Australia, 3168, [2]Department of Anatomy and Cell Biology, Monash University, Clayton, Victoria, Australia, 3800.

Spermatogenesis is well known to be regulated by testosterone (T) acting on androgen receptors in Sertoli cells. Despite the importance of T in spermatogenesis, few androgen-regulated genes have been identified in the seminiferous epithelium. The aim of this study was to identify T-regulated genes in the adult rat testis. Groups of adult (> 90 day old) male Sprague-Dawley rats (n=7/group) received implants of T and estradiol (E) to suppress LH, reduce testicular T to ~3% of normal, and suppress spermatogenesis. Testicular T was then replaced for periods of 0, 6, 12, 24, 48, and 96 hours to restore spermatogenesis. The main effect of T suppression and subsequent restoration using this treatment regime is the inhibition and subsequent normalization of round spermatid-Sertoli cell adhesion and maturation (1). Animals were then sacrificed, testes were removed and frozen prior to extraction of total RNA and analysis by differential-display PCR. Analysis of differential gene expression is being conducted on RNA samples isolated from tissue at three levels; i) from whole testis tissue, ii) from stage-specific seminiferous tubule cross-sections, and iii) from step-specific round spermatids. Cell samples for the latter 2 methods were collected using laser-capture microdissection. These samples each contained cells from 100 seminiferous tubule cross-sections, which yielded approximately 50ng total RNA. To date, 30 PCR products have been identified in whole testis tissue that showed changes in expression levels between treatment groups. These products are currently being identified and their

expression patterns validated using real-time RT-PCR. In conclusion, this study has isolated candidate genes that show evidence of T regulation which may be important in spermatogenesis.

(1) O'Donnell et al. 1996, Biol Reprod, 55:895.

323

ANDROGENS REGULATE ~~2.~~ THE TIGHT JUNCTION PROTEIN CLAUDIN-11 IN RAT SERTOLI CELLS IN VITRO[†]

T.J. Kaitu'u[1], P. Sluka[1,2], C.F. Foo[1], P.G. Stanton[1]

[1]Prince Henry's Institute of Medical Research, Monash Medical Centre, Clayton, [2]Dept of Anatomy and Cell Biology, Monash University, Clayton, Victoria, Australia, 3168.

Claudin-11 and occludin are ~~integral~~ protein components of the~~s~~ tight junctions (TJs) between Sertoli cells that~~-~~ are important~~responsible f~~ for the maintenance of the blood-testis barrier. ~~These junctions form the blood-testis barrier, and provide a microenvironment within the seminiferous tubule which is essential for the initiation and maintenance of spermatogenesis.~~ Formation of the blood-testis barrier occurs during puberty and is believed to be under ~~gonadotrophin~~gonadotropic~~n~~ regulation, however whether~~the extent to which gonadotrophins regulate~~ claudin-11 and occludin are hormonally regulated ~~at Sertoli cell TJs is~~remains unknown. The aim of this study was to investigate~~characterise~~ the regulation of claudin-11 and occludin mRNA expression by FSH and testosterone (T) and their immunolocalisation at rat Sertoli cell TJs *in vitro*. Sertoli cells formed~~ed~~ functional and stable tight junctions within 3 days of plating as assessed by transepithelial electrical resistance (TER). Both T and dihydrotestosterone significantly ($p<0.01$) increased TER 2-fold and claudin-11 mRNA 2-3 fold ~~compared with control~~ within 3 days, while FSH partially stimulated TER and claudin-11 mRNA, and estradiol had no effect. T~~estosterone~~ also stimulated the formation of cytoplasmic droplets of claudin-11 protein, and promoted its immunolocalisation into extensive inter-cellular junctions. Addition of the androgen receptor antagonist, flutamide, to Sertoli cells pre-stimulated with T~~testosterone~~ caused a 2-fold decrease in both TER and claudin-11 mRNA expression, and ~~also p~~promoted the loss of both claudin-11 and occludin from cell junctions.~~–~~ Removal of flutamide led to a reversal of both TER and claudin immunolocalisation responses. In contrast to claudin-11, T~~testosterone~~ and~~or~~ FSH did not change occludin mRNA expression, however T~~testosterone did~~ promoted immunolocalisation of occludin at TJs~~junctions in a similar~~ in a similar manner to claudin-11.~~-~~ It is concluded that androgens i) co-regulate claudin-11 mRNA expression and TER, ~~in parallel~~ implicating claudin-11 in TJ~~tight junction~~ formation, and ii) promote~~.~~ ~~–~~ the ~~These data are supported by the~~ localisation of both claudin-11 and occludin at Sertoli cell TJs~~junctions~~ ~~.following testosterone treatment. – Occludin mRNA expression during formation and maintenance of Sertoli cell TJs was not altered by testosterone, FSH, FSH + testosterone, or estradiol, but testosterone significantly ($p<0.02$) increased claudin mRNA expression 2-3 fold compared with control cultures. Testosterone also stimulated the formation of cytoplasmic droplets of claudin-11 protein, and promoted the localisation of both claudin-11 and occludin into extensive inter-cellular junctions. Addition of the androgen receptor antagonist flutamide to Sertoli cells pre-stimulated with testosterone caused a 2-fold decrease in both TER and claudin mRNA expression to control levels, and also promoted the loss of both proteins from cell junctions. These effects were reversible, as removal of the antagonist and replacement with testosterone restored TER and junctional localisation, although claudin mRNA expression~~

remained at control levels. In cells treated with flutamide at the time of plating claudin mRNA expression remained at control levels although TER was abolished. It is concluded that Sertoli cell tight junction function is comprised of both constitutive and androgen regulated components. The constitutive component comprises both occludin and basal claudin mRNA expression and limited localisation of these proteins at cell junctions, whereas the androgen regulated component involves significantly upregulated claudin 11 mRNA expression and localisation, together with occludin localisation, at Sertoli cell tight junctions. These data suggest that the ability of androgens to maintain spermatogenesis *in vivo* is partly via their effects on TJ proteins and regulation of the expected to be how the f androgens regulate ormation and maintenance of blood-testis barrier, and the role of this junction in spermatogenesis.

†Supported by the NH&MRC of Australia.

324

INSL3 EXPRESSION IN BOVINE FOLLICLES

H.F. Irving-Rodgers, M. Krupa, M.L. Harland, R.A.D. Bathgate*, R.J. Rodgers

Reproductive Medicine Unit, Obstetrics and Gynaecology, University of Adelaide, Adelaide, SA and *Howard Florey Institute, University of Melbourne, Vic.

The precise role of INSL3 (relaxin like factor) in females is not known. In bovine follicles INSL3 immunoreactivity localizes to the steroidogenic ovarian thecal cells, however when follicles enter atresia INSL3 levels decline (1). In bovine follicles < 5 mm in diameter two forms of atresia are recognized. In antral atresia granulosa cells die first nearest the antrum with cell death progressing towards the basal lamina, in basal atresia the converse occurs (2). Healthy follicles also have two phenotypes based upon columnar or rounded shape of the granulosa cells aligning the follicular basal lamina (3). In this study, INSL3 expression was examined in bovine healthy and atretic small antral follicles. Ovaries were obtained from a local abattoir (n = 23) and were perfused fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and embedded in paraffin. Histological sections were used for hybridisation with digoxygenin-labelled sense and antisense RNA probes to part of the bovine INSL3 gene and the signal visualized using alkaline phosphatase activity and detected with NBT/BCIP. Follicles (n = 145 follicles, 2 – 5 mm diameter) were classified as healthy or atretic and microscopic images captured with the VideoPro 32 image analysis system (Leading Edge Pty Ltd). INSL3 mRNA was only detected in the theca layers. Densitometry was performed using the analySIS software (Soft Imaging System GmbH) on an area of the theca interna of approximately 3290-6700μm^2. The levels were similar in both healthy phenotypes and early antral atresia with approximately 10-15% of the thecal area examined being occupied by INSL3 expressing cells. In mid and even late antral atresia the levels declined to only approximately 5-7%. In contrast, basal atretic follicles had almost undetectable levels of INSL3 ($P < 0.001$). In conclusion, the process of basal atresia in particular is associated with a decline in expression of INSL3 in the theca. This is in contrast to an up-regulation of steroidogenic enzymes in granulosa cells of basal atretic follicles and an increased follicular fluid progesterone concentration (4).

(1) Irving-Rodgers et al (2002) Biol. Reprod. 66, 934-943.
(2) Irving-Rodgers et al (2001) Reproduction 122, 761-775.
(3) Irving-Rodgers HF, Rodgers RJ (2000) J. Reprod. Fertil. 118, 221-228.
(4) Irving-Rodgers et al (2003) Biol. Reprod (accepted).

325

CYTOKINE MRNA EXPRESSION BY CD14+ CELLS (MACROPHAGES/ MONOCYTES) IN HUMAN FOLLICULAR FLUID

R. Wu, N.K. Ryan, M.J. Jasper, K.H. Van der Hoek, R.J. Norman

Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, University of Adelaide, The Queen Elizabeth Hospital, South Australia.

Macrophages are found within the ovary and play an important role in the human reproductive system. They secrete numerous cytokines that mediate diverse functions in ovarian processes such as folliculogenesis, tissue restructuring at ovulation and corpus luteum formation and regression. There is evidence that periovulatory human follicular fluid contains tissue macrophages/monocytes and cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10 and granulocyte-macrophage colony stimulating factor (GM-CSF) have been measured in follicular fluid. Follicular fluid from polycystic ovary syndrome (PCOS) patients treated with dexamethasone has reduced TNF-α activity, however another study has shown that TNF-α and IL-1β levels in follicular fluid from PCOS patients were similar to non-PCOS patients. Our aim was to determine whether macrophages/monocytes could be isolated from follicular fluid of PCOS and non-PCOS IVF-ET patients, and whether they express mRNA for the ovarian regulators TNF-α, IL-1β and IL-6. Follicular fluid from IVF-ET patients was collected and macrophages/monocytes were isolated by anti-CD14 magnetic beads separation. 1.2-1.6×10^5 cells were collected per follicular fluid sample with a cell viability of 40-70%. The percentage of macrophages in CD14+ cells was determined by anti-CD206 immunostaining and approximately 33% were CD206+. Quantitative RT-PCR was performed on the CD14+ cells using specific primers for TNF-α, IL-1β and IL-6. Data were normalized relative to β-actin expression. TNF-α, IL-1β, IL-6 mRNA transcripts were detectable in CD14+ cells. Future work will compare cytokine mRNA profiles in follicular fluid derived macrophages/monocytes of PCOS and non-PCOS and may reveal differential expression of cytokine transcripts occurs during folliculogenesis and ovulation in PCOS patients.

