Induction of Endotheilin-2 in bovine and human granulose cells and its role in corpus luteum formation

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Introduction: Endothelins (ETs) are pleiotropic peptides: they play numerous roles both in health and disease. ET-1, ET2, belong to a family of 21-amino acid peptides derived from distinct genes which are expressed in a wide variety of cells. ET-1, the main ET produced in endothelial cells (ECs), was extensively studied and was shown to act as a mediator of luteolysis, however, only little is known of ovarian ET2. Recently, ET2 was proposed to promote follicular rupture in rodents via a progesterone-dependent mechanism. To examine the putative role of ET2 in a monovulatory species, we studied its pattern of expression and roles in the bovine ovary.
Methods: ET2 gene expression was determined in corpus luteum (CL) collected from various stages of the cycle and in follicles during folliculo-luteal transition as follows: GnRH was administered to heifers and follicles were collected before and 4, 10, 20, 25, and 60 h after GnRH treatment. Two cell models were used to study the regulation of ET2 gene expression in vitro: bovine granulosa cells (GCs) of large follicles and human GC line (SVOG). Finally, the effects of exogenous ET2 on GCs proliferation and on VEGF plus Cyclooxygenase-2 (COX-2) expression were measured using XTT, qRT-PCR and western blotting, respectively.
Results: ET2 exhibited a biphasic pattern of expression. It increased moderately at 4 h, post GnRH administration, returned to basal levels, and then dramatically rose again only in the early CL (60 h). ET2 could not, however, be detected in older CL. ET2 in the young CL was identified in luteinizing GCs but not in ECs. Similarly, in preovulatory follicles, higher ET2 levels were observed in the GC layer than in the theca internal layer. Ample ET-converting enzyme-1 expression in GCs enables the cells to secrete mature ET2 peptide. We then studied the in vitro regulation of ET2. In accordance with the in vivo data, GCs incubated for 42 h with LH responded with an increase in ET2. Progesterone itself, or in conjunction with LH, did not further affect ET2 levels. Before completion of angiogenesis, hypoxic conditions prevail in early CL. Therefore, GCs were incubated with a hypoxia-mimicking agent, CoCl2. CoCl2 (50-200 uM) elevated ET2 in GCs in a dose-dependent manner 3h after its addition. Also in SVOG cells incubated in low oxygen tension ET2 was elevated. In both GC and SVOG, hypoxia augmented VEGF levels. Incubation of GCs with ET2 (10-9-10-7 M) induced characteristics of early CL: marked GC proliferation as well as VEGF and COX-2 expressions (mRNA and protein levels).
Conclusions: ET2 in the early bovine CL, triggered by LH surge and hypoxia, may facilitate CL formation by promoting cell proliferation and differentiation.

The role of amygdalar corticotropin-releasing factor receptor type 1 (CRFR1) in the anxiolytic effect of environmental enrichment.

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Introduction: Environmental enrichment (EE) has been shown to be beneficial for the physiological and psychological well being of the animals. Animals maintained under enriched conditions have been shown to express reduced levels of emotionality-related measurements such as defecation and freezing, less stereotyped behaviors and reduced anxiety levels. Although the beneficial effects of EE on the general well being of the animals are well documented, the molecular mechanisms underlying the anxiolytic effect of EE are not fully understood. Among the various peptide and neurotransmitter systems that have been implicated in the regulation of stress, CRF system plays a critical role in initiating the cascade of biological events during the stress response. Data in animal models demonstrating anxiogenic-like behavioral effects of CRF administration and anxiolytic-like activity of CRFR1-selective antagonists led to the suggestion that CRF may be involved in anxiety-related disorders. In the present study we hypothesized that changes in limbic CRFR1 expression may have a crucial role in mediating the anxiolytic effect of EE.

Methods: Enriched cages were specially designed to facilitate enhanced sensory, cognitive, motor and social stimulation relative to standard housing conditions (SC). After 6 weeks of rearing in different housing conditions, mice were tested for anxiety like behavior. Next, the basolateral amygdala and the BNST of EE and SC mice were microdissected and the CRFR1 mRNA expression was assessed by semi-quantitative and real-time PCR. We then designed and constructed a lentiviral vector expressing small interfering RNA (siRNA) against CRFR1 (lenti-siCRFR1) for specific in vivo knockdown of CRFR1.

