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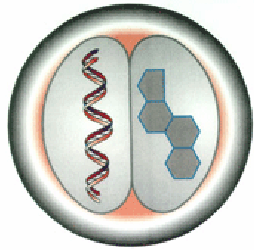
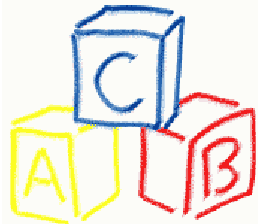
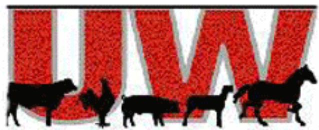


Endocrinology-Reproductive Physiology Training Program

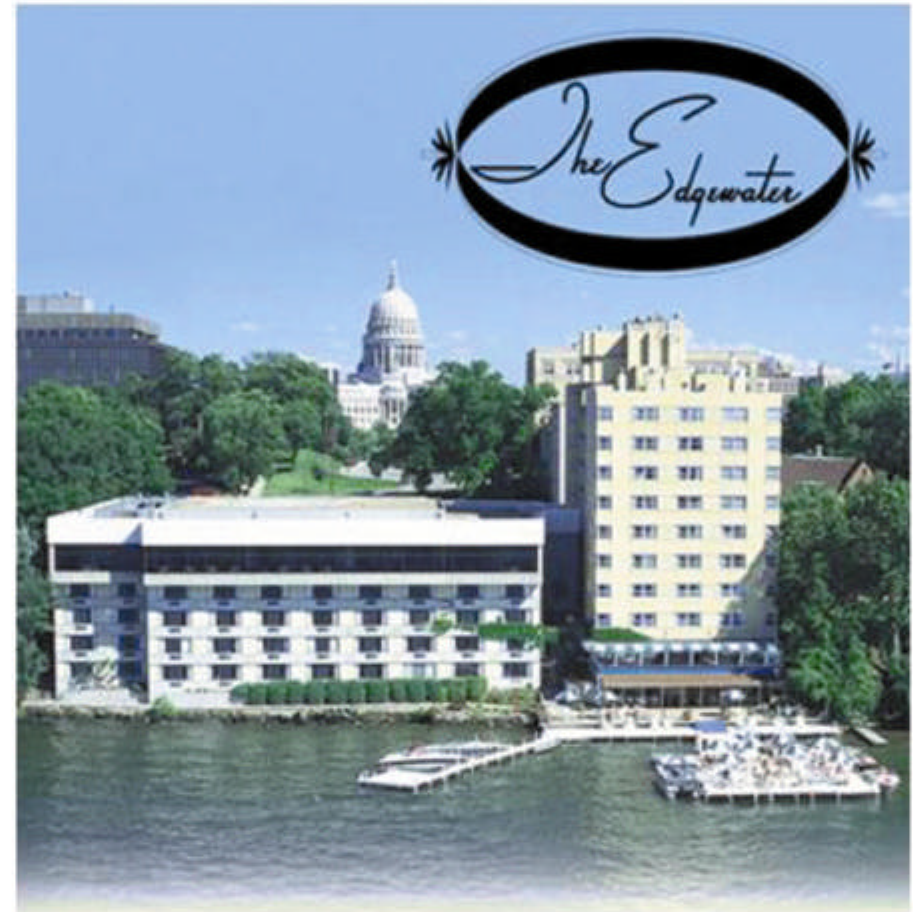


Department of Obstetrics and Gynecology



Animal Sciences Department

Endocrinology-Reproductive Physiology Annual Research Symposium



October 15, 2004
The Edgewater Hotel
666 Wisconsin Avenue

Notes

Schedule

Time	Event
8:00 am – 9:00 am	**Registration, Poster Set-up, and Breakfast** Pacific Room
9:00 am	**Opening Remarks:** Mediterranean Room **Dr. Ian Bird**, Endocrinology – Reproductive Physiology Program Director
9:00 am – 10:00 am	**Opening Lecture:** Mediterranean Room *“Dysfunctional Uterine Bleeding and Angiogenesis”* **Dr. Elizabeth Pritts,** Assistant Professor, Obstetrics and Gynecology, University of Wisconsin - Madison
10:00 am – 10:15 am	**Coffee Break** Pacific Room
10:15 am - 11:15 am.	**Concurrent Oral Sessions I and II:** **Whole Animal Physiology:** Mediterranean Room: Chairs: Dr. Ron Magness, Ob/Gyn Dr. Milo Wiltbank, Professor, Dairy Science 10:15: “*Effects of Endogenous Ovarian versus Exogenous Estrogen on Urogenital TissueBlood Flows*” **Dr. Tova Ablove**, Women’s Health Clinic, UW Madison-Ob/Gyn 10:45: *“Zinc Protoporphyrin/Heme (ZnPP/H) Ratios and Birthweight”* **Kelsey Kleven**, Kling Lab, Pediatrics, UW Madison ***In Vitro* Cell Culture Systems:** Captain’s Table Room Chairs: Dr. Jack Rutledge, Professor, Animal Science Dr. Jing Zheng, Assistant Professor, Ob/Gyn 10:15: “*An* In Vitro *Three Dimensional Model Simulating Primate Embryo Implantation During Early Pregnancy*” **Tien-Cheng Chang**, Golos Lab, ERP Program 10:45: *“Optimization of Culture Conditions for Differentiation of PDX1-positive Cells from Human Embryonic Stem Cells”* **Andrea Forgianni**, Odorico Lab, ERP Program
11:15 am – 11:30 am	**Break**
11:30 am - 12:30 pm	**Invited Speaker’s Lecture:** Mediterranean Room *“Solving the Puzzle: Does PCB=Preterm Child Birth?”* **Dr. Rita Loch-Caruso**, Professor, Environmental Health Sciences, Assistant Scientist, Reproductive Sciences Program, University of Michigan

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Opening Speaker
Elizabeth A. Pritts, M.D.

Elizabeth A. Pritts, M.D. received her Bachelor of Arts Degree from the University of California at Berkeley, where she graduated Cum Laude. She received her Medical degree, graduating in the top 10% of her class from Jefferson Medical College of Thomas Jefferson University in Philadelphia, PA. She did her residency at Yale New Haven Hospital, New Haven CT, and Fellowships at Yale New Haven Hospital and the University of California at San Francisco.

Dr. Pritts' academic appointments include Assistant Professor, Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility at the University of Wisconsin Medical School, Madison WI; Clinical Fellow, University of California at San Francisco; Instructor, Yale University School of Medicine.

Her hospital appointments include Assistant Professor/Attending, UW Hospital, Madison WI; Clinical Fellow/Attending, Moffitt/Long Hospital, San Francisco, CA; Attending Physician, Baylor University Medical Center, Dallas, TX; Attending Physician, Yale New Haven Hospital and Hospital of St. Raphael, New Haven, CT.

Professional Honors include: World congress of Endometriosis Annual Meeting, March 2002, First Prize Paper; Marine Biological Laboratory, One of 16 international scholars for the course "Frontiers in Reproduction" May-July 1999; Yale New Haven Hospital, "Special Excellence in Endoscopic Procedures" award to the outstanding laparoscopist of the graduating class of OB/GYN Residency 1998; Yale New Haven Hospital, "Outstanding Clinician in the class, 1997 and 1998."

She is the author of 13 Abstracts, 23 Publications, and 21 Academic Activities and Presentations. She is also the author and Co-Investigator of several University of Wisconsin Protocols and studies.

Abstract 38

TNF-alpha Inhibits ATP-induced [Ca2+]i by Producing ROS and Inhibiting Mitochondria in Pregnant and Nonpregnant Uterine Artery Endothelial Cells

FX Yi and IM Bird
Obstetrics and Gynecology, University of Wisconsin-Madison, Madison, WI

Pregnancy increases uterine artery (UA) vasodilation to help maintain increased blood flow. The 20- to 50-fold increase in uterine blood flow during pregnancy results in a rise in shear stress. ATP, released from endothelial cell by increased shear stress, may be the most important mediator of this response by activating Ca2+-dependent eNOS. We previously demonstrated that ATP stimulates a more sustained [Ca2+]i response and more NO production in fresh-isolated UA endothelium from pregnant ewes compared with those from nonpregnant ewes. Preeclampsia is characterized by endothelial dysfunction and increases both maternal plasma levels of tumor TNF-a and oxidative stress, which is known to induce endothelial dysfunction. In this study, we examined the effect of TNF-a on reactive oxygen species (ROS) production and on ATP-induced [Ca2+]i response in ovine uterine artery endothelial cells (UAEC, passage 4) from nonpregnant (NP) and pregnant (P) late term ewes. Using the ROS sensitive dye H2DCFDA, we monitored the ROS production with a fluorescence microscope. TNF-a (10 ng/ml) stimulates ROS production in NP-UAEC more than in P-UAEC. ATP (100 uM) continuously induced [Ca2+]i oscillation and multiple [Ca2+]i responses in individual cells over 30 min, and this response was greater in P-UAEC vs NP-UAEC. Pretreatment of TNF-a (10 ng/ml) for 24 hours resulted in a loss of mitochondrial membrane potential (MMP) in both NP- and P-UAEC as measured using MMP-sensitive dye rhodamine 123. Pretreatment of TNF-a also inhibited ATP (100 uM)-induced initial [Ca2+]i peak and decreased the following [Ca2+]i peak numbers. N-acetyl cysteine (NAC, 5 mM), an antioxidant, prevented both TNF-a-induced loss of MMP and inhibition of ATP-induced [Ca2+]i. Both mitochondrial inhibitors CCCP (mitochondrial protonophore, 1 uM) and rotenone (mitochondrial complex I inhibitor, 5 uM) rapidly induced a loss of MMP. Pretreatment of CCCP or rotenone for 30 min significantly inhibited the ATP-induced initial [Ca2+]i peak and abolished [Ca2+]i oscillation and the following [Ca2+]i peaks. Taken together, these data support the hypothesis that TNF-a stimulates ROS production in UAEC, which in turn chronically impairs mitochondrial function, and inhibits ATP-induced [Ca2+]i responses. These findings also provide a potential cellular mechanism for the beneficial effects of antioxidant therapy in preeclampsia.

Concurrent Oral Sessions I and II

10:15 AM – 11:15 AM

Whole Animal Physiology

Mediterranean Room
Chairs: Dr. Ron Magness
Dr. Milo Wiltbank

In Vitro Cell Culture Systems

Captain's Table Room
Chairs: Dr. Jack Rutledge
Dr. Jing Zheng

Abstracts 2-5

Intracerebroventricular (icv) Administration of GnRH II Increases Proceptive and Receptive Sexual Behavior in Female Common Marmosets (Callithrix jacchus)

DK Barnett[1], TM Bunnell[1], AE Carlson[1], RP Millar[4], DH Abbott[1,2,3]

[1]National Primate Research Center, University of Wisconsin, Madison, WI, 53715; [2]Department of Obstetrics and Gynecology, University of Wisconsin, Madison, WI, 53792; [3]Endocrinology-Reproductive Physiology Training Program, University of Wisconsin, Madison WI 53706; [4]MRC Human Reproduction Sciences Unit, Edinburgh, EH3 9ET, Scotland, UK

GnRH II, (pGlu-His-Trp-Ser-Try-Gly-Leu-Arg-Pro-GlyNH2), an evolutionarily conserved member of the gonadotropin releasing hormone family, stimulates reproductive behavior in a number of non-primates (1-3). To explore a role for GnRH II in primate sexual behavior, 8 female common marmosets (age: 2-4 yr) with an indwelling cannula for intracerebroventricular (icv) infusion of GnRH II and the GnRH I antagonist 135-18 (an agonist at the marmoset type II GnRH receptor; 4) were ovariectomized, implanted subcutaneously with silastic capsules (4 empty and 4 with estradiol) and pair-housed with an intact male mate. After icv infusion, of vehicle or peptides, females were placed in a test cage for 90 min out of visual contact with their mate. The male pair-mate then entered the test cage via a remote door and behaviors were scored for 30 min by observers from behind a 1-way mirror. Mean values from each female (3-4 replicate behavioral test/treatment/female) were analyzed by ANOVA with post hoc tests to determine significant differences between means. GnRH II (1 and 10 ug) increased ($p < 0.02$) nearly 4 fold the total number of proceptive (sexual solicitation) behaviors (tongue flicking, proceptive stares and frozen postures) exhibited by females towards their pair-mates compared to vehicle. Effects were already maximal at 1ug. GnRH II (1ug) increased sexual receptivity (females enabling male sexual behavior) by doubling the frequency of intromission demonstrated by males ($p = 0.021$) compared to vehicle without affecting mount or mount attempt frequencies. GnRH II stimulation of proceptive and receptive sexual behavior was not estradiol dependent. The GnRH I antagonist 135-18, which stimulates inositol phosphate production via the marmoset type II receptor, increased the frequency of proceptive behaviors (1ug; $p < 0.02$) similar to the action of GnRH II, but failed to increase the frequency of male intromission ($p = 0.741$) over vehicle. These findings indicate a role for GnRH II in marmoset reproductive behavior which is mediated through the cognate type II receptor. The absence of a functional type II receptor in humans suggests that if GnRH II serves a similar function it does so through the type I receptor which it binds to with high affinity.