326

REGULATION OF ESTROGEN ACTION BY A NOVEL SRA BINDING PROTEIN

Esme C. Hatchell, Shane M. Colley, Andrew D. Redfern, Dianne J. Beveridge, Rainer B. Lanz[1], Lisa M. Stuart, Bert W. O'Malley[1], Peter J. Leedman

Laboratory for Cancer Medicine, Centre for Medical Research, Western Australian Institute for Medical Research, and School of Medicine and Pharmacology, University of Western Australia, Perth, WA, [1]Division of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA.

SRA (Steroid Receptor RNA Activator)[1] plays an important role in regulating estrogen receptor activity. Expression of this non-coding mRNA is both increased and aberrant in many human breast tumours suggesting a potential role in pathogenesis. SRA mRNA has a complex predicted

structure with multiple stem-loops, which are integral to its transactivation capacity[2]. To identify proteins that bind SRA, and could therefore regulate its activities, we employed yeast three hybrid analysis. Using discrete domains of SRA as bait, a human breast cancer cell line library was screened resulting in the isolation of a novel protein, C1. This gene encodes a 109 amino acid protein containing an RNA recognition motif (RRM) distinct from that which characterizes the previously described SRA-binding protein family. We have found variable amounts of C1 mRNA to be present in human tissues and cell lines with the highest levels being detected in skeletal muscle, liver and hormonally responsive tissues. Transient transfection of C1 expression constructs into mammalian cells results in the suppression of SRA-mediated transactivation of an estrogen-responsive reporter. Consistent with its role as a transcriptional regulator, further expression studies revealed that C1 has a nuclear localisation pattern. In summary, we have identified a novel RRM containing SRA-binding protein that represses SRA-potentiated ER-mediated transcription. Further characterisation of this molecule will provide important insights not only into SRA co-regulation of estrogen dependent pathways but also the emerging field of non-coding RNA co-factor activity.

(1) Lanz RB, et al. Cell 1999, 97: 17-27.
(2) Lanz RB, et al. PNAS 2002, 99:16081-6.

327

EXPRESSION OF STEROID RECEPTOR COACTIVATORS IN PAIRED MYOMETRIAL ~~NORMAL~~ AND FIBROID TISSUES~~MYOMETRIUM~~

Sonay Hussein-Fikret[1,2], Caroline E. Gargett[3], Peter A.W. Rogers[3], Peter J. Fuller[1,2]

[1]Prince Henry's Institute of Medical Research, Monash University Departments of [2]Medicine and of [3]Obstetrics and Gynaecology, Clayton, Victoria, 3168.

Uterine fibroids or leiomyomas are benign smooth muscle tumours of the myometrium that affect ~25% of women, cause significant morbidity, and are the most common indication for hysterectomy. Estrogens play a central role in fibroid pathology, since fibroids grow during the reproductive years when estrogen levels are high, and regress in hypoestrogenic states, such as menopause. Estrogen actions are mediated via the two estrogen receptors, ERα and ERβ. Fibroids ~~have been shown to~~ overexpress ER, and ~~more~~ recently we demonstrated that cultured fibroid and myometrial smooth muscle cells (SMC) only express ERα, ~~and~~ while microvascular endothelial cells (MEC) constitutively express ERβ and variably express ERα in these tissues (1). ~~ERβ expression has been examined in the two cell types of normal and fibroid myometrium: the smooth muscle cells (SMC) and the microvascular endothelial cells (MEC).~~ The contribution of ERα and ERβ, in the response to estrogen, at the level of the vascular and muscular components of myometrium ~~normal~~ and fibroids ~~myometrial tissues~~ is likely to be influenced by the relative levels of the steroid receptor coactivator (SRC) family members. The aim of this study was to determine if pure populations of SMC and MEC from paired human myometrium and fibroid samples, used in the ER study, differentially express the SRC-1, SRC-2 and SRC-3 coactivator genes. Using RT-PCR coupled with Southern blot analysis, we demonstrated that both SMC and MEC from myometrium and fibroids expressed SRC-1, SRC2 and SRC-3, but there was no ~~change~~ differential expression between ~~-~~SMC or MEC~~in SRC-1, SRC-2 or SRC-3 expression between cultures of~~ from ~~normal~~ myometrium and fibroid pairs~~in both SMC and~~ . However, SMC expressed higher levels of the SRC family members compared to MEC. Given

the expression profile of ERα and ERβ in SMC, higher coactivator levels in SMC suggest that SRC-1, SRC-2 and SRC-3 may have a role in myometrium and fibroid SMC growth via the action of ERα. Although these coactivators are likely to play a role in the response of the myometrium to estrogens, changes in their levels do not appear to contribute to fibroid pathogenesis. ~~since SMC express only ERα, while MEC constitutively express ERβ and variably express ERα.~~

(1) Gargett C.E. et al, Mol.~~ular~~ Hum.~~an~~ Reprod.,~~uction~~ 8: 770-775, 2002.

328

SECOND MESSENGER CONTROL OF PROGESTERONE RECEPTOR EXPRESSION IN HUMAN MYOMETRIAL CELLS

Gemma Madsen, Tan Erdonmez, Roger Smith, Tamas Zakar, Sam Mesiano

Mothers and Babies Research Center, University of Newcastle & John Hunter Hospital, Newcastle, NSW.

In most mammals progesterone withdrawal initiates parturition. In humans, progesterone withdrawal occurs by decreased myometrial progesterone responsiveness (i.e., functional progesterone withdrawal), achieved by increased expression of progesterone receptor-A (PR-A), a repressor of progesterone actions. As the extent of progesterone responsiveness is inversely related to the amount of PR-A relative to progesterone receptor-B (PR-B), the principal mediator of progesterone actions, it is important to understand the mechanisms controlling the myometrial PR-A/PR-B expression ratio. Therefore, we examined the intracellular signaling pathways that control PR-A and PR-B expression in PHM1-31 cells, an immortalized human myometrial cell line derived from a term non-laboring uterus. PHM1-31 cells were exposed to cyclic-8-bromoadenosine monophosphate (cAMP) and phorbol 12-myristate 13-acetate (PMA) at various concentrations for 24h. Abundance (relative to 18S rRNA) of mRNAs encoding total PRs and PR-B was determined by real-time quantitative RT-PCR. Abundance of mRNAs encoding PR-A and PR-B were coordinately increased by cAMP in a dose dependent manner. Consequently, the PR-A/PR-B expression ratio was not changed by cAMP. In contrast, both PMA and oxytocin dose dependently increased PR-A expression but did not affect PR-B expression. This caused a marked dose dependent increase in the PR-A/PR-B expression ratio. These data indicate that in human myometrial cells, hormonal agents acting through the cAMP/PK-A signaling pathway maintain progesterone responsiveness, whereas those acting through the PLC/PK-C/Ca^{++} signaling pathways decrease progesterone responsiveness. Interestingly, agents that increase cAMP/PK-A relax the myometrium whereas agents that increase intracellular Ca^{++} levels induce contractions. This suggests that a positive feedback loop operates whereby agents that increase myometrial contractions also cause functional progesterone withdrawal by increasing the PR-A/PR-B expression ratio

MOLECULAR LOCALIZATION OF THE HUMAN CALCIUM-SENSING RECEPTOR BINDING SITE FOR L-AMINO ACIDS

A.H. Franks, H-C. Mun, E. Culverston, C. Collyer, A.D. Conigrave

School of Molecular and Microbial Biosciences, University of Sydney, NSW 2006 and Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown NSW 2050.

We demonstrated previously that the calcium-sensing receptor (CaR) is activated allosterically by L-amino acids (1,2). As a result, the CaR's Ca^{2+} sensitivity is markedly enhanced in the presence of physiological concentrations of L-amino acids. This may explain how elevated dietary protein intake influences calcium metabolism by suppressing parathyroid hormone secretion and activating urinary calcium excretion (3) via CaRs in the parathyroid and kidney respectively.

We have now localized the amino acid binding site on the CaR using chimeric receptors constructed from major domains of two homologs – the human CaR and a rat metabotropic glutamate receptor (mGlu-1). Three potential binding regions have been analysed: the N-terminal extracellular Venus Fly Trap (VFT) domain (approximately 500 amino acids), the contiguous Cys-Rich region (CRR; approximately 100 amino acids) and the seven transmembrane domain region. The following chimeric receptors (in the order VFT-CRR-TMR:tail) have been used in this analysis: the wild-type CaR, CaR-CaR-Glu, CaR-Glu-Glu and a functionally active construct lacking both the VFT and CRR. Taken together, the analysis points to an amino acid binding site in the CaR's VFT domain.

Refinement of the amino acid binding site was undertaken using site-directed mutagenesis of conserved residues in the putative VFT binding pocket. This study indicates that two conserved residues in ligand binding lobe-1 (LB-1) of the VFT, T145 and S170 (as revealed by T145A and S170A) are required for amino acid dependent activation. Another conserved residue in LB-1, S147 is required for Ca^{2+}-dependent but not amino acid dependent activation (as revealed by the mutant S147A). Conserved residues in LB-2 including Y218 (as revealed by Y218F) and E297 (as revealed by E297Q) are required for Ca^{2+}-dependent but not amino acid dependent activation. Molecular modelling of the amino acid binding site in the VFT domain led to the further prediction that H41 projects into the side-chain binding region. Consistent with this hypothesis, H41W markedly impaired amino acid-dependent but not Ca^{2+}-dependent activation.

(1) Conigrave, A. D. et al., *Proc. Natl. Acad. Sci. USA, 97*, 4814-4819 (2000).
(2) Conigrave, A. D. et al., *Eur. J. Clin. Nutr., 56*, 1072-1080 (2002).
(3) Kerstetter, J. E., et al., *Am. J. Clin. Nutr., 66*, 1188-1196 (1997).

331

GENERATION AND CHARACTERISATION OF OSTEOCLAST-CRE TRANSGENIC MICE

W.S.M. Chiu[1], R.A. Davey[1], J.F. McManus[1], A.J. Notini[1], A.I. Cassady[2], J.D. Zajac[1]

[1]Department of Medicine, University of Melbourne, Austin Health, Studley Road, Heidelberg, VIC. [2]Institute for Molecular Bioscience, Brisbane, QLD.