Results: EE induced an anxiolytic effect in the light-dark transfer, the elevated plus-maze and the open-field tests. We found that the anxiolytic effect of EE was correlated with a significant downregulation of CRFR1 mRNA expression in the basolateral complex of the amygdala (BLA), a limbic CRFR1 expressing structure that has been implicated as a major anatomical site for the regulation of anxiety-like states and reactions. To genetically mimic the anxiolytic effect of EE, we designed and constructed a lentiviral vector expressing small interfering RNA (siRNA) against CRFR1 (lenti-siCRFR1) for specific in vivo knockdown of CRFR1 in the BLA of adult mice. We showed that similar to the EE effect, knockdown of CRFR1 mRNA in the BLA significantly reduced anxiety-like behavior.

Conclusions: Understanding the molecular mechanisms underlying the anxiolytic effect of EE may improve the ability to design therapeutic interventions for, and thus manage, affective and stress-related disorders. This study demonstrated for the first time the specific role of CRFR1 in the BLA in mediating the anxiolytic effect of EE.

GnRH induces various histone modifications at the chromatin of the gonadotropin genes

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Introduction: Levels of the gonadotropin hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) vary considerably throughout the life cycle and are regulated primarily by the gonadotropin releasing hormone (GnRH) which induces their synthesis and release. We have shown previously that expression of the β-subunit genes of both hormones is repressed in immature gonadotrope cells by the actions of histone deacetylases (HDACs) which cause chromatin compaction so preventing transcriptional activation. Moreover, we showed that GnRH overcomes this repression by causing the removal of several of the HDACs from the LHβ and FSHβ gene promoters. We hypothesized that GnRH also promotes additional specific histone modifications at these gene loci which alter chromatin structure and facilitate gene expression. The aim of this study was to examine these effects and the mechanisms involved.

Methods: Using western analysis and chromatin immunoprecipitation (ChIP) with antisera that recognizes specific modifications on histones H2A, H3 and H4, we have assessed the effects of GnRH on global levels, as well as levels at the gonadotropin gene promoters, of H2B120 ubiquitylation (H2BK120ub), H3K4 trimethylation (H3K4me3), H3S10 phosphorylation (H3S10p) as well as the acetylation of various lysines on H3 and H4.

Results: GnRH was seen to induce global levels of H3S10 and H2BK120ub but not H3K4me3. Both histone acetylation and phosphorylation increase quite dramatically at the LHβ gene promoter following GnRH treatment. The phosphorylation of H3 likely results from GnRH-induced activation of the kinase, MSK1/2, which was seen after 10 minutes treatment. The H3K4 appears to be already trimethylated at the LHβ gene locus, although there does appear to be an increase at the FSHβ gene promoter. However at both promoters, a net loss in H3 was seen after GnRH treatment, indicating nucleosomal repositioning or histone loss.

Conclusions: H3K4me3 is commonly seen at the promoters of actively transcribed genes and thought to be crucial of transcription initiation, it recognized by various families of proteins through specific binding domains. Histone phosphorylation has been noted to be associated with H3K4me3 and histone acetylation at active genes, and several studies indicate the requirement of all three modifications for transcriptional activation to proceed. Our studies have revealed the ability of GnRH to induce several of these modifications in a global and/or gene-specific manner. We are currently working to elucidate the precise mechanisms involved, as well as the direct consequence of the modification in terms of recruitment of other regulatory or modifying complexes.

Glucose augments fatty acid-induced endoplasmic reticulum stress via activation of mTORC1 in beta-cells.

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Introduction: In type 2 diabetes, glucose amplifies fatty-acid toxicity for pancreatic beta-cells, leading to beta-cell dysfunction and death. Why glucose exacerbates beta-cell lipotoxicity is largely unknown. Palmitate is a potent inducer of endoplasmic reticulum (ER) stress in beta-cells. We hypothesized that glucose augments lipotoxicity, through amplification of ER stress. Moreover, it was recently shown that the conserved serine/threonine kinase mTORC1 is an important regulator of ER stress in tumor cells. Others and we have shown that in beta-cells, glucose is a potent inducer of mTORC1, which functions as an important nutrient sensor. Herein, we studied the role of mTORC1 in mediating the effects of glucose and palmitate on ER stress in beta-cells.

Methods: The effects of glucose and palmitate on mTORC1 signaling and ER stress markers was studied in INS-1E beta-cells by Western blot and real-time PCR. Proinsulin and total protein biosynthesis were studied in beta-cells that were incubated at 3.3 mmol/l and 16.7 mmol/l glucose with and without palmitate and/or the mTORC1 inhibitor rapamycin. Proinsulin biosynthesis was analyzed by measuring L-[2, 3, 4, 5-3H]leucine incorporation followed by immunoprecipitation with anti-insulin serum. Total protein biosynthesis was determined by trichloroacetic acid precipitation. Apoptosis was assessed by Western blot for cleaved caspase 3 and quantified using the Cell Death ELISAPLUS assay (Roche Diagnostics, Manheim Germany).