Zinc Protoporphyrin/Heme (ZnPP/H) Ratios and Birthweight

K Kleven, S Blohowiak, PJ Kling

Department of Pediatrics and Center for Perinatal Care, Meriter Hospital & University of Wisconsin, Madison, WI, 53715

Iron deficiency is the most common childhood nutritional deficiency today with many immediate and long-term consequences. Our work and others have shown that infants born to mothers with insulin-dependent diabetes, especially those large for gestational age (LGA) suffer from tissue iron deficiency. The aim of the current study is to investigate whether LGA infants born to mothers without diabetes are also at risk for tissue iron deficiency. All subjects were recruited from Meriter Hospital within two days after delivery. Informed consent was obtained from the infant's mother to use the cord blood and to access infant and mother medical charts. Infant's birth weight, gestation, gender, and medical problems were collected. Tests of iron status were performed on the cord blood, including zinc protoporphyrin/heme (ZnPP/H) ratios. An aliquot of blood was rinsed with saline solution and assayed for the ZnPP/H ratio via a hematofluorometer. This is a relatively novel test measuring incomplete iron incorporation in the protoporphyrin ring of hemoglobin. Sample size estimation indicates that will need to recruit 24 LGA infants and 24 infants born average for gestational age (AGA) born at Meriter Hospital in Madison, WI. ZnPP/H ratios and final data are analyzed with an unpaired t-test. Preliminary data are reported below as mean ± SEM. Interim analysis of current enrollees show that ZnPP/H ratios of LGA infants are 75.2 ± 5.0 µM/M ($n = 11$), compared to historical controls from the University of Arizona that are 67.9 ± 3.7µM/M ($n = 36$), $p=0.3$. The historical controls may not be representative of Wisconsin infants and representative control samples are in the process of being collected at Meriter Hospital. If we determine that LGA infants exhibit elevated ZnPP/H ratios than AGA infants, there may be serious clinical implications. First, more aggressive iron supplementation may be indicated in all pregnancies, as a recent study shows the benefits of iron supplementation in otherwise normal pregnancies. Alternatively, current screening for diabetes during pregnancy may be inadequate, requiring more frequent or different screening techniques to be employed.

Abstract 33

Modeling Placental Development with a Human Embryonic Stem Cell Co-Culture System

LM Pollastrini, B Gerami-Naini, YP Liu, OV Dovzhenko, TG Golos
Wisconsin National Primate Research Center and Dept. Ob/Gyn, University of Wisconsin-Madison, Madison WI 53715

Researchers have only speculated on the earliest events in human placentation because elaborate maternal-fetal interactions make it difficult and generally unethical to study placental development *in vivo*. Currently, *in vitro* culture systems utilize placental villous explants cultured on extracellular matrices, but this system is not appropriate for studying early morphogenic events because many of the major structures of the placenta have already been formed by this stage. The undifferentiated state of human embryonic stem cells (hESCs) make them an appealing system for the study of early developmental events, including placentation. In normal placental development it is believed that invasion of extraembryonic mesoderm into the primary villous structure correlates with increased villous development and placental morphogenesis. We propose that placental mesenchymal cells, such as fibroblasts, and/or endothelial cells provide instructive cues for trophoblast differentiation, villous formation and placental development. To test our hypothesis, we have developed *in vitro* and *in vivo* co-culture experiments with EGFP expressing hESCs and human uterine vein endothelial cells (HUVECs) or placental fibroblasts. An initial experiment with the EGFP hESCs asserted that these cells behave in a manner similar to wild-type hESCs, making them appropriate for use in the co-culture system. Through alterations to the co-culture system we aim to stimulate the formation of the cells and structures of the placenta.

Abstract 34

A study on the effects of oxytocin on GnRH II in rats and salmonoids

SB Ellenberger[1], KE LaZotte[1], AK Schneider[1,2], MJ Woller[1]
[1]Department of Biological Sciences, University of Wisconsin – Whitewater,Whitewater, WI 53190, [2]Life Sciences, New Glarus High School, New Glarus, WI 53574

Oxytocin has been shown to impact the release of Gonadotropin Releasing Hormone (GnRH 1) in several model systems. We are interested in the regulation of GnRH release in fish, specifically salmonoids. Fish express 3 distinct variants of the GnRH molecule: GnRH (mammalian GnRH), chicken GnRH II (cGnRH II), and salmon GnRH (sGnRH). We hypothesize that oxytocin will increase the release of cGnRH II in rats and salmonoids. Both rat and trout hypothalami were removed and placed into an in vitro perifusion system to collect samples linearly for 10-20 hours. Hypothalami were exposed to one of three treatments. Treatment 1: five hours continuous oxytocin. Treatment 2: 10 minute challenge of oxytocin. Treatment 3: 10 minute vehicle challenge. Samples are collected every 10 minutes and GnRH and cGnRH II levels are determined using radioimmunoassay. We have assayed GNRH I in all samples. We are in the process of assaying all samples for cGnRH II as the materials become available. Our preliminary results suggest oxytocin has minimal effect on mGnRH I in the rat.

Funded by an NSF-REU Grant as part of the BEST-TERP summer research program at UWW.

Abstract 5

Optimization of Culture Conditions for Differentiation of PDX1-Positive Cells from Human Embryonic Stem Cells

AE Forgianni[1,2], X XU[1,2], JS Odorico[1,2]
[1]University of Wisconsin School of Medicine, Department of Surgery, Madison, WI 53792, [2] WiCell Research Institute, Madison, WI 53707

With their ability to differentiate into cell types from all three embryonic germ layers, including endoderm from which pancreatic cells develop, human embryonic stem (HES) cells remain an attractive avenue of research into developing treatments for cell loss of function diseases such as type I diabetes. Previous experiments have shown that HES cells can express PDX1, a marker of cells committed to become pancreatic cells. Avenues to enhance and enrich for PDX1-expressing cells may yield the ultimate goal: purified cultures of pancreatic islet cells suitable for transplantation. The present study seeks to begin to define optimal conditions for differentiation of PDX1+ cells from HES cells. We tested a variety of conditions: i) different sources of serum, ii) whether embryoid body (EB) formation is required, and iii) whether addition of FGF10 to the growth medium increases PDX1 expression. First, we replaced fetal bovine serum (FBS), a standard supplement in differentiating HES cell cultures, with bovine calf serum (BCS), which is less expensive and whose components are clearly defined and then compared the number of PDX1+ cells that differentiated. BCS treatment yields an increased number of PDX1+ cells per cover slip and per cluster. In the EB14 +7 cultures 56 versus 142 PDX+ cells were counted on 3 FBS-treated and 5 BCS-treated cover slips, respectively. Second, to determine whether early germ layer interactions within EBs are necessary for PDX1 expression, HES cells were allowed to differentiate either with or without EB formation and the number of PDX1+ cells was compared. Cultures were either allowed to establish 3-dimensional interactions in suspension or were directly plated so contact during differentiation was mostly limited to 2-dimensions. When examined with IHC, cultures without EB formation completely lacked PDX1 expression; cultures in which EBs formed expressed PDX1 throughout EB 14+14. Third, to determine if FGF10 promoted pancreatic lineage differentiation from HES cells, we cultured differentiating HES cells in the absence of FGF10 and in the presence of either 25 or 50 ng/mL FGF10. Treatment yielded increases in PDX1 expression, as determined by QPCR, at EB day 14 and 14+7. At EB14+7 without FGF10 treatment, PDX1 expression was about 5 times that over the baseline conditions of undifferentiating HES cells. PDX1 expression increased about 9.5 and 14-fold with addition of 25 and 50ng/mL of FGF10, respectively. Taken together, use of BCS, allowing EB formation and addition of FGF10 all enhance PDX1 expression in differentiating HES cells.

Abstract 31

Endothelial Differentiation from Human Embryonic Stem Cells (hES cells) Induced by Growth Factors

Y Song, YX Wen, J Zheng
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Human ES cells are able to differentiate to a variety of tissue and cell types including endothelial cell precursors. These differentiated endothelial cells will have enormous potential for clinic applications such as replacing damaged vasculature or correcting dysfunction of endothelial cells occurred in placentas of abnormal pregnancies. Therefore, a better understanding of mechanism controlling endothelial differentiation from human ES cells can lead to better strategies for gene therapies. Method: Human ES cells (H9) were plated on Matrigel coated wells. After cultured overnight ES cells were treated with EBM2 media with or without VEGF, bFGF and two different concentrations of BMP-4 respectively to induce cell differentiation. After 10-21 days of treatment the cells were analyzed for their uptake of LDL and expression of CD31 and VEcad. Results: ES cells treated by EBM2 with a high concentration of BMP4 exhibited more spindle like cells compared to those treated by a low concentration of BMP4 and EBM2 media alone. Low concentration of BMP4 induced more differentiated cells than EBM2 alone group. The spindle- like cells increased through time course in different treatment groups. After 15 days of differentiation more cells showed positive uptake of LDL in high concentration of BMP4 compared to low concentration of BMP4 and EBM2 alone groups. When analyzed by dual staining with LDL and CD31 or VEcad by flowcytometry more dual staining cells were observed in EBM2 with high concentration of BMP4 treated group than EBM2 media alone. LDL uptake showed no significant difference between EBM2 with high concentration of BMP4 and EBM2 alone groups. Conclusion: Combined with morphology, fluorescence microscopy and flowcytometry analysis ES cells treated by high concentration of BMP4 may promote ES cell differentiation toward an endothelial precursor fate. But LDL appeared not to be a suitable endothelial marker for this procedure.

Abstract 6

Solving the Puzzle: Does PCB=Preterm Child Birth?

R Loch-Caruso
University of Michigan

Preterm birth rates in the USA remain unacceptably high after increasing by more than 15% over the past 20 years. The cause of the majority of preterm births is unknown, although a role for environmental exposures has been suggested. Throughout their lives, women are exposed to a large number of chemicals, many of which are stored in the fat. During pregnancy, fat stores release these lipophilic substances, providing an endogenous source of exposure to toxicants. We are investigating whether persistent toxicants of concern may be contributing to women's risk for preterm birth. Our laboratory uses various approaches to study direct toxicant actions on uterine muscle and placenta, including in vitro cell cultures, muscle baths and an in vivo exposure/in vitro assessment protocol, to identify whether selected toxicants stimulate pathways known to activate uterine muscle contraction and labor. Much our work has focused on polychlorinated biphenyls (PCBs), a major class of persistent environmental chemicals of concern.