The Cre-*lox* system is a powerful technique that allows tissue specific deletion of a target gene. The purpose of this study is to generate two transgenic mouse lines that express Cre recombinase specifically in osteoclasts by utilising the tartrate-resistant acid phosphatase (TRAP) and the cathepsin K (Ctsk) promoter. To test the suitability of these promoters, Northern blot analysis was used to determine their mRNA levels in tissues from 4 male and 4 female F1 CBA×C57BL6 mice. TRAP and Ctsk mRNA levels did not differ between males and females. TRAP mRNA levels were significantly higher in bone compared with brain (14 fold), colon (4 fold), heart (25 fold), kidney (5 fold), liver (4 fold), lung (13 fold), muscle (15 fold) and stomach (11 fold) ($P < 0.05$). Ctsk mRNA levels were significantly higher in bone compared with heart (55 fold), lung (12 fold) and muscle (60 fold) ($P < 0.05$). Ctsk was undetectable in brain, colon, kidney, liver and stomach. Two constructs have been made using the TRAP (TRAP-Cre) and Ctsk (Ctsk-Cre). Transient transfection of the constructs into the macrophage cell line, RAW 264.7 cells, showed that the promoters were able to drive Cre expression in osteoclast-like cells *in vitro*. The TRAP-Cre construct was microinjected into C57BL6 oocytes resulting in one male and one female founder. The female founder was mosaic and did not transmit the transgene to her offspring. Heterozygous offspring from the male founder expressed Cre mRNA at high levels in brain and testes and at lower levels in bone, colon, heart, kidney, lung, ovary and stomach. TRAP-Cre homozygotes are currently being generated in attempt to increase Cre expression in bone. In conclusion, TRAP and Ctsk mRNA levels are expressed at much higher levels in bone than other tissues suggesting they are appropriate promoters to drive Cre expression in osteoclasts. A TRAP-Cre transgenic mouse line that expresses Cre in bone has been generated. The Ctsk-Cre construct is currently being microinjected into F1 CBA×C57BL6 oocytes. The resulting transgenic mice with the highest level of Cre expression in bone will be mated with *floxed* mice to delete target genes in osteoclasts.

332

INTERACTIONS BETWEEN THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ) AND PEPTIDE GROWTH FACTOR SIGNALLING PATHWAYS IN PROSTATE CANCER CELLS

A.C. Chew[1], L.M. Brownrigg[2], B.B. Yeap[2]

[1]School of Biological Sciences and Biotechnology, Murdoch University, and [2]School of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, Western Australia.

Aims: To examine the effect of the synthetic thiazolidinedione (TZD) agonist ciglitazone, epidermal growth factor (EGF), and other non-TZD ligands on proliferation and peroxisome

proliferator-activated receptor gamma (PPARγ) activation in human prostate cancer (CaP) cells, and the effect of interactions between EGF and ciglitazone.

Methods: Human CaP cell lines DU145 and PC3 were incubated with either ciglitazone (1-10μM), EGF (2.5-25ng/mL), butyrate (a short chain fatty acid; 10μM), indomethacin (10μM) or vehicle control. Cell proliferation was measured using the thiazolyl blue (MTT) assay. To assess PPARγ transactivation, transient transfections were performed using a luciferase reporter under control of three PPARγ DR-1 response elements.

Results: In DU145 cells, ciglitazone 10μM reduced proliferation by 10% compared to control at 48 hours (*P*=0.02), whilst EGF 2.5 and 25ng/mL increased proliferation by 10% (both *P*=0.01). At 96 hours, ciglitazone 10μM decreased proliferation by 20% (*P*=0.01). Ciglitazone 1μM +EGF 2.5 ng/mL decreased proliferation by 20% (*P*=0.03), compared to no effect on proliferation with ciglitazone 1μM alone. Ciglitazone 10μM+EGF 25ng/mL had the greatest effect, decreasing proliferation by 25% (*P*=0.02). In PC3 cells, ciglitazone 1 and 10μM inhibited proliferation by 10% and 15% at 48 hours (both *P*=0.01), while ciglitazone+EGF inhibited proliferation by 15% and 25% at 48 hours (*P*=0.01 and 0.03). In DU145 cells at 48 hours, butyrate 10μM and indomethacin 10μM caused 10% and 25% reductions in proliferation respectively (*P*=0.01 and 0.03). By 96 hours, butyrate 10μM had reduced proliferation by 30% (*P*=0.001), as had indomethacin 10μM (*P*=0.005). In PC3 cells at 48 hours, butyrate and indomethacin 10μM reduced proliferation by 20%, but these changes were of borderline statistical significance. Ciglitazone 1-10μM increased PPARγ transactivation in a concentration-dependent fashion in DU145 cells, while ciglitazone 10μM activated PPARγ in PC3 cells.

Conclusions: Ciglitazone inhibited DU145 and PC3 cell proliferation within 48 hours. In DU145 cells at 96 hours ciglitazone 10μM inhibited proliferation, and EGF potentiated the effect of ciglitazone 1μM. Both butyrate and indomethacin at 10μM concentration inhibited DU145 proliferation. These data indicate potential roles for combinations of glitazone PPARγ agonists and growth factors, and compounds such as butyrate and indomethacin, to inhibit CaP cell proliferation.

333

ANDROGEN RECEPTOR SUBCELLULAR LOCALIZATION FOLLOWING INTERLEUKIN-6 TREATMENT IN PROSTATE CANCER CELLS

Catherine S-Y. Choong[1], Carmela Ricciardelli[1], Li Jia[3], Ghafar Sarvestani[2], Wayne D. Tilley[1], Gerhard A. Coetzee[3]

[1]Department of Medicine and [2]Detmold Family Trust Imaging Facility, University of Adelaide, Hanson Institute, SA, [3]Department of Preventative Medicine, Norris Cancer Centre, University of Southern California, Los Angeles, CA.

Cross talk between the androgen receptor (AR) and the pleiotropic cytokine interleukin 6 (IL-6) may play a role in AR function. We assessed AR occupancy on the endogenous prostate specific antigen (PSA) promoter/enhancer in response to IL-6 treatment in LNCaP prostate cancer cells. Dihydrotestosterone (DHT) treatment resulted in rapid occupancy of the PSA promoter in chromatin immunoprecipitation (CHIP) experiments. Acetylated histone H3 immunoprecipitation

increased in a qualitatively similar fashion. PSA mRNA levels increased concomitantly with AR promoter occupancy in DHT treated cells. IL-6 treatment inhibited DHT-stimulated PSA expression in LNCaP cells however had little effect on DHT- mediated AR occupancy and failed to stimulate histone H3 acetylation[1] .

Here, we evaluated the effect of IL-6 on AR subcellular localization. LNCaP cells cultured in steroid free medium for 72 hours were treated with IL-6 (10ng/ml) either independently or in combination with 1-10nM dihydrotestosterone (DHT) for 4 and 24 hours. Cultures were scanned using a BioRad Radiance 2100 confocal microscope (Bio-Rad Microscience Ltd, UK) equipped with three lasers, Argon ion 488nm (14mw); Green HeNe 543nm (1.5mw); Red Diode 637nm (5 mw) outputs and Olympus IX70 inverted microscope. Treatment with DHT decreased cytoplasmic AR levels to 60% of control levels ($P<0.0001$, one-way ANOVA). The cellular distribution of AR in cells treated with IL-6 (10ng/ml) alone was not different to that in untreated controls. AR distribution in cells treated with DHT and IL-6 were similar to those treated with androgen alone. In parallel experiments, localization of the expressed fusion protein AR-GFP in transiently transfected LNCaP cells was evaluated. AR-GFP remained cytoplasmic in IL-6 treated cells similar to untreated controls. DHT induced nuclear localisation of AR-GFP which was not prevented by combined treatment with IL-6 and DHT. Our results indicate that nonsteroidal activation of the AR by IL-6 may not occur by direct interaction with the AR rather that indirect mechanisms such as modulation of common coactivators associated with both signalling axes may be involved.

(1) Jia, *et al.* Androgen receptor activity at the prostate specific antigen locus: steroidal and non-steroidal mechanisms, Molecular Cancer Research. *1:* 385, 2003.

334

PREGNANCY-ASSOCIATED PLASMA PROTEIN-A, IGF AND IGFBP-4 EXPRESSION IN HUMAN OVARIAN CANCER

Maria Alexiadis, Simon Chu, Peter Fuller

Prince Henry's Institute of Medical Research, Clayton, Victoria 3168.

Pregnancy-associated plasma protein-A (PAPP-A) is expressed in human ovaries, being restricted to the granulosa cells of healthy antral follicles and the granulosa-lutein cells of the healthy corpus luteum. PAPP-A has recently been identified as a protease that acts on IGFBP-4 to modulate the availability of insulin-like growth factors (IGF). IGF's are important in carcinogenesis and are known to have a critical role in granulosa cell growth and differentiation. We have postulated that the PAPP-A/IGFBP-4/IGF pathway may play a role in the development of granulosa cell tumors (GCT). In order to address this hypothesis, we characterized the expression profiles of the IGF-I, IGF-II, IGFBP-4 and PAPP-A genes in a cohort of GCT and compared these with expression in the normal ovary and epithelial tumors of the ovary. Total RNA was extracted and reverse transcribed for use in RT-PCR assays for the above genes. B_2M microglobulin was used to correct for variability in loading and PCR efficiency. The amplicons were verified by sequence analysis and subjected to semi-quantitative southern blot analysis using gene specific ^{32}P-labelled probes. Elevated PAPP-A expression was observed in the GCT relative to the other tissues, being 3-fold higher than in normal ovary. PAPP-A expression was virtually absent in the serous (SC) and mucinous (MC) epithelial tumors. IGFBP-4 was similarly absent from the epithelial tumors but was expressed at equivalent levels in the GCT and normal

ovary. IGF-I was expressed in all tissues but was also most abundant in the GCT and the normal ovary. IGF-II was expressed with relative abundance: normal ovary > SC > GCT > MC. The relative ratio of IGF-I to IGF-II in the GCT when compared with the normal ovary is consistent with the reported role of IGF-I in malignancy. Although the increase in PAPP-A mRNA levels in the GCT may simply reflect enrichment of a granulosa cell population, the lack of a parallel increase in IGFBP-4 levels may be significant. This suggests that IGFBP-4 proteolysis may be increased with a consequent increase in IGF-I and IGF-II availability.

335

CLINICAL AND BIOCHEMICAL REMISSION OF ACROMEGALY IN PREGNANCY

Sultan Linjawi, Shaun M^cGrath, Roger Smith

Mothers and Babies Research Centre, John Hunter Hospital, Newcastle, NSW.

Pregnancy in acromegalic women is very uncommon. This is in part due to gonadotrophin deficiencies, hyperprolactinaemia and menstrual disturbances. We report on a 19-year-old patient with growth hormone excess due to a large pituitary macroadenoma who underwent spontaneous remission during two sequential pregnancies. At diagnosis her growth hormone (GH) level was 68.5mIU/l with an Insulin-like growth factor-1(IGF-1) of 4.54 U/ml (0.6-1.5). Her initial surgery failed to improve her symptoms and her biochemistry remained abnormal with a GH 12.7mIU/l and IGF-1 2.93U/ml.

Two months following pituitary surgery the patient conceived and during the course of the pregnancy her headaches improved, her clinical features subsided and there was a reduction in her GH levels and normalization of IGF-1 in the second trimester. The GH/IGF-1 profile was typical of normal pregnancy. She delivered a healthy baby and her symptoms returned post partum with biochemistry deteriorating to pre-parous levels.

