Sirt1 is a regulator of osteoblastogenesis and bone mass

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Introduction: The Silent Information Regulator (SIR) family of genes represents a highly conserved group of genes present in the genome of lower species up to mammalians. The encoded SIR proteins are involved in regulation of gene silencing, DNA repair and are key mediators of the beneficial effects of calorie restriction on lifespan. A well-characterized gene in this family is S. cerevisiae Sir2. To date seven mammalian homologues have been identified, where SIRT1 is the closest evolutionary to Sir2. SIRT1 has a NAD-dependent histone deacetylase activity and plays important roles in aging and in age-associated diseases. SIRT1 regulates epigenetic silencing and chromatin modification, partially by direct regulation of modifying enzymes. The sirtuins were connected to several age-related diseases such as neurodegenerative, vascular and metabolic diseases. Their role in osteoporosis hasn't been studied yet.

Patients/ Methods: To investigate the role of SIRT1 in bone we sought to characterize the bone phenotype in adult 3- month-old female haplo-insufficient mice (Sirt1+/-), and compared them to their Sirt1+/+ (WT) littermates. Complete Sirt1 KO is lethal or results in severe post natal malformations. Murine embryonic mesenchymal C3H10T1/2 cells over-expressing Sirt1 were compared to WT C3H10T1/2.We used microCT and histomorphometric analyses to study bone mass, architecture, formation and resorption. Marrow derived mesenchymal stem cells (MSCs) were used to evaluate osteoblastogenesis by ALP activity and mineralizd nodule formation. Osteoclastogenesis was evaluated in primary bone marrow cultures by TRAP staining. Serum 25-OHvitaminD3, E2, IGF-1 PINP and RANKL were determined.

Results: SIRT1 haplo-insufficient mice had a significant reduction in bone mass. There was a 30% decrease in trabecular BV/TV, as a result of a 23% reduction in trabecular number, no significant change in trabecular thickness and a 37% decrease in Conn.D. Decreased osteoblastogenesis was found in Sirt1+/- mice as indicated by reduced calcein labeling, decreased bone formation rate, a 50% reduction in alkaline phosphatase activity and a significantly reduced number of mineralized nodules. A reciprocal result was observed in C3H10T1/2 cells over-expressing Sirt1 in which there was a 50% increase in alkaline phosphatase activity compared to C3H10T1/2 cells. Osteoclast number was lower in Sirt1+/- mice and osteoclast generation upon exposure to RANKL and M-CSF was reduced in Sirt1+/- mice, resulting in smaller osteoclasts with fewer nuclei. Serum markers showed no difference between Sirt1+/- and WT mice.

Conclusions: Our results implicate a major role for SIRT1 in regulating osteoblastogenesis. SIRT1 activators may have a favorable effect inducing osteoblast formation and reducing bone loss for the treatment of osteoporosis.

Truncated beta epithelial sodium channel (ENaC) subunits responsible for multi-system pseudohypoaldosteronism support partial activity of ENaC

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Introduction: The major site of action of aldosterone in electrolyte regulation is epithelial cells where aldosterone induces expression of epithelial sodium channel (ENaC) subunits increasing ENaC activity at the apical cell surface. ENaC is constructed of three homologous subunits. The amino and carboxy terminal domains of the subunits are located in the cytoplasm, while the bulk of their structure is exposed outside of the cell, forming a funnel that directs ions from the lumen into the epithelial cell. Mutations in the alpha, beta and gamma ENaC genes (SCNN1A, SCNN1B and SCNN1G) are associated with multi-system pseudohypoaldosteronism (PHA), and mutations in the PY motif of beta and gamma subunits are associated with Liddle syndrome of hereditary hypertension.

Patients/ Methods: In this study we sequenced segments of the genomic DNA of a female infant diagnosed with multi-system PHA to identify the mutations responsible for her condition. She is the first case of PHA in an Ashkenazi family in Israel. After identifying two mutations in the SCNN1B alleles we generated mutant forms of beta-ENaC cDNA using a site-directed mutagenesis method we recently developed. The cDNAs were transcribed in vitro using T7-RNA polymerase to generate complementary RNAs (cRNAs) for expression studies in Xenopus oocytes. Amiloride-sensitive whole-cell inward Na+ current was measured 2-3 days after cRNA injection using the two-electrode voltage-clamp method while oocytes were clamped at –80 mV.

Results: We identified two frameshift mutations in the SCNN1B alleles of the patient. The p.Glu217fs (c.648dupA in exon 4) and p.Tyr306fs (c.915delC in exon 6) mutations produce shortened beta-ENaC subunits with 253 and 317 residues respectively instead of the 640 residues present in beta-ENaC subunit. Expression of normal alpha and gamma cRNAs together with mutant beta cRNA in Xenopus oocytes showed that the mutations drastically reduce but do not eliminate ENaC activity (3% of normal ENaC). Oocytes injected with both mutant cRNAs showed > 2 fold higher ENaC activity, indicating a synergism between mutant forms. Oocytes injected with alpha and gamma cRNAs without the beta cRNA showed no detectable ENaC activity.

Conclusions: The findings reveal that truncated beta-ENaC subunits are capable of partially supporting intracellular transport of the other two subunits to the membrane and the final assembly of a weakly active channel together with normal alpha- and gamma-ENaC subunits on the oocyte cell surface. Moreover, these results enhance our understanding of the long-term consequences of these types of mutations in PHA patients. The present findings should also be useful for prenatal diagnosis and early
treatment of multi-system PHA.