PCBs were manufactured and used in a wide variety of applications as mixtures, although commercial use of PCBs has been discontinued in most countries since the late 1970s. We found that commercial PCB mixtures (Aroclor) and PCB isomers exert rapid and direct actions on uterine contractions. By using biochemical, fluorometric and pharmacological approaches in myometrial cell culture and tissue contractility experiments, stimulation of phospholipase A_2 (PLA_2) and elevation of intracellular calcium concentration via activation of voltage-operating calcium channels have been linked to the stimulatory activities of Aroclor 1242. These results suggest that some PCBs may directly stimulate uterine contraction by activating arachidonic acid and calcium dependent mechanisms.

Because prostaglandins have a critical role in parturition, recent studies have focused on the arachidonic acid/prostaglandin pathway. PCBs have been reported to activate phospholipase A_2 (PLA_2) to release arachidonic acid from membrane phospholipids. We found that the non-coplanar PCB congener 2,2',4,6–tetrachlorobiphenyl (PCB 50) increases uterine contraction in a gestation day-specific manner that corresponds with increases in calcium-independent and secretory PLA_2 expression from midgestation compared with late gestation. Additionally, we have shown that PCB 50 stimulates arachidonic acid release via activation of these PLA_2. Because release of arachidonic acid is the rate-limiting step for prostaglandin synthesis, these studies suggest that some PCBs may stimulate uterine contraction by increasing PLA_2–mediated release of arachidonic acid.

In other experiments, we have investigated increased response to oxytocin as an estrogenic mechanism by which some PCBs might stimulate uterine contraction. Prolonged exposure to the estrogenic PCB 4-hydroxy-2',4',6'-trichlorobiphenyl increased oxytocin-induced oscillatory uterine contraction frequency in an estrogen receptor-dependent manner, showing the potential for estrogenic environmental chemicals to stimulate uterine contractions via indirect mechanisms involving oxytocin. The environmentally persistent PCB congener 2,4,5,2',4',5'-hexachlorobiphenyl stimulated the frequency and force of uterine contractions, assessed in vitro in muscle baths, of rats exposed in vivo during the latter part of gestation. No further stimulation was observed with in vitro application of oxytocin or prostaglandin, however, indicating that upregulation of these critical pathways did not occur in vivo with this PCB congener.

These experimental approaches and results provide some insight into the possible ways in which PCBs could contribute to a woman's risk for preterm birth by demonstrating PCB stimulation of pathways known to activate parturition.

Investigation of c-Raf-1 Activity in an Ovine Uterine Artery Endothelial Cell Culture Model (UAEC).

JA Sullivan and IM Bird
Dept Ob/Gyn, University of Wisconsin-Madison, Madison

A multitude of changes must occur in and around the utero-placental unit to allow a dramatic increase in blood flow to afford a successful pregnancy, including an increased production of Nitric Oxide (NO) and Prostacyclin (PGI2) from the uterine artery and an increase in angiogenesis of the surrounding vasculature. Whether examining angiogenesis, NO or PGI2 production, the implication for regulation by the protein kinase ERK2 has precedent. In our UAEC model consisting of cells from pregnant (P-UAEC) and non-pregnant (NP-UAEC) ewes, we have observed a general pregnancy enhanced, agonist specific phosphorylation of ERK2 that coincides with a similar general, pregnancy specific increased production of NO and PGI2. To characterize the ERK2 response in our model, we have begun examination of a kinase upstream of ERK2 activation that has been widely implicated as a kinase responsible for phosphorylating MEK1/2 leading to ERK2 activation in numerous cell models, the protein kinase c-Raf-1. To examine the activity of Raf-1 in our model, we employed a two-step radioactive labeling protocol consisting of immunoprecipitating Raf from treated and control cultured cells, incubation with a competent MEK1 and further incubation with a GST-ERK1/2 protein. Preliminarily, we observe no change in substrate 32P incorporation upon agonist stimulation (ATP, AII, bFGF, EGF, VEGF) and control treatments in both P and NP-UAEC. The activity of Raf-1 has been associated with phosphorylation on certain residues of the kinase. Phosphorylation of Ser340/341 on Raf-1 is usually associated with increased activity, whereas phosphorylation on Ser259 is associated with reduced activity. Our early data imply no change in the level of Ser259- P over time upon treatment with a prototypical ERK2 agonist, ATP(100uM). We do, however, observe an increase in 340/341-P in P-UAEC upon ATP(100uM) treatment occurring before the maximum observed time of ERK2 phosphorylation. GW5074, a reasonably selective Raf-1 inhibitor, may allow us to examine Raf-1 involvement in vasodilator production, ERK2 activation and other cellular processes without the use of dominant negatives. We observe 20 minute pretreatment of P-UAEC with GW5074 (.02-2Um) exhibits a trend toward dose dependent inhibition of bFGF(10ng/ml) stimulated ERK2 phosphorylation. This preliminary work illustrates a three-tiered approach toward investigating Raf-1 in our model. Investigation of Raf-1 activity by use of in vitro kinase assay, phospho-specific western analysis and reasonably selective chemical inhibition may permit us to associate changes in Raf stimulation with physiological changes including changes in vasodilator production or angiogenesis. Funded by HL64601 and HD38843.

Polycystic Ovary Syndrome (PCOS): Genomic and Genetic Insights into Pathophysiology

JF Strauss
Center for Research on Reproduction & Women's Health,
University of Pennsylvania Medical Center, Philadelphia, PA, USA 19104

The underlying pathophysiology of polycystic ovary syndrome (PCOS), the most common endocrine disorder of women of reproductive age, remains poorly understood despite the fact that over sixty plus years have passed since PCOS was first recognized as a common entity. Clinicians have entertained the notion for some time that PCOS is a genetic disorder. However, exploration of the genetic basis of PCOS has been hampered by several factors. PCOS is associated with infertility and low fecundity and it is rare to find large pedigrees with multiple affected women with which to perform linkage analysis. There has also been an on-going debate over disease phenotypes. The biochemical evidence for involvement of multiple organ systems in PCOS including hyperandrogenemia of ovarian origin, elevated adrenal androgen production, insulin resistance and abnormal pancreatic ? -cell function raises several important questions: Is PCOS many diseases, or do the factors that influence reproductive function also impact different cell types simultaneously, resulting in the multi-system PCOS phenotype? Are the metabolic abnormalities detected in different cells the result of a shared intrinsic defect, or are they the consequence of exposure to an altered endocrine state (i.e., increased androgen or increased insulin)? The answers to these questions have profound significance when one contemplates the role of genetics in PCOS.

The foundation of genetic studies is the evidence that disease clusters in families. Nearly fifty percent of sisters of women diagnosed with PCOS had elevated total or bioavailable testosterone levels, suggesting that hyperandrogenemia is a dominant trait. The contribution of genetics to blood androgen levels was recently substantiated in a large population study that was not dealing with PCOS per se. Freshly isolated theca cells collected from ovaries of PCOS women studied in short term culture and propagated PCOS thecal cells grown through multiple population doublings display greater steroidogenic activity than theca cells collected from normal ovaries. The increased steroidogenic activity is due to increased transcription of genes encoding steroidogenic enzymes as reflected by enhanced promoter activities in cultured PCOS theca cells and increased levels of steroidogenic enzyme mRNAs in thecal tissue. The biochemical phenotype displayed by PCOS theca cells is consistent with the findings from family studies indicating genetic control of androgen production. Moreover, the elevated androgen production by theca cells appears to provoke stromal hyperplasia. The stromal cells produce growth factor binding proteins which may contribute to follicular maturation arrest.

While the studies described above have identified important correlations between increased steroidogenic enzyme gene expression and increased androgen biosynthesis in PCOS theca cells, they have not disclosed the upstream genes that are important for increased transcription. Furthermore, the global changes in theca cell gene expression or the alterations in gene networks or signal transduction cascades that may play an important role in the manifestation of other PCOS theca cell phenotypes, which may contribute to arrested follicular growth have not been defined. In order to define the genes that are differentially expressed in PCOS theca cells and to identify new candidate genes that may contribute to the etiology of PCOS, we compared gene expression profiles of normal and PCOS theca cells using Affymetrix oligonucleotide microarray chips. Our analysis revealed that PCOS theca cells have a gene expression profile that is distinct from normal theca cells. Included in the cohort of genes with increased mRNA abundance in PCOS theca cells was the transcription factor, GATA6 and the regulator of the AKT/protein kinase B (PKB) signaling pathway, cAMP-GEF-II. Changes in cAMP-GEF-II and other genes increase the sensitivity of theca cells to insulin activation of AKT/PKB. We demonstrated that GATA6 increased the expression of enzymes involved in androgen biosynthesis, providing a functional link between altered gene expression and intrinsic abnormalities in PCOS theca cells. The elevation of GATA6 and cAMP-GEF-II in PCOS theca cells appears to be the result of increased mRNA half-life, which may be controlled by a gene on chromosome 19p13.2, a locus shown to be linked to PCOS in our genetic studies. *ELAVL1,* a gene that encodes and RNA binding protein that regulates mRNA stability is located in this region. Our analyses have (1) defined a stable molecular phenotype of PCOS theca cells; (2) suggested new mechanisms for excess androgen synthesis by PCOS theca cells; and (3) identified candidate genes that may be involved in the genetic etiology of PCOS and its metabolic phenotype.

Abstract 27

Endothelial Estrogen Receptors and VEGF Interactions in Ovine Uterine Arteries

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Estrogen levels and uterine blood flow (UBF) are elevated during the high estrogen states (follicular phase and pregnancy). Magness et al reported (SSR 2003) that E2 -induced, Follicular and Pregnancy rises in UBF are inhibited, in vivo, by estrogen receptor (ER) antagonist ICI 182,780. Although estrogen acts by binding to its receptors in target cells, ER and ER expression, their cellular localization (endothelial vs. VSM) and regulation in uterine arteries (UAs) is unknown. Methods: UAs were fixed and paraffin-embedded sections prepared for studying cellular localization of ER and ER mRNA by in situ hybridization (ISH) using [35S]-labeled riboprobes or protein localization using immunohistochemistry (IHC). Endothelial-isolated proteins were mechanically isolated from luteal phase (high Prog), follicular phase (high estrogen), and pregnant (gestational age 120-130d; high estrogen and Prog) sheep (n=6-8), comparing compartmentalization of ER and ER in endothelium (UAendo) with intact uterine artery (UA+), and VSM (UA- or denuded vessel) using Western analysis. Endothelial protein samples were also collected from reproductive and nonreproductive vessels to compare ER and ER protein levels to UAendo. Results: ISH demonstrated the presence of ER and ER mRNA in both the UAendo and UA-. ER protein distribution by IHC was similar to that of the mRNA. When studying ER regulation in luteal, follicular, and pregnant ewes, UAendo ER , but not ER , expression was elevated 1.5 fold (P=0.05) in the follicular compared to luteal samples; however, UA- VSM ER was unaltered. In contrast, ER , but not ER , protein expression in pregnant UAendo (P=0.004) and UA- (P=0.008) showed 1.6 and 3.2 fold respective rises over luteal controls. ER was not substantially altered in other reproductive [Mammary (MA) and Placental (PA)] or nonreproductive [Omental, Renal, Coronary (CA)] endothelium. In contrast, MAendo ER increased 1.5 fold (P=0.008) by pregnancy, PAendo ER was 3 fold greater than luteal UAendo and 1.5 fold greater then pregnant UAendo. The nonreproductive CAendo ER was elevated 1.4 fold (P<0.05) by pregnancy. Conclusion: ER and ER mRNA and protein are both expressed in UAendo and UA- indicating potential targets for endogenous estrogen in vivo. While both ER and ER are differentially regulated by follicular endogenous estrogen in the UAendo, ER regulation mainly confined to the UAendo more so than VSM. ER is elevated by pregnancy in UAendo and other reproductive and nonreproductive endothelium, suggesting a putative role in blood flow regulation. The relative role(s) of UAendo vs. VSM ERs in controlling UBF remains to be determined. NIH HL49210, HD33255, HL57653, HL70563, HD388.