Six months later the patient conceived for a second time and this pregnancy was also associated with a reduction in her GH and normalization of IGF-1 levels with a subsequent improvement in her clinical symptoms. Again following the delivery of a normal baby there was a return of her symptoms and an elevation of her biochemical markers and she began treatment with long acting intramuscular octreotide.

There are a number of possible explanations for this phenomenon but we postulate that in this case placental growth hormone is partially inhibiting GH production by the pituitary tumour but more importantly providing competitive inhibition at the level of the hepatic GH receptor, thereby decreasing IGF-1 levels. Further understanding of the molecular mechanisms of this process may offer an alternative method of growth hormone control in acromegalic patients.

INTRA-AND INTER-SUBJECT VARIABILITY AND ANALYTIC IMPRECISION OF MEASUREMENTS ON GH-REGULATED PROTEINS: IMPLICATIONS FOR MEASUREMENT OF BIOMARKERS IN SPORT

Tuan Nguyen[1] , Chris Howe[2], Kevin Hardman[3], Sridevi Meka[3], Anne Nelson[1], Kin Leung[1], Graham Trout[2], Robert Baxter[3], David Handelsman[4], Minoru Irie[5], Ray Kazlauskas[2], Ken Ho[1]

[1]Garvan Institute; [2]ASDTL, Pymble; [3]Kolling, St Leonards; [4]ANZAC Research Institute, Concord, Sydney; and the [5]Japan Institute of Sports Science, Tokyo, Japan.

Assessment of IGF-I system components are subject to random measurement error and biological variation. These effects need to be understood if related markers are used for detecting GH use in sport. We investigate the analytic imprecision, intra- and inter-subject variability in a large data set of 1087 international elite athletes. Samples were obtained on at least three successive occasions. Serum IGF-I, IGF binding protein-3 (IGFBP-3), and acid-labile subunit (ALS) levels were measured in duplicate by radioimmunoassay. A random-effects model was used to estimate four components of variance, expressed relative to the mean as coefficients of variation: intra-assay, (CV_a), biological (CV_b), intra-subject (CV_i), and inter-subject (CV_t). Index of individuality (*II*) was defined as the ratio of intra- over inter-subject variation (CV_i/CV_t). A value of <0.6 suggests that the population-based reference intervals are of limited value in detecting unusual results for an individual. The analytical imprecision ranged from 4.6% to 6% for the three markers. The CV_i for IGF-I was 21%, nearly two-fold higher than that for IGFBP-3 and ALS, with the major component being biological. CV_t for IGF-I was also significantly higher than for IGFBP-3 and ALS. *II* was > 0.6 for all three measures.

Measurement	Mean	CV_a	CV_b	CV_i	CV_t	*II*
IGF-I (µg/L)	161.8	5.9	20.0	20.8	30.8	0.67
IGFBP-3 (mg/L)	3.9	4.6	9.7	11.3	15.9	0.71
ALS (nmol/L)	321.0	5.4	10.1	11.6	18.7	0.62

Substantial biological variation of IGF-I markers exists, rendering it important to obtain multiple measurements for characterising diagnostic status, particularly in the context of detecting doping in sport. This variability should be built into defining the positive predictive value of a test.
Supported by the World Anti Doping Agency.

MULTIPLE ENDOCRINE NEOPLASIA SYNDROME PRESENTING WITH UPPER GASTROINTESTINAL BLEEDING

H.V. Nguyen[1], L.C. Mollison[2], R. Weedon[3], B.B. Yeap[1,4]

[1]Department of Endocrinology and Diabetes, [2]Department of General Medicine, and [3]Department of Surgery, Fremantle Hospital, Western Australia, and [4]School of Medicine and Pharmacology, University of Western Australia.

Case history: We report a probable index case of MEN-1 syndrome in a 37 year-old Caucasian female who presented with upper gastrointestinal bleeding. She had recently undergone a three-gland parathyroidectomy for hyperparathyroidism presenting with significant hypercalcemia, with parathyroid hyperplasia confirmed histologically. Gastroscopy revealed a bleeding ulcer in the distal duodenum and multiple other small shallow ulcers. Fasting plasma gastrin was elevated at 4,554 pmol/L (N 0-43). Octreotide scan showed lesions in the head of pancreas, duodenum and spleen. CT abdomen depicted a 2.4*1.7cm lesion in the pancreas and a less discrete hypodensity in the tip of the spleen. Her admission was complicated by pulmonary embolism and low grade fever.

Family history: her younger sister was on cabergoline therapy for a pituitary macroprolactinoma and her mother underwent partial parathyroidectomy in her late 20's.

Other investigations: included normal VIP, IGF-1 and fasting BSL. C-peptide was mildly elevated, and prolactin level was 1100 mU/L.

Points for discussion: Optimal management of multifocal gastrinoma without liver metastases. Likely benefits and risks of medical versus surgical management will be reviewed (1,2,3,4). Unusual features of the case will also be discussed such as the presence of splenic metastasis, not commonly seen with this tumour (5,6), and the occurrence of a paraneoplastic syndrome more typical of epithelial cell tumours encompassing fever and a hypercoagulable state leading to pulmonary emboli.

(1) Brandi ML, Gagel RF, Angeli A et al. JCEM 2001;86:5658-5671.
(2) Veldhuis JD, Norton JA et al. JCEM 1997;82:357-364.
(3) Gibril F, Venzon DJ et al. JCEM 2001;86:5282-5293.
(4) Proye CAG, Nguyen HH. Aust NZ J Surg 1999;69:106-116.
(5) Azimuddin K, Chamberlain RS. Surg Clin North Am 2001:511-523.
(6) Jensen RT. J Intern Med 1998;243:477-488.

338

GHRELIN AND MEASURES OF SATIETY ARE ALTERED IN POLYCYSTIC OVARY SYNDROME BUT NOT AFFECTED BY DIET COMPOSITION

L.J. Moran[1,2], M. Noakes[2], P.M. Clifton[2], G.A. Wittert[3], N. Luscombe[3], C. Galletly[4], L. Tomlinson[1], R.J. Norman[1]

[1]Depart. Obstetrics and Gynaecology, University of Adelaide, [2] CSIRO Health Sciences and Nutrition, South Australia, [3] Depart. Medicine, University of Adelaide, [4] Depart. Psychiatry, University of Adelaide.

Polycystic ovary syndrome (PCOS) is a common endocrine condition in women of reproductive age. Ghrelin is a hormone implicated in appetite regulation which may be dysregulated in PCOS. The effect of varying dietary composition on ghrelin is additionally unknown. This study examined the effects of a standard protein (SP) (55 % carbohydrate, 15 % protein) or high protein (HP) diet (40 % carbohydrate, 30 % protein) on weight loss, body composition, glucose and insulin homeostasis, fasting and post-prandial ghrelin and satiety by visual analogue scores (VAS) in overweight women with (n = 20, n = 10 SP, n = 10 HP) or without PCOS (n = 12, n = 6 SP, n = 6 HP) matched for BMI. The intervention consisted of 12 weeks energy restriction (~ 6000 kJ/day) followed by 4 weeks weight maintenance. Post-prandial insulin, ghrelin and satiety were assessed after a representative meal tolerance test (MTT). Decreases in weight (7.5 %), total fat mass (13.4 %), abdominal fat mass (13.3 %), fasting insulin (18.7 %) and MTT insulin (25 %) occurred with no differential effect of diet composition or PCOS status. Diet composition had no effect on fasting or post-prandial ghrelin or measures of satiety. Compared to subjects with PCOS, subjects without PCOS had higher fasting baseline plasma ghrelin (60.8 ± 6.8 pM vs 103.6 ± 17.2 pM, p = 0.011), a greater increase in fasting ghrelin with weight loss (16.5 ± 5.1 pM vs 47.9 ± 16.7 pM, p = 0.033) and a greater improvement in MTT ghrelin with weight loss (-8.5 ± 4.1 pM vs -42.7 ± 17.3 pM, p = 0.02). Satiety was not related to ghrelin or diet composition, however PCOS subjects were significantly hungrier at both week 0 and week 16 than non-PCOS subjects (p = 0.003). Measures of satiety and ghrelin homeostasis are significantly impaired in subjects with PCOS with no differential effect of diet composition. These results suggest that appetite regulation may be impaired in PCOS.

339

SALIVARY CORTISOL ASSAY– ADAPTATION OF THE ROUTINE SERUM ASSAY

Max Goodwin, Mairead Harrop,

Peter Colman Departments of Biochemistry and Diabetes & Endocrinology, Royal Melbourne Hospital, Grattan St, Parkville, 3050.

Diagnosis of Cushing's syndrome remains one of the most challenging aspects of Endocrine practice. Measurement of salivary cortisol at 2300h has recently been suggested as a sensitive and specific screening test. Adoption of this cortisol measurement creates a number of challenges. Firstly, cortisol in saliva is unbound and thus in low concentration and secondly collection of saliva samples is not routinely used. However, cortisol stability potentially allows sampling of saliva within the patient's home with samples transported to the laboratory the next

day. Our objective was to develop a method suitable for small numbers of patient samples which could be quickly and easily measured for cortisol using our routine assay, the Diagnostic Products Corporation (DPC) Immulite 2000 cortisol assay. The required limit of detection was less than 3.6nmol/L (upper limit of 2300h reference range (1)). Results were compared to a commercially available RIA, (Coat-a-Count Cortisol, Los Angeles, CA, (DPC) using Tunn's modification (2). 45 people without pituitary or adrenal problems collected saliva into plain "Salivettes", Sarstedt Australia Pty Ltd., SA. The device consists of a cotton swab within 2 plastic tubes. Subjects were asked to collect saliva at 0800h and 2300h, to store samples at 4°C until transported to the laboratory. Centrifuged samples were stored at -20°C until assay. Salivary cortisol was extracted using equal volumes of ethyl acetate and saliva. The ethyl acetate extract was reconstituted with DPC cortisol diluent to attain a 10-fold concentration and maintain a suitable matrix for analysis on the Immulite 2000. The ranges for salivary cortisol are shown below.

	0800h	2300h
Immulite2000	4.6-24.6 nmol/L(n = 35)	<6.2 nmol/L(n = 43)
RIA	3.4-23.6 nmol/L(n= 32)	0.15-4.1 nmol/L(n = 40)
Correlation (r)	0.89342	0.84598

Modification of the currently used assay to measure salivary cortisol provides comparable results to the commercially available salivary cortisol assay.

(1) Raff et al., JCEM 83(8): 2681.
(2) Tunn et al, Clin Chem. 1992;38:1491-94.

340

THE EFFECTS OF DENSITY ON TADPOLE GROWTH, DEVELOPMENT AND METAMORPHOSIS: MEDIATION VIA ENDOCRINE PATHWAYS

J. Taylor[1], J. Clulow[1], J. Fitter [2], N. Plant[2], R. Smith[2]

[1] Department of Biological Sciences, University of Newcastle, Callaghan, [2] Mothers and Babies Research Centre, HMRI, John Hunter Hospital, Newcastle.