Results: We found that glucose amplifies palmitate-induced ER stress, which was accompanied by a robust and rapid activation of the JNK pathway leading to increased beta-cell apoptosis. Moreover, glucose increased mTORC1 activity, and its inhibition by rapamycin decreased palmitate-induced ER stress and beta-cell apoptosis and prevented the amplification of palmitate toxicity by glucose. Activation of mTORC1 augments protein synthesis, which may increase the ER client protein load, leading to exacerbation of ER stress. However, palmitate completely abolished the glucose stimulation of proinsulin and total protein biosynthesis and rapamycin did not further decrease protein synthesis in palmitate-treated INS-1E cells. This indicates that rapamycin inhibition of the ER stress response to glucolipotoxicity is not mediated through attenuation of global protein or proinsulin biosynthesis. Preliminary results show that rapamycin decreased the expression of IRE1alfa, thereby reducing the phosphorylation of JNK. Moreover, studies in TSC2 deficient mouse embryonic fibroblasts, in which mTORC1 is constitutively active, show that mTORC1 regulate the stimulation of JNK by ER stressors, but not in response to anisomycin, which activates JNK independent of ER stress. Finally, we found that JNK inhibition decreased beta-cell apoptosis under conditions of glucolipotoxicity.

Conclusions: Our findings suggest that stimulation of mTORC1 constitutes the molecular basis of hyperglycemia-amplified lipotoxicity, acting through activation of ER stress and JNK. Thus, mTORC1 is an important mediator of beta-cell glucolipotoxicity in type 2 diabetes. Rapamycin alleviates the ER stress response to glucose and palmitate by inhibiting the IREalfa-JNK pathway. This may have important implications for understanding the pathophysiology of beta-cell dysfunction and the treatment of type 2 diabetes.

Aldosterone's action in vascular smooth muscle cells in madiated, in part, through activation of angiotensin in ll receptor type 1 (AT1R)

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Introduction: Aldosterone excess is associated with coronary inflammation and fibrosis, endothelial dysfunction, increased large artery rigidity and overall cardiovascular mortality. In the present study we examined potential interactions between mineralocorticoid receptors (MR) and angiotensin II type 1 receptor (AT1R) in vascular smooth muscle cells (VSMC).

Methods: We assessed four different genes as potential vascular targets for Aldo's action: 12-lipoxygenase (12LO), 15LO type 2, AT1R and LOX- 1 (Lectine -Like- Oxidized LDL receptor 1, a scavenger receptor for oxidized-LDL).

Results: First, aldosterone (1nmol/l) (24 hours) increased the expression of 12LO mRNA by 70 %, which was blocked by co-treatment with 100 fold excess of losartan, a specific inhibitor of AT1R. Losartan also inhibited the 3 fold aldosterone –induced rise in 12HETE, a product of both LO isoforms. Second, aldosterone induced a 2 fold increase in 15LO type II mRNA expression as well as in the generation of 15HETE (a product of 15LO action, 100% increase), both of which were also blocked by the AT1R inhibitor losartan. Third, aldosterone increased the expression of LOX-1 (~2 fold increase, $P=0.03$) and stimulated LDL oxidation as measured by TBARS method. Both effects were significantly blocked by losartan. Fourth, aldosterone increased AT1R mRNA expression by 2.5 fold ($P<0.001$), an effect which was likewise inhibited by losartan. While both angiotensin II and aldosterone each elicited a rapid rise in VSMC ERK1/2 phosphorylation, (~ 2 fold, $p<0.001$), the stimulatory effect of aldosterone was blocked not only by the MR antagonist spironolactone, but also by the AT1R antagonist losartan. Finally, aldosterone (1nmol/l) induced a rapid increase (5 min) in AT1R phosphorylation, as detected by immunoprecipitation with phosphotyrosine antibody (exposure to AT1R antibody, 60% increase, $p<0.02$). Significantly, however, aldosterone-induced phosphorylation of AT1R was inhibited by either the MR receptor antagonist, spironolactone (100 nmol/l) or the AT1R antagonist losartan (100nmol/l).

Conclusions: Thus, aldosterone increases the expression of four different genes in VSMC, the well known ERK1/2 phosphorylation and the novel AT1R tyrosine phosphorylation and each of these effects can be inhibited by angiotensin receptor blockade. These results suggest that aldosterone's effects in VSMC are mediated in part via transactivation of angiotensin II receptors, but the precise mechanism/s involved require further investigation.