Abstract 8

Differential Timing in cAMP Regulation of Cyclins, Aromatase, and LH Receptor in Cultured Bovine Granulosa Cells from 5mm Follicles

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Follicular development involves a complex interplay of proliferation and differentiation pathways regulated by multiple intracellular effector systems. This study evaluated regulation by the cAMP pathway which is know to mediate many of the actions of FSH and LH in granulosa cells. Three experiments were done with granulosa cells collected from 5mm bovine follicles (n = 4-5 granulosa cell preparations per experiment with each treatment evaluated in triplicate wells), an FSH-dependent follicle stage that is about 3-4 days before selection of a single dominant follicle. In expt. 1, granulosa cells were treated with different doses of forskolin (1, 3, 10, 30? M) for 24 hours. Steady state concentrations of cyclin D2 and cyclin A mRNA were increased by lower doses (1µM=139% and 3? M=178% of controls for cyclin D2; 1µM=152% and 3? M=178% of controls for cyclin A) but not higher dose of forskolin. Forskolin induced a dose-dependent increase in aromatase mRNA (1µM=350%; 3µM=517%; 10 µM=914%; 30 µM=1199%) and aromatase activity (1µM=244%; 3µM=336%; 10 µM=481%; 30 µM=560%). In expt. 2, cells were treated with 3µM forskolin for 1, 6, or 24 h. Forskolin acutely increased phosphorylation of CREB (1h=181%; 3h=227%; 24h=210%) and cyclin D2 mRNA (1h=137%; 3h=206%; 24h=159%) with slightly later induction (no increase at 1 h) of cyclin A (1h=116%; 3h=171%; 24h=155%), aromatase mRNA (1h=165%; 3h=698%; 24h=1003%), and aromatase activity (1h=91%; 3h=161%; 24h=370%). No changes in LH receptor mRNA were observed at any time point or dose of forsklin in expt 1 and 2. In expt. 3, cells were treated with 3 µM forskolin for 1, 3, 5, or 7 days. Forskolin increased aromatase mRNA (d1=673%; d3=638%; d5=1140%; d7=1157%) and activity (d1=418%; d3=254%; d5=346%; d7=475%) at all times. Forskolin increased LH receptor mRNA but with a substantial delay (3d=154%; 5d=396%; 7d=741%). Thus, cAMP rapidly induces pathways involved in granulosa cell proliferation (cyclins) and estradiol production. The delay in induction of LH receptor by cAMP suggests different induction mechanisms that may be critical in selection of a single dominant follicle.

Abstract 25

Comparison of Plasma and Blood Viscosity During the Ovine Ovarian Cycle and Pregnancy

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Shear stress is the most potent physiologic stimulus for elevating endothelial cell nitric oxide production and inducing flow-mediated vasodilatation. Shear Stress is equal to 4*Blood Flow * viscosity/ r3. At a given vascular cross sectional area, shear stress is directly proportional to elevations in both blood flow and viscosity. Substantial elevations in uterine blood flow (UBF) observed during the follicular phase and pregnancy undoubtedly are responsible for rises in shear stress. However, changes in blood viscosity may also contribute to increases in uterine shear stress during these physiological states of elevated estrogen. We tested the hypothesis that viscosity at equivalent hematocrits will increase during the follicular phase and pregnancy. Methods: Heparinized jugular blood was collected from luteal phase (Lut; Day10-11, n=12), follicular phase (Fol; periovulatory Day 0, n=11) and pregnant (126 1 days of gestation, n=18) sheep. Because blood is a non-Newtonian fluid and hematocrit plays a major role in controlling this property, viscosity was analyzed in plasma (0% hematocrit) and whole blood at endogenous (30-40%), low (15-29%) and high (41-55%) hematocrits using a torque viscometer. Studies were performed on each sample at 7 shear rates (6 134/sec) to partly simulate low to high flows and establish potential changes in the rheological properties of blood. Results: Endogenous hematocrit was not significantly altered by the ovarian cycle (Lut=36 0.9%; 33-41% , Fol=34.7 1.6%; 30-48%) or pregnancy (38.7 1.0%; 29-44%). Viscosity of blood samples was increased as hematocrit was elevated ($P<0.05$). Viscosity was inversely related across this range of shear rates in all samples, regardless of hematocrit ($P<0.05$). Plasma viscosity and viscosity at hematocrits below physiologic levels were unaltered by the stage of the ovarian cycle or gestation. In contrast, viscosities of blood samples within ranges of hematocrits at or above physiological levels (31-55%) were greater in Fol and Pregnant sheep ($P<0.05$) than Lut sheep. Conclusions: When compared to the progesterone dominated luteal phase, both the follicular phase and pregnancy (physiological states of high estrogen) significantly elevated blood viscosity at physiologic hematocrits and higher. These data suggest estrogen-mediated increases in blood viscosity partly contribute to elevations in uterine artery and possibly systemic artery shear stress during the ovarian cycle and pregnancy. NIH HL49710, HD33255, HL57653, HD38843.

Abstract 10

Gonadotropin Releasing Hormone (GnRH) Associated Peptide (GAP) Does Not Affect GnRH Release In Perifused Rat Hypothalami.

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Gonadotropin Releasing Hormone (GnRH) is the principle molecule controlling reproductive function in vertebrates. The mechanism of coordinated release by GnRH neurons is a central target for our research group. It has been shown that GnRH can influence GnRH release through autocrine/paracrine effects, but the role of the peptide synthesized with GnRH, GnRH Associated Peptide (GAP), has not been determined. We conducted a preliminary investigation to evaluate the role of GAP in the coordination of GnRH release events or pulses using an in vitro perifusion approach. Rat hypothalami were dissected from adult male rats, cut down midline to produce 2 hemihypothalami, and the tissues were placed in a perifusion system and maintained for 10 hours. Samples were collected every 10 minutes and GnRH concentration was determined by radioimmunoassay. The protocol involved treatment of all tissues with media for 3h, then treatment with GAP 10-8M, GAP 10-10M, or vehicle (media) for 3h, and then 3h media again. Comparison of treatments vs. prechallenge or postchallenge blocks produced p values greater than 0.8, indicating little support for an effect of GAP on coordinating GnRH release. This work was supported by an NSF-REU grant supporting the BEST-TERP summer research program at UWW

Abstract 23

A New Model for Neonatal Iron Distrubution and Tissue Iron Deficiency

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Premature infants have low tissue iron stores. Treatment of anemia in premature infants with recombinant erythropoietin (rEpo) shows only marginal efficacy and further depletes tissue iron. Tissue iron deficiency in early development is particularly problematic as it results in long-term cognitive impairment. Thus it is critical to examine iron delivery to brain in a model of iron deficiency. We previously showed mild iron deficiency anemia after rEpo therapy in suckling rats and more severe anemia after feeding artificial formula. We hypothesized that brain and body iron concentrations are lower in anemic rats fed "low iron" artificial formula than control rats. After dam feeding or artificial feeding through a gastrostomy tube, brain and total body iron content was measured by atomic absorption after nitric acid/perchloric acid digestion. Results are reported as mean ± SEM, and analyzed by unpaired t tests. Data are expressed as mean ± SEM. Untreated Sprague Dawley rats were studied at postnatal day 4, 8 & 12 to determine iron accretion and best timing of feeding intervention. Between postnatal day 4 and 8, no net iron accretion was observed (0.59 ± .01 mg and 0.57 ± .01, p=.5) mg despite growth (11.5 ± .4 g to 16.0 ± .5 g, p<0.0001), but that accretion occurs between postnatal day 8 and 12 (0.89 ± .03 mg of iron for 24.9 ± .4 g weight), p<0.0001. We concluded that studies perturbing iron status should begin at day 4 to optimize tissue iron differences. Rats were fed low iron formula vs. by dam for 8 total days. At 12 postnatal days, low iron rats had lower body iron concentration (0.027 ± .002 mg/g tissue) than control (0.036 ± .002 mg/g tissue), p<0.0015. Low iron rats exhibited lower brain iron concentration (14.7 ± 0.6 µg/g tissue) than control (18.4 ± 1.0 µg/g tissue), p<0.002. We conclude that the suckling rat's limited ability to accrete iron normally, and impaired iron accretion after iron deficient feeding allow it to be an excellent model for continued iron deficiency studies. Studies examining tissue iron expression and iron transport are underway.

Poster Session

Pacific Room

4:00 PM – 5:00 PM

Abstracts 12-38

Prenatal Androgen Excess Alters Body Fat Distribution in Female Rhesus Monkeys

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Prenatally androgenized (PA) female rhesus monkeys exhibit metabolic abnormalities similar to those of polycystic ovary syndrome (PCOS), the most common endocrinopathy of reproductive-aged women. Female rhesus monkeys exposed to testosterone in early gestation (PAE) exhibit insulin resistance, impaired pancreatic beta-cell function and diabetes mellitus, while those exposed in late gestation (PAL) develop supranormal insulin sensitivity that declines with age and body mass index (BMI). To determine whether PA females have altered body fat distribution and to validate use of computed tomography (CT) in assessing body composition in rhesus monkeys, 5 PAE, 5 PAL and 5 control (C) adult female rhesus monkeys underwent somatometrics, dual x-ray absorptiometry (DXA) and abdominal CT. There were no differences in age (PAE: 21.19 ± 0.67, PAL: 18.96 ± 0.97, C: 18.39 ± 0.92 yrs), weight (PAE: 8.59 ± 0.59, PAL: 8.57 ± 0.33, C: 8.28 ± 0.30 kg), BMI (PAE: 37.59 ± 1.26, PAL: 36.06 ± 0.82, C: 36.33 ± 1.63 kg/m2) or somatometrics. DXA-determined total body fat (PAL: 2647.50 ± 150.53, PAE: 2023.73 ± 152.43, C: 1844.77 ± 149.58 g;), percent body fat (30.30 ± 1.81, PAE: 24.46 ± 1.83, C: 21.86 ± 1.80 %), total abdominal fat (PAL: 1319.09 ± 88.16, PAE: 983.15 ± 89.27, C: 940.57 ± 87.60 g) and percent abdominal fat (PAL: 44.68 ± 2.39, PAE: 36.28 ± 2.42, C: 33.66 ± 2.37 %) were significantly greater in PAL than PAE or C females. PAL females also had significantly more CT-determined non-visceral abdominal fat than PAE or C females (PAL: 618.68 ± 61.86, PAE: 363.51 ± 62.64, C: 364.51 ± 61.47 cm3), while proportionate amounts of non-visceral fat were similar. Absolute and proportionate amounts of visceral fat were similar among the 3 groups. PAE females alone had a significant positive relationship between visceral fat and BMI ($R2=0.94$, $p<0.01$), while C females alone had a significant positive relationship between non-visceral fat and BMI ($R2=0.98$, $p<0.001$). CT and DXA measurements of abdominal fat were strongly correlated ($R2=0.916$). Increased total body fat in PAL females and increased BMI-related visceral fat in PAE females may contribute to metabolic abnormalities typically found in PCOS.

The Physiological Effects of Genistein on Smoltification and Estrogen Metabolism in Atlantic Salmon (Salmo salar)

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In the aquaculture industry, feed cost is generally the highest annual variable costs;therefore, ingredient availability is a serious concern. Growth in the aquacultural production requires high quality feed ingredients that contain relatively high levels of crude protein and a favorable essential amino acid balance. Soybean meal contains these qualities, and cheaper than current fish based meal. Thus, it is a logical ingredient to include in or replace the existing diets to the industry. However, soybean contains high level of isoflavones, with genistein as the primary isoflavones. Genistein has both estrogenic and antiestrogenic effects, which may have stimulatory or suppressive effects on reproductive function and development of fish. Therefore, the goal of this study is to investigate the physiological effects of genistein on smoltification and estrogen metabolism in atlantic salmon. Smoltification is the physiological metamorphosis from a freshwater fish to a saltwater fish. In this study, atlantic salmon parr were fed various levels of genistein (0 ppm, 500 ppm, 1000 ppm, 3000 ppm) and 20 ppm estrogen as positive control. The fish were put under photoperiod and water temperature manipulation to induce them to smolt. Smoltification was judged by putting the fish under 24 hr saltwater challenge test, osmolality test, plasma chloride test, and Na, K, ATP-ase gill test. In the second study, we investigate the mechanism of action of genistein. Kidney and liver tissue were cultured and subjected to different levels of genistein (10-9 M to 10-5 M). So far, the result suggested that genistein inhibits estrogen metabolism in liver but not the kidney.