The life cycle of anurans involves the transformation of aquatic larvae into semi-terrestrial frogs by a process termed metamorphosis. Anuran larvae are known to exhibit adaptive plasticity in size at metamorphosis and duration of the larval period when subjected to environmental changes. Density stress is well documented to reduce larval survivorship, growth rate, and body size at metamorphosis. Though not well understood, the regulation of metamorphosis is via the hypothalamic-pituitary-thyroid axis. It is unclear whether the effects of density are related to total water volume or number of tadpoles per unit volume. To explore the relationships between these two variables we examined density effects on the Australian species *Limnodynastes peronii* through metamorphosis, with regard to body weight and length. In preliminary growth studies, tadpoles were grown in a 2x4 factorial design with two water volumes (shallow and deep) and four tadpole densities (0.088 tadpoles/L to 4 tadpoles/L). At the same density (tadpoles per litre), deep water treatments produced metamorphs of a larger size than shallow water. Shallow water treatments revealed a negative correlation between size at metamorphosis and tadpole density, and a negative correlation between time to metamorphosis and tadpole density. We hypothesise

that an increase in density is associated with an increase in CRH levels, which in turn stimulates thyrotropin-stimulating hormone and adrenocorticotropin production, leading to higher levels of TH and corticosterone than in control animals at low density. Higher levels of TH and corticosterone may account for the reduction in growth rate and time to metamorphosis observed at high densities.

341

THE IMPACT OF HABITAT ON THE METAMORPHOSIS OF THE AUSTRALIAN ANURAN LIMNODYNASTES PERONII

John Clulow[2], Katrina J. Pirlo[1], Robert Denver[3], John T. Fitter[1], Michael J. Mahony[2], Natalie Plant[1], Roger Smith[1]

[1]Mothers and Babies Research Center, Hunter Medical Research Institute, JHH, Newcastle, NSW, [2]Australian Amphibian Research Group, University of Newcastle NSW, and [3]University of Michigan, Ann Arbor, USA

A number of species of North American anurans respond adaptively to habitat changes (such as pond drying) by accelerating metamorphosis. We manipulated swimming volume independently of total water volume using an internal cage. We grew 10 day old *Lim. peronii* tadpoles in three treatments with total volume/swim volume as: large/large; large/small; small/small. Tadpoles reared in large/large had a long larval period, were the greatest size at metamorphosis, and exhibited a strong relationship between the weight of animals and their developmental stage (r=0.868). Tadpoles reared in large/small had a long larval period, were small at metamorphic climax and the relationship between weight and development stage was reduced (r=0.694). Tadpoles reared in small/small had a short larval period, were small at metamorphic climax, and showed less correlation between weight and stage (r=0.529).

These results suggest that large water bodies with lower tadpole density result in a long larval period, where metamorphosis is closely linked to tadpole weight. Reduced swimming volumes and higher tadpole density result in a small size at climax for *Lim. peronii* tadpoles. Nearly 2.5 times more tadpoles reared in small/small reached metamorphic climax within the experimental period compared to large/large and large/small. Stress elicits a plastic reaction in phenotype with production of rapid metamorphosis regardless of weight. In the absence of stress, the phenotype demonstrates a close relation between weight and metamorphic stage.

342

HABITAT EFFECT ON THE ENDOCRINOLOGY OF THE ANUSTRALIAN ANURAN, *LIMNODYNASTES PERONII* DURING METAMORPHOSIS

Katrina J. Pirlo[1], John Clulow[2], Robert Denver[3], John T. Fitter[1], Michael J. Mahony[2], Natalie Plant[1], Roger Smith[1]

[1]Mothers and Babies Research Center, Hunter Medical Research Institute, JHH, Newcastle, NSW, [2]Australian Amphibian Research Group, University of Newcastle, NSW, [3]University of Michigan, Ann Arbor, USA.

Anuran species exhibit phenotype plasticity in growth and time to metamorphosis in stressful environments. We hypothesised that tadpole larvae brain corticotrophin releasing hormone (CRH) concentrations would reflect an integrated measure of larval stress and correlate with growth, time to metamorphosis and concentrations of thyroid hormone. Overcrowding and reduced water volumes are known stressors for tadpoles. To test our hypothesis *Lim peronei* tadpoles were grown in large and small containers with food ad libitum. Larvae were randomly harvested from each condition every second day from 30 to 50 days post spawning. Larval stage was recorded using the Gosner Staging table, plus larval weight and length. Brain CRH was assayed using a CRH radioimmunoassay previously validated for this species. Whole body T_4 and T_3 was assayed using an extraction procedure described by Denver et al., and radioimmunoassay. Larvae grown in restricted water exhibited rapid metamorphosis and reduced weight at metamorphosis. Brain CRH concentrations were detectable but did not change with metamorphic stage or between groups. Whole body T_4 did not change with metamorphic stage or across treatment groups. T_3 increased with metamorphic stage but did not vary between treatment groups.

Conclusions: this study did not support the hypothesis that CRH drives changes in metamorphosis in the stressed developing larvae of the Australian anuran *Lim peronei.* Increasing T_3 with metamorphosis supported the involvement of this factor in regulating metamorphosis but no similar evidence was produced for T_4. In *Lim peronei* regulation of thyroid hormone action may occur at the level of T_4 to T_3 formation by de-iodinases.

343

EFFECTS OF GHRH AND GHRP-2 TREATMENT IN VITRO ON LEVELS OF GH, PIT-1, GHRH-R, GHS-R, SST-R MRNAS AND GH SECRETION IN OVINE PITUITARY CELLS

Chen Chen, Ming Yan, Maria Hernandez, Ruwei Xu

Endocrine Cell Biology, Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Victoria 3168. <chen.chen@med.monash.edu.au>

The GH-releasing hormone (GHRH) and GH-secretagogues (GHS) stimulate the release of GH through their specific receptors on somatotropes. In clinical diagnosis, the combined GHS and GHRH administration has been suggested as the new "gold standard" test of GH deficiency with or without pituitary dysfunction. Therapeutically, administration of synthetic small molecular GHS may be preferable to the direct administration of GH due to an increase in pulsatile GH

secretion without change of tough GH levels. The molecular and cellular mechanism of GHS action on the function of somatotropes is not clear. In the current experiments, primary cultured ovine somatotropes were used to measure GH secretion and the expression of mRNAs encoding for GH, Pit-1, GHS-R (GHS-R), GHRH-R, somatostatin receptors (sst-1 and sst-2) after GHRH and GHRP-2 treatment of cells for 0.5, 1, 1.5 and 2 h. The concentration of GH in the incubation medium was measured by radioimmunoassay. GH, Pit-1, GHS-R, GHRH-R, sst-1 and sst-2 mRNA levels were assessed using semi-quantitative RT-PCR methods. GHRH (10 nM), GHRP-2 (100 nM), and combined GHRH/GHRP-2 increased the levels of GH mRNA and GH release from 0.5 to 2 h in a time-dependent manner. The levels of Pit-1, GHRH-R and GHS-R mRNA were increased after 0.5 h treatment of cells with GHRH and GHRP-2. The levels of sst-1 but not sst-2 mRNA were significantly increased after 0.5 and 1h of GHRH treatment. In contrast both sst-1 and sst-2 mRNA expression was inhibited after 0.5 to 2h of GHRP-2 treatment. These data demonstrate a direct in vitro modification of ovine somatotropes by GHRH and GHRP-2 on GHRH-R, GHS-R, Pit-1, sst-1, sst-2, and GH gene expression, which may underlie the regulatory effects of GHRH and GHRP-2 on GH secretion. This study represents an essential step forward in understanding the influence of GHRH and GHS on pituitary somatotropes and will aid the development of new GHS with high efficacy. Supported by Australian NHMRC.

344

HISTO-ANATOMICAL ANALYSIS OF NEURONAL SYSTEMS THAT REGULATE GROWTH HORMONE RELEASING HORMONE (GHRH) AND SOMATOSTATIN (SRIF) CELLS

Javed Iqbal, Iain J. Clarke

Prince Henry's Institute of Medical Research, P0 Box 5152, Clayton, 3168, Australia.

Central mechanisms controlling secretion of GHRH and SRIF are not well defined. We have used immunohistochemistry and confocal microscopy to determine the extent of colocalisation of peptides/neurotransmitters within GHRH and SRIF cells and afferent inputs to these cells in food restricted (Lean) and Normal animals.

TABLE 1: Mean (±SEM) number of GHRH and SRIF cells with terminals from various neuronal systems in close apposition.

Type of input	GHRHcells (Lean)	SRIF cell (Normal)
SRIF	31.7 ± 1.5	-
Enkephalin	20.2 ± 3.0	0
GHRH	-	26.5 ± 8.0
Neuropeptide Y	0	20.6 ± 6.5
Orexin	0	14.0 ±4.8
Galanin	0	15.6 ± 5.5
Noradrenergic	29.5 ± 4.0	28.2 ± 3.0

The number of GHRH cells (mean ± SEM) was higher in Lean animals (115±14) than in Normal animals (63.7±6.6), but the number of SRIF cells in the periventricular nucleus was similar in Lean (230±21) animals and Normal (196.25±17.5) animals. To optimise the number of cells under study, we examined GHRH cells in Lean animals and SRIF cells in Normal animals. Double-labelling immunohistochemistry and confocal microscopy showed that subpopulations of

GHRH cells co-stain for neuropeptide Y (17%), tyrosine hydroxylase (dopaminergic) (23%), galanin (9%) and galanin-like peptide (2%). In the periventricular nucleus, 8% of SRIF neurons also immunostained for enkephalin. Input to the GHRH and SRIF cells from various neuronal systems was quantified by counting cells for which close contacts were seen with immunoreactive terminals and the data are shown in Table 1.

We conclude that various neuronal systems regulate GH secretion by acting on GHRH and SRIF. A novel finding is that a substantial level of input to both cell types is noradrenergic.

345

PROLACTIN REGULATION OF TYROSINE HYDROXYLASE ACTIVITY AND EXPRESSION IN CULTURED HYPOTHALAMIC DOPAMINERGIC NEURONS

F.Y. Ma, D.R. Grattan, S.J. Bunn

Department of Anatomy and Structural Biology and Centre for Neuroendocrinology, University of Otago, Dunedin, New Zealand.

Dopamine released from the hypothalamic dopaminergic neurons is the major inhibitory factor in the control of prolactin secretion. The activity of these neurons is regulated by prolactin negative feedback. To determine the mechanism by which prolactin mediates this action, we have investigated the activity and expression of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis, using cultures of fetal rat medial basal hypothalamic neurons. Prolactin was found inducing a time- and concentration-dependent increase in TH activity (1μg/ml for 60 minutes raising the response to 126±4 % of basal). This response was abolished by inhibitors of protein kinase A (10 μM H89 but not H85), protein kinase C (3μM bisindolylmaleimide I but not V) or the MAP-kinase pathway (50μM PD 98059). In contrast the CaMKII inhibitor KN-93 (3 μM) but not KN-92, produced only a partial inhibition. Prolactin also raised the TH mRNA expression, with 4 hr incubation (1 μg/ml) increasing TH mRNA levels to 161±13% of basal. In contrast to TH activity this response was not inhibited by any of the protein kinase inhibitors mentioned above.