Bioinformatics and experimental approaches to analyze Prokineticin Receptors signaling

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Introduction: Prokineticin (PK) 1 and 2 are two novel secreted proteins with diverse biological functions. PKs are the cognate ligands for two homologous G protein-coupled receptors termed PKR1 and PKR2. We have previously shown that both PKR1 and PKR2 are involved in luteal endothelial cell (EC) proliferation and survival. In addition, PKR2 mediates selective microvascular responses such as increased permeability and ability to withstand stress. This is supported by recent reports demonstrating that PKR1 signaling enhances proliferation of myocardial EC and tube-like formation, while PKR2 induces fenestrations of EC and vascular leakage. The diversity in signaling between PKR1 and PKR2, and the molecular mechanisms involved are still unknown. We hypothesized that coupling to G-proteins as well as distinct phosphorylation sites may be responsible for these differences.

Methods: Prediction of G protein coupling specificity was performed using the PRED-COUPLE web server and by multiple sequence alignments of PKRs to known functional residues in Rhodopsin. Prediction of potential phosphorylation sites in PKR1 and PKR2 was conducted using the GPS, PPSP, NetPhos and NetPhosK web servers. Only sites predicted to be phosphorylated by all methods were considered as putative phosphorylation sites. EC expressing PKRs were utilized to study the effects of PKs on adenylyl cyclase (AC), MAPK cascade activation and gene expression (Cyclo-oxyganase 2, COX-2 and endothelial nitric oxide synthase, eNOS).

Results: Sequence analysis of PKRs in various species and consensus phosphorylation sites revealed that PKR1 and PKR2 differ in putative phosphorylation sites, mainly in the first and second intracellular loops and in the cytoplasmic tail. PKRs are predicted to couple to Gαs, Gαi and Gαq. Treatment of luteal ECs with PKs activated p44/p42 MAPK cascade and elevated COX2 and eNOS mRNA levels. Similar effects were observed in cells treated with TPA, a protein kinase C (PKC) activator. MAPK, COX2 and eNOS are downstream targets of PKC, triggered by receptor coupling to Gαq. Treatment of luteal ECs with PKs alone elevated basal cAMP levels, however, when stimulated with the β2-adrenergic agonist, Isoproterenol, PKs decreased cAMP production. Thus, at basal conditions PKRs may couple to Gαs and activate AC, but with massive Gαs recruitment such as occurs by β2-adrenergic stimulation, PKs may signal via Gαi, as predicted.

Conclusions: Experimental data and bioinformatics predictions show that PK may signal via different G proteins. The question whether different G protein coupling determines the distinct biological functions of PKR1 vs. PKR2 and the role of phosphorylation are currently under study.

The role of Urocortin peptides in regulating the central stress response: Evidence from a novel Urocortin triple knockout mouse model

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Introduction: The CRF-peptide family includes corticotropin releasing factor (CRF) and Urocortins (Ucn) 1, 2 and 3. These peptides integrate the neuroendocrine, autonomic and behavioral responses to stressors through selective activation of two receptors, CRFR1 and CRFR2, both widely expressed in stress-related nuclei in the brain. CRF activates CRFR1, Ucn1 activates both CRFR1 and CRFR2, whereas Ucns 2 and 3 activate CRFR2. Accumulating evidence suggest opposing roles in regulating the central stress response for these two CRF receptors systems, the CRF-R1 system was found critical for initiating stress responses while the CRF-R2 system appears principal for reestablishing homeostasis.

Methods: This study evaluated the role of Ucn 1, 2 and 3 in regulating the central stress response by utilizing a novel knockout mice model we generated, lacking all three known Ucns (tripleUcnKO). We compared anxiety indices of tripleUcnKO mice with those of wild-type mice (WT), obtained from the same colony, under basal conditions and both immediately and 24 hours following exposure to an acute stressor.

Results: Under basal conditions and immediately following exposure to acute stress, tripleUcnKO mice did not differ from WT mice in most anxiety indices of the Open- Field and Dark- Light transfer tests. However, 24 hrs following the exposure to stress tripleUcnKO mice exhibited increased levels of anxiety compared to WT mice. Furthermore, tripleUcnKO mice continued to appear anxious even 10 days following the stress exposure as indicated by increased freezing in the fear conditioning context test. Furthermore, tripleUcnKO mice exhibited an altered corticosterone profile in response to restraint stress.

Conclusions: Collectively, the results suggest an important role for endogenous CRFR2 ligands in mediating the coping mechanisms following stressful experience and support the tripleUcnKO mouse line as a useful stress sensitive mouse model. Further elucidating the role of central Ucns in mediating the central stress response may provide new insights for developing therapeutic tools for stress- related psychopathologies.