Association Between Free Fatty Acids and Insulin Resistance in a Nonhuman Primate Model for Polycystic Ovary Syndrome

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Polycystic ovary syndrome (PCOS) is the most common cause of infertility in young women. PCOS is characterized by anovulation, LH hypersecretion, hyperandrogenism and insulin resistance. To investigate the mechanisms underlying insulin resistance, we employed a nonhuman model for PCOS: prenatally androgenized (PA) female rhesus monkeys, exposed *in utero* to fetal male levels of testosterone for 15-35 days during early (starting on gestation days 40-44; n=5), mid- (starting on gestation day 60; n=1) and late gestation (starting on gestation days 100-115; n=2). These monkeys exhibit adiposity-dependent hyperinsulinemia, hyperandrogenism and anovulation. Elevated circulating levels of free fatty acids can impair insulin action and secretion. Reducing the circulating levels of free fatty acids should increase insulin sensitivity, thereby reducing circulating insulin levels. Five control and 8 PA female monkeys, similar in age and body mass index, underwent oral administration of placebo followed by pioglitazone (3 mg/kg). Each treatment lasted for 6 months with an intervening non-treatment period of at least 90 days. Insulin sensitivity was determined during month 4 from a frequently sampled, i.v. tolbutamide-modified glucose tolerance test (FSGTT), after analysis using the Modified Minimal Model of Bergman. Serum levels will also be analyzed for circulating androgen levels and assessing ovulatory cyclicity. We propose that the application of pioglitazone should increase insulin sensitivity, lower circulating levels of free fatty acids, improve glucose regulation in both control and PA females, but should only normalize ovulatory cycles in PA females alone.

Characterization of Intracellular Calcium Response to Ionomycin in the Equine Oocyte at Different Stages of Nuclear Maturation: Preliminary Data

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Sperm-induced [Ca2+]i oscillations at the time of fertilization are a required signal for activation in most mammalian oocytes. In these species the ability to generate normal [Ca2+]i oscillations is acquired during final stages of oocyte maturation. In equine oocytes, the injection of homologous sperm extract induced [Ca2+]i oscillations; however, the injection of a single spermatozoon failed in consistently initiate that response. When compared to mature bovine oocytes, mature equine oocytes showed attenuated [Ca2+]i response to ionomycin. The objective of this study was to characterize the [Ca2+]i response to ionomycin in equine oocytes at different stages of nuclear maturation. Materials and Methods: Oocytes were collected from slaughterhouse ovaries. Follicles smaller than 20mm were sliced and contents were recovered with bone curettes. Cumulus oocyte complexes were placed in maturation media (TCM 199, 10 % FCS, Pyruvate, FSH, LH, IGF, EGF, Estradiol and Gentamycin) at 38,5° C in 5 % CO2 in air for either 28 or 40 h. Oocytes were separated from cumulus cells by gentle pipetting in D-PBS-Hyaluronidase solution. If necessary, incubation for 2 min in D-PBS-Trypsin solution was used. Oocytes were loaded with AM-Fura 2 (2μM in D-PBS-BSA) for 50 min and then incubated for 30 min in Fura-free media. Fluorescence intensity of oocytes was recorded and analyzed by computer software (Meta Fluor) that average 16 image frames captured by a CCD camera, connected to an epifluorescence microscope, into one digital image. Images were obtained every 5 seconds: excitation wavelengths 340/380nm, ND 2 filter, 400nm dichroic mirror, and 510nm emission filter. Calibration standards were used to translate the 340/380 fluorescence ratio into [Ca2+]i. Ten images were initially obtained to establish baseline concentration. Ionomycin was added to the media to obtain 10μM final concentration and 100 images were recorded. Immediately after image recording oocytes were fixed in paraformaldehyde. To determine nuclear maturation stage, oocytes were permeabilized with Triton X for 6 h and stained with Hoesch 33342. Results: No differences were found in [Ca2+]i response to ionomycin between MI and MII oocytes. These are preliminary data; we plan on gather further information from GVBD and aged oocytes to complete this study.

Evaluation of cAMP Effects on Aromatase mRNA and Activity, Steroidogenic Factor- 1 (SF-1) mRNA, and cAMP Response Element Binding Protein (CREB-1) mRNA and Phosphorylation in Granulosa Cells from 5 mm Bovine Follicles

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The gonadotropins, FSH and LH, regulate follicular function primarily through the cAMP pathway through mechanisms that are still being defined. Previous studies using follicles from rats have concluded that cAMP regulates aromatase gene expression through the transcription factors SF-1 and CREB-1. In the bovine, follicular waves are initiated by a surge in FSH that causes follicles to grow from 4 mm up until the time of selection of a single dominant follicle (diameter deviation) at about 8-9 mm, when there is an increase in circulating estradiol, a nadir in circulating FSH, and regression of all non-dominant follicles from that follicular wave. In this study, we evaluate if continuous activation of the cAMP pathway would lead to differential regulation of expression of SF-1, CREB-1, and aromatase depending upon the length of granulosa cell culture. Granulosa cells (GC) were collected from 5 mm bovine follicles, a FSH-dependent stage that is about 3 days before follicular deviation (n = 4 different cell preparations with each treatment done in triplicate in each preparation). GC were treated with 3 mM of forskolin, an agonist of adenyl cyclase, for 1, 3, 5 or 7 days. Real time PCR was used to determine mRNA concentration of CREB-1 and SF-1, Western blot was used to evaluate phosphorylated CREB-1 (only on day 1) and ELISA was used to determine 17beta-estradiol concentrations in media samples. On all culture days forskolin increased aromatase mRNA (d1= 673%, d3= 638%, d5= 1140%, d7= 1157%) and activity (d1= 418%, d3= 254%, d5= 346%, d7= 475%). Forskolin did not change steady-state concentrations of mRNA for CREB-1 (d1= 94%, d3= 100%, d5= 112%, d7=104%) or SF-1 (d1=117%, d3=107%, d5=135%, d7=91%) on any day of GC culture, but forskolin acutely increased phosphorylation of CREB-1 (d1= 210%). Thus, cAMP increases expression of aromatase mRNA and aromatase activity similarly at all times during 7 days of GC culture. This increase is independent of changes in CREB-1 and SF-1 expression but is probably reliant on activation of CREB-1 by phosphorylation through the cAMP/protein kinase A intracellular effector system.

Distinct regulation of matrix metalloproteinases and tissue inhibitors of metalloproteinases in porcine corpora lutea before versus after acquisition of luteolytic capacity

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Prostaglandin F2a (PGF2a) causes luteolysis in pig corpora lutea (CL) only after Day 12 of the estrous cycle. Before Day 12, CL lack luteolytic capacity and do not regress after a single PGF2a treatment. Luteolysis involves degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are enzymes that collectively degrade components of the ECM. Tissue inhibitors of metalloproteinases (TIMPs) inhibit the action of MMPs. We hypothesized that MMPs would be induced and TIMPs inhibited by PGF2a only in CL with luteolytic capacity. CL (n=4/5) on Day 9 or Day 17 were collected 10 h after saline or 500 µg PGF2a analog (cloprostenol) and were frozen in liquid nitrogen for analysis of specific mRNA using semi-quantitative RT-PCR. Values are the ratio of target band intensity to G3PDH band intensity. All data were analyzed by single factor ANOVA and considered significantly different at $P < 0.05$. MMP-2 and TIMP-2 mRNA were not affected by PGF2a in either Day 9 or Day 17 CL. There was a dramatic increase in MMP-1 (1353%, $P<0.01$) and MMP-9 (409%, $P<0.01$) mRNA at 10 h after PGF2a treatment of Day 17 CL (with luteolytic capacity) but no change in MMP-1 or MMP-9 at 10 h after PGF2a treatment of Day 9 CL (without luteolytic capacity). Furthermore, PGF2a decreased TIMP-1 mRNA (53% of control, $P<0.05$) only in Day 17 CL and not in Day 9 CL (72% of control, $P=0.269$). These results indicate that acquisition of luteolytic capacity is associated with the ability of PGF2a to induce MMP-1 and MMP-9. PGF2a treatment also decreased TIMP-1 mRNA and this response also only occurred in CL with luteolytic capacity. TIMP-1 normally inhibits MMP-9 activity and therefore this change could lead to greater MMP-9 activity after PGF2a treatment. It seems likely that these changes would increase degradation of the ECM, a process that is likely to be critical for completion of luteolysis. Furthermore, the differences between Day 9 versus Day 17 CL in regulation of MMP-1, MMP-9, and TIMP-1 indicate that there are important alterations in the PGF2a-induced intracellular effector systems that control these gene products after acquisition of luteolytic capacity.

Enhanced Bovine Embryonic Development After Microfluidic Cumulus Cell Removal Post Fertilization

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Microfluidic technologies are increasingly being used in cell biology and embryology research. In order to manipulate an embryonic environment microfluidics take advantage of miniscule media amounts. By using pressure heads and laminar flow profiles, a presumptive zygote can be gently manipulated in the device to remove the supporting cumulus cells post fertilization. Presumptive embryos were assigned at random to three cumulus removal treatments at 48 hours post fertilization: vortexing (3 min), handstripping (with 135µm ID stripping pipette) and microfluidics. Blastocyst rates were determined through day eight post fertilization. Rates were analyzed by GENMOD procedure in SAS, accounting for replicates and treatment. Cumulus removal by vortexing was done on 521 embryos, yielding 44 blastocysts at day eight for an 8.4% development rate. The handstripping procedure was done on 644 embryos, yielding 99 blastocysts for a 15.4% development rate. Microfluidic cumulus removal was done on 529 embryos, yielding 135 blastocysts for a 25.5% development rate. The developmental rate resultant from the handstripping treatment significantly differed from that of the vortex treatment ($p=0.0004$). The developmental rate resultant from microfluidic treatment significantly differed from both the vortex and handstripping treatments ($p<0.0001$). Kinetics of development were also impacted as larger proportions of embryos in the microfluidic group reached the blastocyst stage before embryos of the vortex or hand stripping treatments. These data suggest that cumulus cell removal in a gentle fashion is associated with enhanced embryonic development in the bovine.

Adrenal hyperandrogenism is induced by fetal androgen excess in a rhesus monkey model of polycystic ovary syndrome

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Adrenal androgen (AA) excess is found in 25-50% of women with polycystic ovary syndrome (PCOS). To determine whether AA excess is exhibited by a nonhuman primate model for PCOS, the prenatally androgenized (PA) female rhesus monkey, 6 PA and 6 control (C) females of similar age, body weight and BMI were studied during days 2-6 of two consecutive menstrual cycles or anovulatory 30-day periods. The pre-dexamethasone adrenal steroid levels were assessed in the first cycle (cycle 1) after an overnight fast. In the next cycle (cycle 2), adrenal steroids were determined immediately prior to [post-dexamethasone levels] and following an i.v. injection of 50 mg ACTH 1-39, given 14.5-16.0 h after an i.m. injection of 0.5 mg/kg dexamethasone. While serum levels of cortisol, corticosterone, dehydroepiandrosterone (DHEA) and testosterone (T) were all suppressed to a similar degree by dexamethasone in both PA and C females, androstenedione (A) and 17a-hydroxyprogesterone (17OHP) levels were not. Nevertheless, when compared to pre- and post-dexamethasone serum DHEA levels in C females, DHEA levels in PA females were elevated. Following ACTH injection, PA females exhibited higher circulating levels of DHEA, A and corticosterone, but comparable levels of 17OHP, cortisol, DHEA-S, and T, to C females. Furthermore, the increase in DHEA and A values in response to ACTH were augmented in PA females. Such augmented androgen responses to ACTH in PA females may reflect up-regulation of 17,20 lyase activity in the adrenal zona reticularis resulting in AA excess comparable to that found in PCOS women. These findings open the possibility for fetal androgen excess programming of adrenal hyperandrogenism that could have genetic, epigenetic or environmental origins.