Prolactin mediates its actions via type I cytokine receptors which couple to the JAK/STAT signaling pathway. *In vivo* data from our laboratory indicates a selective role for STAT5b in these neurons. Immunocytochemistry revealed that prolactin caused STAT5b (but not STAT1, 3 or 5a) to undergo nuclear translocation and phosphorylation in some but not all of the TH-positive neurons in culture. Using an antibody against phospho-STAT5 this response was detectable after 5 minutes (approx. 30% of TH positive neurons) and reached a maximum between 15 to 30 minutes (60%) but then declined by 1 hour (40%) to reach near basal levels by 4 hours (little detectable staining). As with the increase in TH mRNA expression prolactin-induced STAT5 phosphorylation and nuclear translocation was unaffected by the protein kinase inhibitors mentioned above. Our results suggest the involvement of multiple protein kinases (protein kinase A, protein kinase C, MAP-kinase and perhaps CaMKII) in prolactin-induced TH activation in dopaminergic hypothalamic neurons. In contrast these protein kinases are not required for prolactin-induced TH mRNA expression. While the mechanism for this latter response is unknown our data suggest a possible role for STAT5b.

346

HIGH RESOLUTION SEPARATION OF PREGNANCY TRANSCORTIN GLYCOFORMS

E. Mitchell[1,3], D.J. Torpy[1,2], C.J. Bagley[1,3]

[1]Department of Medicine, University of Adelaide, [2]Endocrine and Metabolic Unit, Royal Adelaide Hospital 3. Division of Human Immunology, Hanson Institute.

Transcortin or corticosteroid-binding globulin (CBG) is a 52-56KDa glycoprotein, which has a role in cortisol transport and tissue activity (1,2). CBG levels increase at least 3-fold during pregnancy, and high MW glycan isoforms (glycoforms) of CBG variant (pregCBG) appear. PregCBG comprises at least 10% of circulating CBG during pregnancy and may have altered tissue binding affinity. CBG glycoforms may provide specificity in cortisol transport – each glycan isoform preferentially binding certain tissues (3-5).

CBG glycoforms, including the pregCBG variants, have previously been resolved with lectin affinity purification and SDS-PAGE (4). To achieve high resolution CBG glycoform separation, we employed affinity chromatography, electrophoresis and immunoblotting on serum from women at 16 and 28 weeks gestation and control samples. Each sample was subjected to albumin depletion, using affinity chromatography on Cibacron Blue F3G-A matrix columns, two-dimension electrophoresis, and then Western blotting immunodetection with a commercial polyclonal antibody (Nordic Labs, Tilburg, Denmark). Western blotting clearly revealed several pregnancy specific glycoforms of CBG and their relation to the non-pregnant glycoforms. Determination of the pattern of CBG glycan isoforms, relationship to gestational age, and ultimately their precise sugar moiety distribution, is an important step towards understanding the physiological role of pregCBG.

(1) Hammond GL. *Endocr Rev*, 1990; 11: 65-79.
(2) Ballard PL, New York: Springer-Verlag, 25-48, 1979.
(3) Avvakumov GV, Strel'chyonok OA. *Biochim Biophys Acta*, 1988; 938: 1-6.
(4) Avvakumov GV, Hammond GL. *Biochemistry*, 1994; 31; 5759-65.
(5) Misao R, Iwagaki S, et al. *Horm Res*, 1999; 51: 162-7

347

PATTERN OF GENE EXPRESSION FOR LABOUR IN THE HUMAN MYOMETRIUM

E.-Cheng Chan, Andrew Bisits, George S.H. Yeo[1], Kenneth Kwek[1], Kylie Brown, Roger Smith, Sam Mesiano

Mothers and Babies Research Centre, Hunter Medical Research Institute, The University of Newcastle, John Hunter Hospital, Lookout Road, New Lambton Hts NSW 2305, Australia, [1]Singapore KK Women's and Children's Hospital, Bukit Timah Road, Singapore.

Normal human birth requires the activation of physiological systems that lead to coordinated uterine contractions and full cervical dilatation. Current data suggest inflammatory factors and a functional progesterone withdrawal are key mediators of these essential changes. This study aims to elucidate the relationships among these genes that are implicated in human parturition.

Myometrial strips from the lower uterine segment were collected from women who underwent elective (n=12) and emergency (n=12) Caesarean sections. Total RNA was extracted, reverse-transcribed and quantitative real-time PCR (QRT-PCR) performed to measure the relative expression of progesterone receptors A and B (PR-A and PR-B), estrogen receptors α and ß, oxytocin receptor (OTR), connexin-43 (CX-43), cyclooxygenase-2, (Cox-2), manganese superoxide dismutase (MnSOD), interleukin-8 (IL-8), ß2-microglobulin (ß2µ), hoxA10 and 18S ribosomal RNA (used as reference gene for these assays). The means and medians of all the genes except OTR, ß2µ and hoxA10 were significantly elevated in the labouring tissues (Mann-Whitney U-test). Strong correlations existed among the ratio of PR-A/PR-B and ERα in a non-labouring cohort as well as between ERα and MnSOD, Cox-2, in the labouring group. Using confirmatory factor analysis (STATA), inflammatory scores were calculated for each woman using the 4 genes — IL-8, MnSOD, Cox-2 and ß2-µ. These scores were used in path analysis (AMOS). Path analysis indicated that it was highly probable that the inflammatory genes may drive alterations in PR-A/PR-B ratio that leads to changes in ER and contraction- associated proteins, CX-43 and OTR. These data suggest that immune activation and inflammatory factors may bring about a functional progesterone withdrawal through alteration of the PR-A/PR-B ratio and initiate human parturition.

We conclude that current technology which enables mapping of large numbers of relevant molecular factors can be boosted by multi-variate analysis to reveal networks of gene action that are not apparent from univariate analyses. This provides an approach that circumvents the ethical and practical limitations to understanding human parturition.

348

LOCALISATION AND EXPRESSION OF THE RELAXIN RECEPTOR (LGR7) IN THE CERVIX AND VAGINA OF THE MOUSE

Irna Grace T. Reytomas[1,2], Helen M. Gehring[2], Daniel J. Scott[1], Ross A.D. Bathgate[1], Geoffrey W. Tregear[1], Laura J. Parry[2]

Howard Florey Institute[1]; Department of Zoology[2], University of Melbourne, Victoria, 3010.

Relaxin (Rlx) has important functions in the reproductive tract to ensure safe and rapid delivery of offspring. It targets the cervix and vagina to promote growth and remodelling of the stroma, and proliferation of the epithelium. However, the mechanism by which Rlx contributes to these processes is poorly understood. The recent cloning of the Rlx receptor (LGR7) provides a new tool to identify specific target cells and tissues for Rlx action (1). The aims of this study in pregnant mice were to I) investigate the distribution of Rlx receptors in the cervix and vagina, II) determine changes in LGR7 gene expression throughout gestation and III) examine differences in LGR7 gene expression between $Rlx^{+/+}$ and $Rlx^{-/-}$ mice. Tissues were obtained from C57/Blk6J $Rlx^{+/+}$ mice on days 7.5, 10.5, 14.5, 17.5, 18.5 post coitum (pc) and $Rlx^{-/-}$ litter mates on days 14.5 and 18.5 pc. The murine LGR7 gene was identified in the genome databases by BLAST searching using the human LGR7 sequence. This sequence was used to design specific primers for both RT and real-time PCR for the identification and quantification of mouse LGR7 mRNA in the cervix/vagina of $Rlx^{+/+}$ and $Rlx^{-/-}$ mice. LGR7 mRNA expression was detected by RT-PCR in the cervix/vagina, in all stages examined. Quantitative analysis confirmed there was a significant ($p<0.05$) increase in the expression of the LGR7 transcript in cervix/vagina at the end of gestation. However, there was no difference in LGR7 gene expression between $Rlx^{+/+}$ and Rlx^{-}

$^{/-}$ mice. Using autoradiography, with radioactively labelled synthetic human Rlx2, Rlx binding sites were detected in the luminal region of the cervix and vagina. *In situ* hybridisation confirmed that LGR7s were expressed in epithelial cells lining the lower reproductive tract. In summary, the increase in cervix/vagina LGR7 gene expression at term is associated with an increase in proliferation of the luminal epithelium. In the absence of Rlx, LGR7 expression was unaffected, thus implying a Rlx-independent regulation of its receptor.

(1) Hsu SY *et al* (2002) Science 295:671-74.

349

DIFFERENTIAL GENE EXPRESSION BETWEEN LABOURING AND NON-LABOURING HUMAN MYOMETRIUM

Kellie-Ann Taylor, George S.H. Yeo, Kenneth Kwek, Roger Smith, E-Cheng Chan

Mothers and Babies Research Centre, Hunter Medical Research Institute, The University of Newcastle, John Hunter Hospital, Lookout Road, New Lambton Hts, NSW 2305, Australia

Singapore KK Women's and Children's Hospital, Bukit Timah Road, Singapore.

The current understanding of the initiation and maintenance of human labour is incomplete. To characterize the labour-associated changes in gene expression, we have previously performed RNA subtraction studies using suppression subtractive hybridization (SSH). Two subtracted cDNA libraries enriched for 'labouring' and 'non-labouring' genes respectively were obtained. Preliminary screening of these libraries by dot blots and Southern blots of PCR products indicated a number of genes involved in inflammatory or immune function were up-regulated.

The current study aims to confirm the differential gene expression reported in our published SSH studies (1). Total RNA was extracted from biopsies of the lower uterine segment of women in labour (n=50) and not in labour (n=50). The myometrial RNA was then reverse transcribed and assayed using the sensitive technique of quantitative real-time RT PCR on an ABI 7700 thermal cycler (Applied Biosystems). Specific primers and 5'-FAM-labelled MGB probes (Applied Biosystems) were designed to span exon-intron junctions of target genes while 5'-VIC-labelled probes were used to measure the reference genes, 18S ribosomal RNA and GAPDH. The results confirm that the levels of gene expression of NFKB subunit p65 (*RelA* Accession No. M62399), KIAA0062 (D31887), CRSP6 (AF105421) and MMP9 (NM_004994) are higher in the labouring compared to non-labouring women. Both *RelA* and MMP9 have inflammatory roles and their increase at labour is consistent with the earlier SSH finding that inflammatory genes form a major proportion of labour-up-regulated genes. CRSP6, also called vitamin D receptor interacting protein, is a transcriptional cofactor for Sp1, and is implicated in activation of calcium channels — a role consistent with its probable action in the contracting myometrium. KIAA0062 is one of a family of genes with unascribed function.