Abstract 12

The Effects of Polyhalogenated Aromatic Hydrocarbons on Estrogen Metabolism in Lake Trout

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Polyhalogenated aromatic hydrocarbons (PHAHs), and their hydroxylated metabolites (PHAH-OHs), are persistent environmental pollutants capable of interfering with reproductive and endocrine function in Great Lakes fishes. Many of these compounds can act as environmental estrogens and disrupt physiological function in estrogen-responsive sites such as reproductive organs, liver, and neuroendocrine centers. Little is known about the mechanism by which these compounds act, but it is known that binding affinities of some of these compounds for the estrogen receptor are low. Recent evidence in human liver microsomes and in fish suggests that some PHAH-OHs may act by inhibiting the metabolism of endogenous E2. In fish, endogenous estrogen is initially undergoes phase I metabolism through cytochrome P450 addition of a hydroxyl group. Subsequent phase II conjugation adds either a glucuronic acid or a sulfate group. Lake trout (Salvelinus namaycush) is a top trophic level predator fish native to the Great Lakes. Problems with reproduction in Great Lakes lake trout populations have been apparent for over 50 years. Lake trout have high concentrations of PHAH-OHs in their tissues because of bioaccumulation. The purpose of our research is to evaluate the effect of various PHAH-OHs on estrogen metabolism in lake trout kidney and liver in vitro. Our initial studies demonstrated that estrogen glucuronosyltransferase predominates in the liver and sulfotransferase in the kidney of lake trout. Subsequently, 10 PHAHs were screened as inhibitors of estrogen metabolism in the lake trout. Eight of these compounds inhibited the formation of water-soluble estrogen metabolites at the 100 uM dose. The most potent inhibitors were 3,3',5,5'-PCB,4,4'-OH, bisphenol-A, 2,4,6-tribromophenol, 2,4,6-trichlorophenol, tetrachlorobisphenol-A, and tetrabromobisphenol-A. These compounds reduced the formation of water-soluble estrogen metabolites by 60-85%. Our future studies will identify the metabolic pathways and products of estrogen metabolism in both the kidney and liver of lake trout, and determine whether PHAH-OHs are inhibiting phase I or phase II metabolism. In a related study, the inhibition of human estrogen sulfotransferase was shown to be the most potent biological mechanism described to date regarding the endocrine-disrupting activity of PCBs and their metabolites. Accordingly, we hypothesize that PHAH-OHs are inhibiting lake trout estrogen metabolism via the phase II conjugation reaction.

Abstract 21

A Proposed Model to Study the Affects of Endometriosis on Oocyte Quality

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Endometriosis is a gynecologic disease which affects an estimated 5-10% of reproductive age women. Generally, it is classified as the growth of endometrial glands and stroma outside the uterine cavity, most commonly found within the peritoneal cavity. The most common manifestations of endometriosis are non cyclic pelvic pain, dysmenorrhea and infertility. As of now, the relationship between endometriosis and infertility remains unclear. Several studies have proposed a correlation between the two by using information gathered through egg donation programs. It was found that women with advanced stages of endometriosis have similar success with IVF when oocytes from disease free women are used. Also demonstrated is that disease free recipients undergoing donor IVF show decreased pregnancy rates when oocytes are used from women with endometriosis. These observations are what motivate the proposed study.

Abstract 22

ER beta expression in rhesus monkey endometrium

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OBJECTIVES: The effects of estrogen are now known to be mediated by estrogen receptor (ER) and -beta (ER) receptor subtypes. While ER has a well known distribution in endometrium, the presence of ER is controversial. Previous data suggest ER mRNA to be present in endometrial stroma, epithelium, and accompanying vasculature. However, little data exist showing the presence of the protein in these cells. The goal of this study is to investigate the distribution of ER in normal endometrial samples obtained from rhesus macaques. METHODS: Four samples of endometrium from normally cycling Rhesus macaques were selected for immunohistochemical analysis using a monoclonal antibody generated against the entire splice sequence of the ER molecule. RESULTS: No staining was apparent in either endometrial epithelium or stroma in any of the samples. In each sample, staining was positive for ER protein in the endothelial cells and smooth muscle cells of the endometrial vasculature. CONCLUSIONS: Despite prior evidence of ER mRNA in all cells of the primate endometrium, our results suggest that ER expression in the macaque endometrium may be limited to the supporting vasculature.

Insights into Intra-Adrenal Zonation From the Marmoset Social Subordination Model

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The key proteins required for adrenal C19 steroidogeneis and a functional zona reticularis (ZR) are P450c17, 3B-HSD, cytochrome b5 (cytb5) and P450reductase (reductase). We have previously shown that adult male marmosets do not express a functional ZR in adulthood even though their adrenal development *in utero* is similar to humans and non-human primates. Our purpose in this study was to determine whether female marmosets express a ZR and whether ovariectomy and/or social subordination had an effect on ZR expression and function. Paraffin embedded sections were stained using immunohistochemistry (IHC) for P450c17, 3B-HSD, cytb5 and reductase in cycling (n=6), ovariectomized (ovx; n=6) and subordinant (sub; n=3) female adrenals. Microsomes from whole adrenals were analyzed by SDS-PAGE and immunoblotting for P45017 and cytb5 expression. They were also assayed for 17,20-lyase activity (radio-metric assay) and total enzyme pathway metabolism (radio-HPLC). Additionally, *in vivo* steroids were measured in dominant (dom; n=6), ovx (n=6) and subordinate (n=8) females upon ACTH challenge. While cytb5 is barely detectable by western and IHC in males, cycling females exhibited cytb5 expression in the area of a ZR, a result that was further enhanced by ovariectomy ($p<0.05$). Although no significant differences were observed in 17,20-lyase activity, ovx females tended to have higher activity than cycling females and males. Analysis of the *in vivo* steroids revealed that sub females have a significantly increased efficiency for glucocorticoid synthesis ($p<0.05$) and ovx females had the greatest capacity for C19 steroid (ZR) responsiveness to ACTH challenge ($p<0.05$). Our preliminary hypothesis is that cytb5 expression is necessary for 17,20-lyase activity in marmosets, especially in the presence of 3B-HSD, although it's presence won't guarantee C19 steroid biosynthesis. What is becoming apparent is the relative size of the zona fasciculata to the ZR (glucocorticoids vs. C19 steroids) and the efficiency of the steroidogenic enzymes in those zones is more predictive of the measured steroid output than the absolute expression of those same enzymes. Further work needs to be done to investigate the role of ovarian hormones and signaling factors on the structure and function of the adrenal gland. *This work was supported by NIH grants MH060728, RR000167, HL056702, HD036913, and HD041921.*

Enteral Erythropoietin Stimulates Erythropoiesis in Suckling Rats

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Mammalian milk contains biologically relevant amounts of erythropoietin (Epo), the primary hormone regulating erythropoiesis. In suckling rats, milk borne Epo remains intact and exerts mitogenic effects on gastrointestinal tract, but no sustained stimulation of erythropoiesis was previously found. Aggressive iron supplementation must generally accompany parenteral rhEpo treatment in order to observe sustained erythropoiesis. We hypothesized that the combination of supplemental iron with enteral Epo therapy would stimulate erythropoiesis in suckling rats. To investigate the effects of oral Epo (EntEpo), we performed gastrostomies on 4 day-old Sprague Dawley rats, feeding artificially with rat formula for 8 days. The artificial formula was devoid of growth factors. Rats were fed formula (control), EntEpo (430 U/kg/d), or EntEpo+iron (6 mg/kg/d orally). After treatment for 8 days, blood was collected to measure hemoglobin, red cell indices and reticulocytes. Results are reported as mean ± SEM, and analyzed by factorial ANOVA, with Fisher post hoc testing. Hemoglobin levels differed ($p<0.0001$). Although Hb levels in control (8.4±0.2 g/dL) were similar to EntEpo (8.5±0.3 g/dL), Hb levels in EntEpo+iron (10.4±0.3 g/dL) were higher than either control or EntEpo group ($p<0.0001$). Absolute reticulocytes differed ($p<0.0006$); and were higher in EntEpo+iron ($p<0.006$) than control or EntEpo. Epo fed to suckling rats stimulates erythropoiesis when administered in combination with oral iron supplementation, but not by itself. Iron plays a role in the sustained erythropoiesis seen with enteral Epo feeding.

Growth Factors Stimulate and Inhibit Ca2+ Mobilization in Uterine Artery Endothelial Cells Derived from Pregnant Ewes

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Ca2+ signaling regulates many aspects of endothelial function. Both ERK and Ca2+ regulate endothelial nitric oxide synthase (eNOS) activation and consequently nitric oxide production, particularly during pregnancy when augmented vasodilation supports a 10-fold increase in uterine blood flow. We previously showed that, within 5 minutes, single isolated uterine artery endothelial cells from pregnant ewes (P-UAEC) respond to ATP (but not bFGF, EGF, or VEGF) with an instant peak in intracellular free Ca2+ concentration ([Ca2+]i) followed by a sustained phase. While all these agonist are capable of stimulating ERK activation, only ATP, VEGF, and to a lesser extent bFGF stimulated eNOS activation as measured by L-arg to L-citr conversion assays. Recently, video-imaging equipment has permitted the study of the effects of cell density on agonist-induced Ca2+ mobilization simultaneously in numerous P-UAEC over 30 minutes. The studies showed that with increasing cell density more cells responded to ATP (Table) and at confluence there was a dramatic increase in the number of oscillations, which were often synchronous. Thus, at the physiologic state, the cells may be more capable of propagating an ATP-induced response upstream and/or downstream. The trends for bFGF mimicked the response to ATP, yet the number of cells responding was lower. VEGF treatment showed the opposite trend; less cells responded at higher densities. Cell density did not affect the response to EGF and the low response may explain the lack of eNOS activation. Of interest, if ATP was added after a growth factor, the ATP-induced response was inhibited. For VEGF, there was a significant decrease in the number of bursts, but bFGF or EGF treatment delayed or abolished the ATP-induced response. At confluence, 91.4% of P-UAEC respond to ATP but only 35% and 26% of the cells responded after EGF and bFGF pretreatment, respectively. Thus, growth factors, while capable of stimulating a rise in [Ca2+]i, inhibit ATP-induced Ca2+ mobilization. This may have profound implications in the study of diseased pregnancies because circulating levels of VEGF and bFGF are increased during pre-eclampsia. Funded by HL64601, HD38843, and 0315191Z.

Estrogen Receptors and VEGF Modulation of Angiogenic and Nitric Oxide Responses in Ovine Uterine Artery Endothelial Cells

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Uterine blood flow (UBF) is substantially elevated during high estrogen states such as the follicular phase and pregnancy. Follicular and Pregnancy rises in UBF are inhibited, in vivo, by estrogen receptor (ER) antagonist ICI 182,780 and NOS inhibitor L-NAME demonstrating endogenous E2B-induced increases in UBF via a NO mediated mechanism. Although both NO-mediated vasodilatation and VEGF-associated angiogenesis appear to control UBF, the mechanistic basis and interactions for this are still uncertain. We used in situ hybridization (ISH) ([35S]-labeled riboprobes) and immunohistochemistry (IHC) for mRNA and protein localization. The presence of both ERa and ERB mRNA and protein was identified in uterine artery endothelium ex vivo. In passage 4-5 UAECs both ERa and ERB mRNA and protein were also observed, suggesting that UAECs may be a good model to evaluate direct actions of E2B on the uterine vascular bed. We observed that E2B dose dependently elevated VEGF secretion by cultures of UAECs with maximum responses of 3-4 fold at 10nM ($P<0.001$). This E2B-mediated VEGF secretion was associated with elevations in UAEC angiogenesis using a capillary-like tube formation assay. E2B-induced rises in VEGF and angiogenesis were blocked by ICI 182,780 demonstrating ER receptor involvement. Since the E2B-induced rises in VEGF may also increase vasodilatation, we tested the hypothesis that NO production by UAECs in response to E2B would be similar to VEGF responses. Employing a newly developed real-time intracellular NO assay using DAF-FM DA in a fluorescent plate reader, we observed that in UAECs both E2B (10nM) and VEGF (10ng/ml) increased NO production 2.0 and 1.5 fold, respectively above control; the combination was neither synergistic nor additive. Since the slope of the time course rise in NO with E2B was much steeper than the observed rise with VEGF, it is unlikely that the E2B-induced rise in NO is via stimulation of VEGF. In contrast both sFlt-1 and Flt-1 blocking antibody inhibited the E2B-mediated rise in angiogenesis. Thus based on this in vitro UAEC model E2B-associated rises in VEGF appear to help modulate angiogenesis, but not NO-mediated vasodilatation.
NIH HL49210, HD33255, HL57653, HD38843, HL64703, & HL64601.

Concurrent Oral Sessions III and IV

2:45 PM – 3:45 PM

Cell Signaling

Mediterranean Room
Chairs: Dr. Paul Bertics
Dr. Linda Schuler

Hypothalamus-Pituitary-Adrenal Axis

Captain's Table Room
Chairs: Dr. Ei Terasawa
Dr. David Abbott

Abstracts 8-11

MEK/ERK1/2 Inhibition Alters eNOS Activity in an Agonist-Specific Manner in Endothelial Cells and COS-7 Cells

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Endothelial production of nitric oxide (NO) is critical for vascular homeostasis in many physiologic states. During pregnancy, the substantial increase in blood flow to the uterus is in part due to increased vasodilation as a result of increased production of NO. Endothelial nitric oxide synthase (eNOS) is regulated by acylation, intracellular protein trafficking, protein-protein interactions, changes in intracellular calcium concentration ([Ca2+]i) and phosphorylation. In addition, eNOS phosphorylation may render eNOS more or less sensitive to [Ca2+]i. The MEK/ERK1/2 pathway has been implicated in the regulation of eNOS activity in several studies. Activation of the MEK/ERK1/2 pathway may be required for full eNOS activation by HDL, adenosine, estrogen and ischemia in certain endothelial cell types. In contrast, inhibition of MEK/ERK1/2 in bovine aortic endothelial cells results enhances bradykinin-stimulated eNOS activity and ERK1/2 directly phosphorylates eNOS in vitro on an unidentified amino acid residue, decreasing eNOS activity. We determined whether inhibition of MEK/ERK1/2 signaling influences eNOS activity in response to Ca2+ mobilizing agents in COS-7 cells expressing ovine eNOS (oeNOS) and in human umbilical vein endothelial cells (HUVEC-CS). COS-7 cells do not express any NOS isoforms, but contain caveolae, calmodulin, and Hsp90, important for eNOS trafficking and activation. HUVEC-CS or COS-7 cells transiently transfected with oeNOS (COS-7/oeNOS) were used to determine eNOS activity by 3H-arginine to citrulline conversion assay in intact cells. Cells were pretreated with control or 10 uM U0126 and then treated with either 10 uM A23187 (COS-7/oeNOS) or 100 uM ATP (HUVEC-CS and COS-7/oeNOS). ATP, which mobilizes Ca2+ from intracellular stores, stimulated approximately 15% greater eNOS activity in U0126-pretreated HUVEC-CS compared to control pretreatment. A23187, a calcium ionophore, stimulated about 35% greater eNOS activity in COS-7/oeNOS pretreated with U0126 compared to cells pretreated with control; conversely, ATP-stimulated eNOS activity was reduced by about 20% of control-pretreated COS-7/oeNOS cells. It remains to be determined whether manipulation of eNOS activity by inhibition of MEK/ERK1/2 occurs by direct modifications of eNOS (such as phosphorylation by ERK1/2) or indirect changes in the intracellular environment (such as phosphorylation of scaffolding proteins, altered [Ca2+]i, or changed activity of eNOS interacting proteins).

Keynote Speaker
Jerome F. Strauss, III, M.D., Ph.D.

Jerome F. Strauss, III is the Luigi Mastroianni Jr. Professor and founding Director of the Center for Research on Reproduction and Women's Health and Associate Chairman of the Department of Obstetrics and Gynecology at the University of Pennsylvania. He received the B.A. degree with Honors in Biology from Brown University and the M.D. and Ph.D. degrees from the University of Pennsylvania. He served as a house officer in Obstetrics and Gynecology at the Hospital of the University of Pennsylvania and later joined the faculty in the Departments of

Obstetrics and Gynecology, rising to the rank of Professor in 1985. He is concurrently Director of the NICHD-sponsored National Cooperative Center in Infertility Research at the University of Pennsylvania. His past administrative experience includes nearly a decade as Associate Dean in the School of Medicine and P.I. and Program Director of the University of Pennsylvania's Medical Scientist Training Program. He serves as a member of the National Advisory Child Health and Human Development Council of the National Institutes of Health and on the Board of Directors of the Burroughs Wellcome Fund. Dr. Strauss's research interests include the regulation of steroid hormone biosynthesis; the genetics of polycystic ovary syndrome; trophoblast differentiation and placental endocrine function; and the biology of fetal membranes, a molecular control of sperm motility. Dr. Strauss has served as an editor or on the editorial boards of the *Journal of Lipid Research*; *Endocrinology*; the *Journal of Steroid Biochemistry and Molecular Biology*; *Biology of Reproduction and Placenta*. He currently sits on the editorial boards of the *Journal of Women's Health and Gender-based Medicine*; *Journal of the Society for Gynecologic Investigation*; *Journal of Endocrinology*, *Trends in Endocrinology and Metabolism,* and *Seminars in Medicine*. He is an Associate Editor of *Human Reproduction Update* and an Editor of *Steroids*. Dr. Strauss's honors include the President's Achievement Award from the Society for Gynecologic Investigation, of which he is past President; the Society for the Study of Reproduction Research Award; election to the Institute of Medicine, National Academy of Sciences; and the Transatlantic Medal of the British Endocrine Society.

Exogenous Nitric Oxide-Stimulated Cell Proliferation Via Activation of MEK/ERK1/2 Pathway In Ovine Feto-Placental Artery Endothelial (OFPAE) Cells

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Depts [1]Ob/Gyn, [2]Pediatrics, and [3]Animal Sci, University of Wisconsin - Madison

Nitric Oxide (NO), a local vasodilator, is increased dramatically during pregnancy and plays a role in regulating placental vasodilation. We have shown that bFGF dose-dependently increases endothelial NO synthase (eNOS) expression via activation of MEK/ERK1/2, and also increases cell proliferation in OFPAE cells. Hypothesis: Exogenous NO stimulates cell proliferation via activation of the MEK/ERK1/2 cascade. Methods: For cell proliferation assays, OFPAE cells were treated with sodium nitroprusside (SNP; 1nM-100mM), an NO donor. After 72 hr the number of cells was determined. Additional cells were treated with 1 mM SNP in the absence or presence of PD98059 (a specific MEK inhibitor; 2.5-40 mM) in a similar fashion. To determine whether SNP activates the ERK1/2 cascade, cells were treated with 1 mM SNP for 0, 1, 5, 10, 30, or 60 minutes. Cells were then analyzed for ERK1/2 phosphorylation using immunocytochemistry and Western analysis. Results: SNP dose-dependently stimulated ($p<0.05$) cell proliferation with a maximal effect at 1 mM SNP (3.6 fold). PD98059 dose-dependently inhibited ($p<0.05$) SNP-induced cell proliferation, beginning at 5 mM and reaching a maximum at 20 mM. SNP at 1 mM also time-dependently phosphorylated ERK1/2. The ERK1/2 phosphorylation first appeared after 10 min of treatment, reached a maximum at 30 min, and then remained elevated through 60 min. Conclusions: These data indicate that exogenous NO stimulated cell proliferation is mediated via activation of the MEK/ERK1/2 signaling pathway. These data also suggest that bFGF-induced cell proliferation may in part be mediated through an NO/MEK cascade. Supported by NIH grants HL64703, HL57653, HL38843.

Invited Speaker
Rita Loch-Caruso, Ph.D.

Rita Loch-Caruso is Professor of Toxicology and Interim Chair of the Department of Environmental Health Sciences at the University of Michigan in the School of Public

Health. She is also currently serving as the Toxicology Program Director of the Department of Environmental Health Sciences and the Director of the interdepartmental Environmental Toxicology Research Training Program. She received her Ph.D. in Toxicology from the Department of Environmental Health of the University of Cincinnati in 1984. She was Assistant Professor of Pediatrics & Human Development at Michigan State University before joining the Toxicology Program at the University of Michigan. Her research focus is female reproductive toxicology, emphasizing mechanisms of toxicity at the molecular, biochemical and cellular levels of uterine muscle, and integrating this knowledge at the tissue and whole animal levels. She has a particular interest in environmentally persistent chemicals such as polychlorinated biphenyls (PCBs), lindane and DDT. Major projects involve development of models for reproductive toxicity study, mechanisms of disruption of uterine muscle gap junctions, toxicant-induced alteration of myometrial and placental intracellular signaling (phosphorylation, calcium, phospholipids), and endocrine-mediated modulation of uterine contraction. She is active in local, state and national activities related to environmental health, such as NIH grant review committees and the City of Ann Arbor Environmental Commission.

Effect of Extracellular Matrix in Directing Embryoid Bodies Derived From Human Embryonic Stem Cells into Trophoblast Progenitor Cells

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During decidualization, the structure of the uterine epithelial extracellular matrix consists primarily of collagen IV, laminin, fibronectin and perlecan. In order to better understand the importance of cell-matrix interactions during the early stages of embryo implantation and to understand the control of differentiation of embryonic cells, we propose using human embryonic stem cells differentiated into embryoid bodies (EB) as an in vitro model. We exploited the effect of extracellular matrix in directing EBs to differentiate into trophoblast-like cells by culturing the EBs on different types of matrices. We harvested culture medium following the transfer of EBs into Matrigel-coated or gelatin-coated plates. Our studies show that EBs grown in suspension and transferred to Matrigel-coated plates or gelatin-coated plates adhered to the culture surface and hCG secretion increased within approximately 2 weeks. The hCG secretion levels from EBs in Matrigel-coated were significantly higher than levels from EBs in gelatin-coated plates for up to 22 days. Yet, by day 25 of culture, there was no significant difference between the hCG secretion of EBs grown on of the two groups. In addition, we determined progesterone and estradiol-17beta levels in selected samples from both sets of plates. By day 25, the levels of progesterone and estradiol-17beta secretion by cells in both Matrigel-coated plates and gelatin-coated plates were substantially elevated over control medium. This triad of hormones suggests a high level of trophoblast activity. In addition, cellular outgrowths in both groups contained multinucleated cells that strongly resembled syncytiotrophoblast structure. Passages derived from the cellular outgrowths on the Matrigel plates became confluent more slowly than the cellular outgrowths on gelatin-coated plates. The hCG, progesterone and estradiol-17beta secretion levels in both Matrigel-coated and gelatin-coated plates declined with each consecutive passage. We conclude that gelatin, a type I collagen, can support EB differentiation into trophoblast-like cells.