These results suggest that these genes have a role in the initiation and maintenance of labour. Further investigation is required to determine whether these changes occur as a cause or consequence of labour.

(1) Chan EC, *et al*. 2002 J Clin Endocrinol Metab 87 (6) 2435-2441

350

FOLLISTATIN IMMUNOACTIVE PROFILES ACROSS PARTURITION IN EWES USING DIFFERENT ASSAYS

Kym Rae, Yin Xia, Tim O'Shea, James R. McFarlane

Physiology, University of New England, Armidale NSW 2351 Australia.

Follistatin binds activin with high affinity and modulates its biological activity. A number of studies have shown that parturition is associated with an increase in activin A. We have previously reported that follistatin rises on the day of parturition in sheep, indicating that follistatin may also be important. However, the data was based on daily samples without the exact time of parturition being recorded, and a more intensive study was required to closely monitor the changes of follistatin around the actual time of birth. Blood samples were collected from pregnant Merino ewes daily and every 6 hr around parturition. Follistatin concentrations were measured using two different radioimmunoassays (RIA). One assay used a rabbit antiserum (#204) raised against purified 35 kDa bovine follistatin and human recombinant follistatin (FS288) was used as both standard and tracer (1). The other assay used a chicken antiserum raised against a follistatin peptide sequence from aa121-133 and the peptide was used as both standard and tracer. Follistatin in daily samples measured using the #204 assay remained unchanged from day –10 to the day of parturition, and then increased by approximately 2-fold on the day following parturition similar to our previous report (1). Follistatin (#204) in the 6 hr samples declined from –9 hr to –3 hr, while cortisol reached the highest level at –3 hr before the completion of lamb delivery. Follistatin (#204) levels were elevated from 15 to 45 hr with peak levels at 27 hr after parturition. Follistatin measured using the peptide assay showed no change before parturition, but there was a significant elevation peaking at 36 hr post birth. The drop in follistatin (#204) levels a few hours before parturition may be involved in the mechanism of normal parturition by increasing activin bioavailability, while the rise in both forms of follistatin after parturition is likely to be linked to the tissue injury during delivery. However, the difference between the two assays suggests that follistatin isoforms may be differentially regulated with different functions.

(1) O'Connor et al 1999 Serum activin A and follistatin levels during human pregnancy: a cross-sectional and longitudinal study. Human Reproduction 14: 827-832.

(2) McFarlane et al 2001 Follistatin levels during the oestrus cycle and across gestation in merino sheep. Reproduction 124:259-265.

351

ESTROGEN MEDIATED REPRESSION OF PLACENTAL CRH GENE EXPRESSION REQUIRES THE CAMP REGULATORY ELEMENT

Xin Ni*, Mark A. Read, Bruce R. King, Roger Smith, Richard C. Nicholson

Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, Australia, & *Department of Physiology, Second Military Medical University, Shanghai, P.R.China.

CRH is the principal mediator of the HPA response to stress, stimulating pro-opiomelanocortin gene expression and ACTH release in the anterior pituitary, which, in turn, stimulates glucocorticoid release from the adrenal cortex. However, during pregnancy the placenta becomes

the major source of CRH production. Placental corticotropin-releasing hormone (CRH) and estrogens, produced by placental trophoblasts, have been suggested to play pivotal roles in the control of human parturition. It is known that estrogen stimulates hypothalamic CRH expression. Therefore, we have evaluated 17β-estradiol (E2) in the regulation of CRH gene expression in placental trophoblast cells in culture. We found that E2 inhibits CRH mRNA expression in a dose-dependent manner, which is paralleled by a decrease in CRH protein levels in culture media. A putative 'pure' estrogen receptor (ER) antagonist ICI182780 not only blocked repression of CRH mRNA levels by E2, but up-regulated CRH mRNA and protein synthesis. The partial estrogen agonist 4-hydroxytamoxifen also down-regulated CRH gene expression. We have observed that placental trophoblasts express predominantly the ERα form of the receptor. Transient transfection assays conducted in the choriocarcinoma cell line JEG-3 cells demonstrated that E2 repressed CRH promoter activity, whereas ICI up-regulated CRH promoter activity, when ERα was cotransfected. Through deletion and mutagenesis analyses we have determined that ERα dependent inhibition of the CRH gene expression is mediated through the cAMP regulatory element (CRE). The CRE is capable of conferring ERα mediated inhibition upon a heterologous promoter, and the A/B domain in ERα is not essential for the estrogen suppressive action. These studies demonstrate that E_2 represses placental CRH gene expression through an ERα mechanism and ERα mediated repression requires the CRE site in the CRH promoter region. Estrogen may therefore modulate placental CRH production, influencing the rate of rise of maternal plasma CRH concentrations and potentially the length of gestation.

352

PRODUCTION OF TRANSTHYRETIN IN PLACENTAL TROPHOBLASTS

Melanie Tom[1], Brett McKinnon[1], Robin Mortimer[1,2,3]

1Conjoint Endocrine Laboratory, Royal Brisbane Hospital Research Foundation, Bancroft Centre, QLD, [2]Department of Endocrinology, Royal Brisbane Hospital, & [3]University of Queensland.

Thyroid hormones, being hydrophobic, require facilitated transport through aqueous environments. Placental trophoblasts separate the circulatory systems of mother and fetus. The fetal thyroid does not develop until the second trimester of pregnancy but thyroid hormones are required for early brain development indicating that there is hormone transfer early in pregnancy. Normal levels of T_4 are found in babies with thyroid agenesis indicating that maternal thyroid hormone also can cross placenta in late pregnancy. The mechanisms mediating thyroid hormone transfer are as yet unclear.

Cytosol thyroid hormone binding proteins are found in several tissues and mediate intracellular hormone transport. To date no thyroid hormone binding proteins have been found in placenta. Transthyretin (TTR) is a major thyroid hormone transport protein that circulates in blood as a tetramer. Circulating TTR is primarily made in hepatocytes, although it is also made in choroid plexus where it carries thyroid hormone into the cerebral-spinal fluid, human retinal pigment epithelium and in low levels in pancreas and kidney. Choroid plexus cells and human retinal pigment epithelium both act as barriers for microenvironments, as does the placenta. **Objectives:** To investigate the presence of TTR in placenta. **Methods:** We performed reverse

transcriptase polymerase chain reaction for TTR in placenta and choriocarcinoma cell lines (BeWo and JAR). Using a polyclonal antibody against TTR we immuno-stained placental sections. **Results:** TTR mRNA was detected in all tested placental samples and in the BeWo but not JAR cell line. By immunohistochemistry TTR was shown to be present and localised in placental trophoblast cells. **Discussion:** Our study shows that TTR is produced and present in placental trophoblast cells. The presence of TTR in the placenta may begin to explain the mechanism by which thyroid hormone is transported across the placental barrier.

353

REGULATION OF CRH EXPRESSION IN THE PLACENTA AT THE ECRE AND ½ PPRE

Natalie A. Plant, Roger Smith, Richard C. Nicholson

Mothers & Babies Research Centre, Hunter Medical Research Institute, Newcastle NSW.

It has been hypothesised that placentally-derived corticotrophin-releasing hormone (CRH) controls the timing of birth. The molecular mechanisms controlling expression of the CRH gene in the placenta are therefore likely to play a major role in parturition. Our group has previously shown that mutations of the CRH promoter at approximately –260bp enhanced CRH gene expression in the placenta. This site is part of a consensus EcRE – a response element for an insect hormone not present in humans. Another region of the CRH promoter, a ½ PPRE, is also of interest to us as ligands of the PPAR include derivatives of prostaglandins which are known to form a positive feedback loop with CRH during labour. We have hypothesised that PPAR, RAR and RXR are the main proteins binding to and therefore regulating CRH gene expression in the placenta via these two response elements. An RT-PCR expression survey of term and preterm placentae from both labouring and non-labouring deliveries showed that α, β and γ isoforms of the PPAR, RAR and RXR were present at birth in all placental tissues, regardless of gestational age or labouring status. An electrophoretic mobility shift assay showed that placental nuclear protein extracts bind to the CRH promoter at both the EcRE and ½ PPRE. We hypothesise, based on these findings, that the nuclear proteins binding the EcRE and ½ PPRE of the CRH promoter include PPAR, RAR and RXR, and that these proteins are involved in regulation of CRH gene expression in the placenta.

354

COORDINATED REGULATION OF PGHS EXPRESSION IN THE HUMAN AMNION AND CHORION AT LATE GESTATION AND LABOUR

R. Johnson[1], C. Mitchell[1], W. Giles[1,2], W. Walters[1,2], T. Zakar[1,2]

[1]Mothers and Babies Research Centre, University of Newcastle and [2]Division of Obstetrics and Gynaecology, John Hunter Hospital, Newcastle, NSW, Australia.

The levels of prostaglandin H synthases -1 and -2 (PGHS-1 and -2) in the amnion and chorion determine the overall capacity of intrauterine tissues to produce labour-promoting prostaglandins. We have tested the possibility that PGHS expression is regulated coordinately in the amnion and chorion before and during labour.

Amnion and chorion membranes were collected after elective Cesarean section at term (CS, n=27), pre-term (PCS, n=18) and following spontaneous term labour (SL, n=26). PGHS-1 and PGHS-2 mRNA abundance was determined using quantitative real-time RT-PCR (Q-RT-PCR). PGHS-2 gene activity was assessed by measuring PGHS-2 heterogeneous nuclear RNA (hnRNA) by Q-RT-PCR.

Median PGHS-2 hnRNA abundance was not different in the three groups either in the amnion or in the chorion; however, there was correlation ($p<0.01$) between amniotic and chorionic PGHS-2 hnRNA levels in individuals in all three groups. Median PGHS-2 mRNA in the chorion was higher in the PCS than in the CS and SL groups, and median PGHS-2 mRNA in the amnion was higher in the SL than in the PCS and CS groups, as expected. PGHS-2 mRNA abundance in the amnion and chorion correlated ($p<0.01$) in individuals in the PCS and SL groups, but not in the CS groups. PGHS-1 mRNA abundance was low, but correlated ($p<0.001$) between the two tissues in the PCS and SL groups, and not in the CS group. Thus, PGHS-2 gene activity is maintained coordinately in the two fetal membranes before and during term labour. The resulting coordination of PGHS-2 mRNA levels, however, is disrupted before labour by mechanism(s) that are likely post-transcriptional. The consequences of this disruption are the increase of amniotic PGHS-2 expression during and a decrease of chorionic PGHS-2 expression before labour. PGHS-1 mRNA may be regulated in a way that is analogous to PGHS-2 mRNA. The factors responsible for the coordination and its pre-labour disruption remain to be established.