An *In Vitro* Three Dimensional Model Simulating Primate Embryo Implantation During Early Pregnancy

T Chang, B Gerami-Naini, J Drenzek, O Dovzhenko, M Durning, M Garthwaite, T Golos
National Primate Research Center, UW-Madison

The early initiation of embryo invasion into the endometrium and formation of the placenta from trophoblast and mesenchyme are critical toward establishment of a successful pregnancy. The specific cellular localization and mechanisms of action during embryo implantation and placentation are yet to be well studied due to lack of tools to visualize in vivo and simulate in vitro the process of primate implantation and maternal-embryo interactions. Here we report a novel three-dimensional method of utilizing Matrigel embedded non-human primate embryos to begin to simulate the development occurring at implantation and post-implantation stages. Rhesus monkey (macaca mulatta) embryos generated from IVF procedure were used for producing blastocysts. Expanded blastocysts were embedded into a three dimensional environment of Matrigel and cultured in Buffalo rat liver cell-conditioned medium. Hatching of the blastocysts occurred and cell outgrowths and cystic formation were observed beginning at day 5 to 6 post embedding following a rapid development of cellular-like protrusions on the surface of blastocyst implant. In addition, branch-like projections which grew into the three-dimensional environment similar to in vivo placental structures were observed. Immunoassay indicated a curve of elevation and decline in CG secretion similar to the post-implantation secretion of monkey CG in early pregnancy in vivo. Using non-human primate embryos embedded in Matrigel as a model to study implantation and post-implantation stage maternal-embryo interaction could facilitate our understanding of primate and human reproductive physiology during early pregnancy. This model may have potential to accelerate the development of therapy and treatment for women suffering from early pregnancy loss.

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Expression of Indoleamine 2,3-Dioxygenase in Rhesus Monkeys and Common Marmosets

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During pregnancy, tryptophan is catabolized by indoleamine 2,3-dioxygenase (IDO). IDO is the rate-limiting enzyme in the catabolism of tryptophan along the kynurenine pathway. Tryptophan is an essential amino acid that is important in the activation of a T cell response. IDO causes T cell cycle arrest in the G1 phase of T cells. IDO is expressed in macrophages, dendritic cells, and trophoblasts and is also highly expressed in lung and intestinal tissue. It is hypothesized that IDO maintains immune tolerance during pregnancy because a microenvironment is created in which T cells cannot proliferate and kill fetal tissues. The objective is to investigate if IDO is expressed in reproductive and lymphoid tissues of the rhesus monkey and common marmoset. Methods: Immunohistochemistry was done with a mouse anti-IDO monoclonal antibody on 7um cryosections and paraffin sections of rhesus monkey spleen, lymph node, uterus, and placenta and marmoset spleen, lymph node, placenta, uterus, and nonpregnant uterus. IgG3 was used as a negative control. Results: IDO expression was found in rhesus spleen, lymph nodes, day 36 placenta, term placenta, and uterine endothelial cells and blood vessels. IDO was also expressed in marmoset spleen, lymph node, day 128 placenta, day 100 placenta, pregnant uterus, and nonpregnant uterus. Conclusions: Confirmation of IDO expression in rhesus monkey and common marmoset tissue, especially the placenta, supports the hypothesis that this enzyme prevents a maternal T cell response during pregnancy due to catabolism of tryptophan. Nonhuman primates may provide a model with distinct similarities to human placentation to study the role of IDO in maternal-fetal immune tolerance.

Effects of Endogenous Ovarian versus Exogenous Estrogen on Urogenital Tissue Blood Flows

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Urogenital tissue function is highly sensitive to menopause related falls in ovarian hormones, they are particularly responsive to the vasodilator responses of exogenous estrogen. In contrast, it is unknown if there are equivalent cyclical changes in tissue perfusion to both the reproductive (uterine, vagina, vulva) and the lower urinary tract (bladder) tissues. Objective: To determine the effects of endogenous ovarian-derived estrogen versus exogenous estrogen on blood flow to the urogenital tissues. Methods: Uterine, vaginal, vulvar, and bladder blood flows were measured using radiolabeled microspheres in 26 chronically instrumented ewes. We compared urogenital blood flows of Luteal (low estrogen) to Follicular (Preovulatory high estrogen) phase ewes. In oophorectomized (Ovx; 10-15 days post surgical menopause) ewes, we also compared effects of acute (5ug/kg 120 min) and prolonged (6ug/kg/day for 10 days) exogenous estrogen to vehicle infusions.

Results:

Intact follicular vs luteal

Uterus 120%*, Vagina170%**, Vulva 50%*, Bladder-12%NS

Ovx vs Inact luteal

Uterus -47%**, Vagina -65%**, Vulva -40%*, Bladder -72%**

Ovx: acute E2B vs vehicle

Uterus 1900%**, Vagina 1150%**, Vulva 570%**, Bladder 480%**

Ovx: Prolonged E2B vs vehicle

Uterus 270%**, Vagina 440%**, Vulva 490%**, Bladder 130%*

Values describe % change; *P<0.05,**P<0.01.

During the Follicular phase of the ovarian cycle, increases in uterine, vaginal, and vulvar, but not bladder blood flows were observed. Urogenital blood flows were reduced in all of these tissues in Ovx vs Intact Luteal phase ewes. Acute and prolonged estrogen treatment substantially increased urogential blood flows (Acute > Prolonged all tissues, but vuvlar). Conclusions: Ovx (surgical menopause) lowers blood flow to the uterus, vagina, vulva, and bladder, thus demonstrating the essential role of the ovaries in maintaining urogential tissue perfusion. However we observed Follicular phase estrogen-induced rises in only uterine, vaginal, and vulvar tissues, with no increase in bladder blood flow. All of these tissues including the bladder were highly responsive to both acute and prolonged exogenous estrogen administration. Therefore it is possible that higher endogenous ovarian-derived estrogens are delivered to reproductive tissues, but not the bladder through local transport pathways. Alternatively, threshold response differences between reproductive tissues and the bladder relate to higher estrogen receptor numbers or sensitivities at the endogenous and exogenous levels of estrogen studied. Support NIH HL49710, HD33255, HL57653, and HD38843.

Estrogen Increases the Frequency of [Ca2+]i Oscillations and Frequency of [Ca2+]i Synchronizations in LHRH Neurons Derived From Monkey Olfactory Placode

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We have previously shown that estrogen (E2) induces a rapid increase in the frequency of [Ca2+]i oscillations in the GT1-7 LHRH-secreting neuronal cell line. In the present study we further examined whether similar effects of E2 occur in primary LHRH neurons derived from monkey olfactory placode using [Ca2+]i imaging and patch-clamp recording. Results: 1) Ten min infusion of 17b-estradiol (E2, 0.1 to 1 nM) rapidly increased the number of LHRH neurons exhibiting [Ca2+]i oscillations and the frequency of [Ca2+]i oscillations in a subset of LHRH neurons within a few minutes after the initiation of E2 infusion; 2) E2 also increased the frequency of the synchronization of [Ca2+]i oscillations; 3) similarly, BSA conjugated 17b-estradiol increased the number of LHRH neurons exhibiting [Ca2+]i oscillations as well as the synchronization of [Ca2+]i oscillations; 4) ICI182,780, an estrogen receptor antagonist, did not block the E2-induced synchronization of [Ca2+]i oscillations in LHRH neurons, whereas it reduced the E2-induced increase in the number of [Ca2+]i oscillations. Brief infusion of E2 (2-3 min) also increased the firing number and burst duration of LHRH neurons with a latency of 60-120s lasting for at least 25 min. ICI182,780 blocked the E2-induced increase in the firing activity. These results suggest that 1) E2 causes a rapid stimulatory action in primate LHRH neurons; 2) this E2 action appears to be mediated by non-nuclear receptors, such as putative membrane receptors and/or a non-receptor mediated mechanism, and 3) there are at least two mechanisms of the rapid E2 action in primate LHRH neurons; one is ICI sensitive and the other is ICI insensitive. Supported by NIH grants HD15433 and HD11355.

Abstract 1

Dysfunctional Uterine Bleeding and Angiogenesis

EA Pritts
University of Wisconsin - Madison

Angiogenesis is the process in which new blood vessels develop from pre-existing vessels. They accomplish this through a variety of methods, including sprouting, intussusception, and vessel elongation. There may also be a role in angiogenesis for circulating endothelial progenitor cells.

Endometrial angiogenesis and ultimately blood vessel development depends upon intricate interactions between the endometrial glands and stroma, the basement membranes of the vessels and the vessels themselves. These interactions are orchestrated through the secretion of a variety of angiogenic factors and growth factors.

By far the most important components involved in the monitoring of angiogenesis are Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF) and the angiopoietins (Ang). However, multiple other angiogenic factors may play a role in the menstrual cycle. Each year, putative factors are being added to that list, including the Matrix Metalloproteinases (MMPs), the prostanoids, Endothelin, Urokinase-type Plasminogen Activator (u-PA), Angiogenin, and Thrombin.

Dysfunctional uterine bleeding is defined as irregular menstrual bleeding in women with known ovulation. To confirm the diagnosis, the menstrual effluent must measure more than 80 milliliters per month. It must not be due to pregnancy, endocrine factors, coagulation abnormalities, intra-uterine polyps or fibroids.

Preliminary studies regarding women with dysfunctional uterine bleeding have pointed to aberrant growth of endothelial cells and aberrant maturation of the vessel tubules. Gross examination of the endometrium of these women has shown increased vessel fragility and increased vessel branch patterns of growth.

In this presentation, the audience will learn the basics of angiogenesis, the human menstrual cycle and the profound interactions between the two. A review of the literature (both in vitro and in vivo) will be presented regarding what is known about this disorder and new research will be presented.

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Schedule Continued

12:30 pm – 1:30 pm **Lunch**
Pacific Room

1:30 pm – 2 :30 pm **Keynote Lecture:**
Mediterranean Room
"Polycystic Ovary Syndrome (PCOS): Genomic and Genetic Insights into Pathophysiology"
Dr. Jerome Strauss, Luigi Mastroianni, Jr. Professor, Associate Chairman, Obstetrics and Gynecology, Director, Center for Research on Reproduction and Women's Health, University of Philadelphia-Pennsylvania, President, Society for Gynecologic Investigation, 2003-2004

2:30 pm – 2:45 pm **Break**

2:45 pm – 3:45 pm **Concurrent Oral Sessions III and IV:**

Cell Signaling:
Mediterranean Room
Chairs: Dr. Paul Bertics, Professor, Biomolecular Chemistry
Dr. Linda Schuler, Professor, Comparative Biosciences
2:45: *"Differential Timing in cAMP Regulation of Cyclins, Aromatase, and LH Receptor mRNA in Cultured Bovine Granulosa Cells from 5mm Follicles"*
Dr. Wenxiang Luo, Wiltbank Lab, Dairy Science, UW Madison
3:15: *"Growth Factors Stimulate and Inhibit Ca^{2+} Mobilization in Uterine Artery Endothelial Cells Derived from Pregnant Ewes"*
Shannon Gifford, Bird Lab, ERP Program

Hypothalamus-Pituitary-Adrenal Axis:
Captain's Table Room
Chair: Dr. David Abbott, Professor, Primate Center and Ob/Gyn
Dr. Paul Fricke, Assistant Professor, Dairy Science
2:45: *"Gonadotropin Releasing Hormone (GnRH) Associated Peptide (GAP) Does Not Affect GnRH Release In Perifused Rat Hypothalami"*
Dr. Mike Woller, Woller Lab, UW Whitewater
3:15: *"Insights Into the Regulation of Intra-Adrenal Zonation From the Marmoset Social Subordination Model"*
J Christina Pattison, Bird Lab, ERP Program

3:45 pm – 4:00 pm **Break**

4:00 pm – 5:00 pm **Poster Session**
Pacific Room

5:00 pm **Closing Remarks**
Pacific Room
Adjournment to the Great Dane Pub & Brewery (123 E. Doty St.)

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*** ERP Program Training Grant Recipient**

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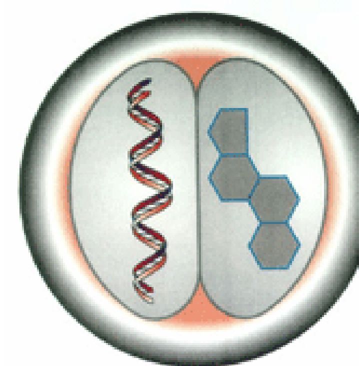
Please join us next year for the 2005 Annual Endocrinology - Reproductive Physiology Scientific Symposium



Friday, October 14, 2005

University of Wisconsin - Madison