355

DIFFERENTIALLY EXPRESSED GENES IN AMNION OF SHEEP INDUCED WITH BETAMETHASONE OR SPONTANEOUS DELIVERIES USING SUPPRESSION SUBTRACTIVE HYBRIDISATION

Sarah Fraser[1], Graham Jenkin[2], Ross Young[2], Robert Fairclough[1]

[1] School of Molecular Sciences, Victoria University, Victoria 3029, [2] Department of Physiology, Monash University, Clayton, Victoria.

Premature rupture of fetal membranes is a major problem for pregnant women as it accounts for some 30-40% of preterm deliveries. However despite considerable research the causes of premature rupture are not completely understood. In order to investigate the nature of genes that are involved in fetal membrane rupture, suppression subtractive hybridization was used to identify genes that were differentially expressed in sheep given betamethasone to induce premature delivery. Amnion samples were collected from a sheep at day 130 of gestation, before and 56 hours after administering betamethasone to induce premature delivery. TRIZOL was used to extract total RNA which was used for cDNA synthesis. Two different cDNA libraries were established and used for suppression substractive hybridization.

As a result of this study a number of genes were identified as being differentially expressed at betamethasone-induced labor. Expression of these genes was analyzed by Northern blotting and real time PCR. These genes included the extracellular matrix protein tenascin-C. The mRNA expression of tenascin-C was then determined by Northern blotting in samples collected from sheep before (n=4) and after (n=4) the administration of betamethasone and at day 136 of gestation (n=4) and at term(n=3). The results showed that there was an increase in the mRNA

expression of tenascin-C at betamethasone-induced delivery and over the last week of gestation ($P<0.05$). Given the role of tenascin-C in the regulation of cell adhesion, this molecule may have a role in the regulation of fetal membrane function in the amnion of sheep near term.

356

PROSTAGLANDIN DEHYDROGENASE IN THE INTRAUTERINE TISSUES OF THE GUINEA PIG DURING LATE GESTATION AND LABOUR

Toni Welsh[1], Sam Mesiano[1], Tamas Zakar[1, 2]

[1]Mothers and Babies Research Centre, University of Newcastle, NSW. [2]Division of Obstetrics and Gynaecology, John Hunter Hospital, Newcastle, NSW.

Prostaglandin dehydrogenase (PGDH) is the key enzyme of prostaglandin (PG) inactivation. In women, intrauterine PGDH expression decreases before labour, which results in elevated PG levels that initiate and maintain labour. The mechanisms that regulate PGDH are largely unknown. Our objective was to develop an animal model of intrauterine PG inactivation. The endocrine characteristics of pregnancy in the guinea pig are similar to women, hence the aim of this study was to determine the level of PGDH mRNA in guinea pig gestational tissues during late pregnancy and labour, in order to evaluate the guinea pig as a model of PG inactivation during gestation.

METHODS: Amnion, chorion, placenta and myo-endometrium were collected from six groups of guinea pigs at late gestation (term: 58-72d). Group 1 (n=6) were at 45d; Groups 2 (n=6) and 3 (n=6) were at 52-58d before and after chorion-uterus attachment, respectively; Group 4 (n=6) were collected on the first day and Group 5 (n=6) on the fifth day of pubic relaxation, and Group 6 (n=3) were labouring. PGDH mRNA was measured by real-time RT-PCR.

RESULTS: PGDH mRNA was most abundant in the chorion, where levels decreased at term, but increased more than 2-fold during labour. PGDH mRNA levels in the amnion were relatively low and decreased with gestational age. PGDH mRNA was highly expressed in the placenta and increased 3-fold with labour. In the myo-endometrium, the lowest levels of PGDH mRNA were seen at term before labour.

CONCLUSIONS: Intrauterine metabolism of PGs in the guinea pig takes place predominantly in the chorion. A fall in PGDH expression in the chorion and myo-endometrium at term may allow PGs from the fetal membranes to cross the chorion and affect the myometrium. High expression of PGDH in the placenta may maintain separation of maternal and fetal PGs. These changes in PGDH mRNA expression are analogous to those seen in human gestational tissues, making the guinea pig a promising animal model to investigate the regulation of intrauterine PG metabolism at labour.

357

OPTIMISATION OF SOLID PHASE EXTRACTION PROCEDURE FOR PROSTAGLANDINS AND METABOLITES

S. Hubbard, T. Welsh, C. Mitchell, T. Zakar, P. Zarzycki

Mothers and Babies Research Centre, The University of Newcastle and John Hunter Hospital, Newcastle, New South Wales 2310, Australia.

Prostaglandin (PG) E_2 and $F_{2\alpha}$ are paracrine mediators involved in many physiological and pathophysiological processes. Pre-purification is critical for the reliable separation and accurate quantification of PGs and metabolites in biological fluids and tissue extracts. We conducted systematic studies to establish a solid phase extraction (SPE) method for isolating PGE_2, $PGF_{2\alpha}$, and their major enzymatic and non-enzymatic metabolites from aqueous solutions.

The stationary phase of the SPE system consisted of octadecylsilane (C-18, 500mg, 6ml Supelclean^{TM} cartidges, Supelco), and the binary mobile phases were combinations of methanol and water (20%-100%, v/v) applied using a vacuum manifold (Visiprep^{TM} Supelco). One ml fractions of the effluent were collected and dried in a centrifugal evaporator. PGs in the fractions were determined by quantitative thin layer chromatography.

Breakthrough volumes for $PGF_{2\alpha}$, PGFM, 15-keto-PGE_2, PGEM, and bicyclo-PGE_2 were determined using purified standards. Based on the similar breakthrough curves, we selected 15-keto-PGE_2 as the model compound to determine extraction recoveries using methanol-water mixtures (20%-60%, with 10% increments) as mobile phases. A recovery curve exhibiting a range from 100% to 13% recovery was obtained, allowing the establishment of a preliminary extraction protocol. Accordingly, SPE cartridges are equilibrated with 5ml of 1% methanol in water. After sample application, the cartridge is washed with 5 ml of 20% methanol-water mixture and dried. PGs are eluted with 2x1 ml of 100 % methanol. We are extending these studies to PGE_2, PGA_2, 15-keto-$PGF_{2\alpha}$, tetranor-PGFM, and tetranor-PGEM to verify the procedure for a comprehensive range of tissue and urinary metabolites of PGE_2 and $PGF_{2\alpha}$. The final SPE procedure will be widely applicable in studies of prostaglandin biosynthesis and metabolism *in vivo* and *in vitro*.

358

OPTIMISATION OF PROTEIN EXTRACTION FROM HUMAN MYOMETRIUM

D. MacIntyre, E.C. Chan, R. Smith

Mothers and Babies Research Centre, The University of Newcastle, Callaghan 2308, NSW, Australia.

Parturition encompasses the stages leading up to and including the activation of the uterine tissues that culminate in labour and birth. Despite the importance of parturition, the physiological regulation of this event remains largely unknown. To advance our knowledge we wish to determine the change in the myometrial proteome that occurs as the uterus is activated. The aim of this study was to compare three methods of protein extraction and isolation from human myometrium and determine which of these was most effective and appropriate for further use in

protein-based studies. Human myometrium samples isolated from the fundal region were treated with either TRIZOL® LS Reagent (n=3, Invitrogen); a mono-phasic solution of phenol and guanididine isothiocyanate, guanidium thiocynate (GTC) extraction media (n=3); or 2D-electrophoresis lysis buffer consisting of 5M Urea, 1M Thiourea, 1% CHAPS and 1% Triton X-100 (pH 7, n=3). Following extraction, the concentration of protein in aqueous solution was estimated colorimetrically using either the bicinchroninic acid (Pierce) or Bradford (Biorad) protein assay kits. The extracted proteins were then investigated by one-dimensional SDS-PAGE. A total of 2µg of protein was loaded onto NuPAGE® pre-cast Bis/Tris polyacrylamide gels (Invitrogen), which were subsequently silver stained. Both TRIZOL® LS Reagent and GTC extraction media produced a similar profile of proteins with approximately 10-20 major protein bands identified that exhibited broad molecular weights ranging between ~10-250 kDa. Of these major proteins, two dominant bands with molecular weights similar to that of human serum albumin (HSA- 66 kDa) and gamma-immunoglobulin (IgG-55 kDa) were observed. This was of interest as high concentrations of these proteins can impede the detection of the remaining proteins in the sample that are present in lower concentrations. In comparison, 2D-electrophoresis lysis buffer generated a greater spectrum of proteins with up to 35 major proteins observed. Similar to TRIZOL® LS Reagent and GTC extraction media, two major protein bands with molecular weights of ~66 kDa and ~55 kDa were identified, however, the staining intensity of these proteins relative to the remaining major proteins was considerably less. Our data suggests that compared to TRIZOL® LS Reagent and GTC extraction media, 2D-electrophoresis lysis buffer is a superior extraction media for human myometrium protein-based studies.

359

NEONATAL BACTERIAL ENDOTOXIN EXPOSURE ALTERS GLUCOSE METABOLISM, LEPTIN PRODUCTION AND BODY MASS INDEX ACROSS DEVELOPMENT IN THE FISCHER 344 RAT

Frederick R. Walker, Nicolette Tape, Deborah M. Hodgson

Laboratory of Neuroimmunology, School of Behavioural Sciences, University of Newcastle, and The Mothers and Babies Research Centre, John Hunter Hospital, NSW, Australia.

Neonatal exposure to gram negative bacterial endotoxin has been shown to alter the functional dynamics of neuroendocrine-immune interactions. In the rat, exposure to endotoxin during the neonatal period alters the development of the hypothalamic-pituitary-adrenal (HPA) axis causing it to become hyper-responsive in later life. What consequences this alteration may have on metabolic function are largely unknown. Evidence from human research has shown, however, that hyperactivity of the HPA axis is associated with abdominal obesity which is in turn is associated with insulin resistance, dyslipidemia and hypertension. The current study assessed the effect of neonatal bacterial endotoxin exposure on metabolic function in adulthood. Fischer 344 rat pups were treated with bacterial endotoxin (50 µg/kg Salmonella enteritidis (SE), i.p.) or the vehicle (phosphate buffered saline) on postnatal days 3, and 5. Circulating insulin, glucose, and leptin levels were assessed in response to a standard glucose challenge test (2g/kg), on postnatal days (PNDs) 36, 90, and 400. Additionally body mass index was monitored at each of these time points. Circulating levels of leptin, insulin, and glucose were found to diverge significantly in the neonatally endotoxin-treated group from controls at each of the time points measured ($p < 0.05$). Body mass index was also found to be significantly reduced in endotoxin treated animals ($p<.05$).

Neonatal corticosterone and leptin levels were also assessed on PND 3 and 5 and corticosterone levels were found to be significantly elevated while leptin levels were significantly decreased in endotoxin-treated pups compared to controls (both, $p<.05$). These findings suggest that early life immune stimulation has long-term metabolic consequences that extend into adult life and may predispose to pathology in later life.