Expression of microRNA 21 in mammary stem cells is controlled by cytokine-STAT5 signaling

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Introduction: In the developing mammary gland, the transcription factors STAT5A/B initiate cascades of events that range from the specification of alveolar progenitors to the establishment of functional alveoli during pregnancy. Although many STAT5 target genes have been identified, the complexity of STAT5s function is still an enigma.

Results: MicroRNAs have emerged as another means of controlling the physiology of cells as each member can regulate multiple mRNAs. However, the transcriptional regulation of microRNA genes is poorly understood. Here we use ChIP-seq and expression analyses to define those microRNAs that are controlled by cytokines through STAT5A/B. ChIP-seq established cytokine-induced STAT5A binding to the miR21 gene promoter and expression analyses confirmed loss of miR21 expression in cells lacking STAT5. Notably, we determined that miR21 expression in mammary stem cells and alveolar progenitors was dependent on the presence of STAT5A.

Conclusions: Current experiments address the role of miR21 in mammary alveologenesis.

Calcitriol stabilizes cyclooxygenase-2 mRNA in epidermal keratinocytes

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Introduction: The biosynthesis of prostaglandins from arachidonic acid is mediated by the rate-limiting enzyme, cyclooxygenase (COX). Two isoforms of COX are known. COX-1 is expressed in many tissues under basal conditions, while COX-2 can be induced by various agents such as growth factors, cytokines, pro-inflammatory agents, and tumor promoters. We have previously shown that treatment with the hormonal derivative of vitamin D, calcitriol, increases PGE2 production by human epidermal keratinocytes and that this increase is due to up-regulation of COX-2 expression. The aim of this study was to explore the mechanism responsible for this effect of the hormone.

Patients/ Methods: The non-tumorigenic immortal HaCaT keratinocytes were employed as an experimental model. Cultures in the absence of serum, growth factors or active mediators were exposed to calcitriol for 2 and 16 hours. mRNA levels were quantified by real time PCR and protein levels by western blot analysis. The rate of mRNA degradation was followed after exposing the cells to the transcription inhibitor Actinomycin D for 30, 60, 90 and 120 minutes.

Results: Exposure of HaCaT cells to calcitriol for both 2 and 16 hours, brought about a similar marked and consistent increase of almost 4 fold in COX-2 mRNA levels. The maximal effect was attained already at 1nM of the hormone. While the half-time of the COX-2 transcript was around 20 minutes in control cultures there was not detectable decay of mRNA levels for 1 hour in calcitriol-treated cultures. The stabilizing effect of calcitriol was not associated with increased levels or changes of cellular localization of the mRNA stabilizing protein, HuR. Using specific pharmacological inhibitors to signaling pathways known to be affected by calcitriol in keratinocyte we found that activity of Src kinase family member(s) and PKC are obligatory for the stabilizing effect of calcitriol.

Conclusions: These findings demonstrate that treatment with calcitriol results in up regulation of COX-2 mRNA in keratinocytes, and that this effect is at least partially due to mRNA stabilization. This increase in COX-2 could provide an explanation to the dual effect of calcitriol on epidermal inflammation: pro-inflammatory in healthy skin and anti-inflammatory in inflamed skin and may contribute to the known anti-apoptotic effect of calcitriol on epidermal keratinocytes.

Differential role of PKC isoforms in GnRH and PMA activation of extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK)

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Introduction: Gonadotropin releasing hormone (GnRH) is the first key hormone of reproduction. Signaling of GnRH in pituitary gonadotropes include sequential activation of phospholipase Cβ (PLCβ), PLD and PLA2, enhanced phosphoinositide turnover, Ca2+ mobilization and influx, activation of PKC and the MAPK cascades (ERK, JNK and p38) and formation of prostaglandins and leukotrienes, culminating in gonadotropin (LH and FSH) synthesis and release. Protein kinase C (PKC) is a serine/threonine lipid-activated kinase family. The many and sometimes opposing functions elicited by the PKC family suggest that different isoforms may mediate its diverse functions. Although PKC is implicated in MAPK activation by some GPCRs in general and by GnRH in particular, the nature of the PKCs mediating GnRH activation of the MAPKs is still unknown. Here we demonstrate for the first time the relative contribution of specific PKCs to ERK and JNK activation by GnRH and PMA in αT3-1 and LβT2 cells.

Results: The role of PKC isoforms (PKCs) in GnRH-stimulated MAPK (ERK and JNK) was examined in the αT3-1 and LβT2 gonadotrope cells. Incubation of the cells with GnRH resulted in a protracted activation of ERK1/2 and a slower and more transient activation of JNK1/2. Gonadotropes express conventional PKCs (cPKC) α and βII, novel PKCs (nPKC) δ, ε and θ, and atypical PKC (aPKC) ι/λ. The use of GFP-PKCs constructs and their translocation to membranes as a measure of activation, revealed that GnRH induced a rapid translocation of PKCα and PKCβII to the plasma membrane, followed by their re-distribution to the cytosol. PKCδ and PKCε localize to the cytoplasm and Golgi followed by rapid re-distribution by GnRH of PKCδ to the perinuclear zone, and PKCε to the plasma membrane. Interestingly, PKCα, PKCβII and PKCε translocation to the plasma membrane was more pronounced and more prolonged in PMA than in GnRH-treated cells. The use of selective inhibitors and dominant negative plasmids for the various PKCs has revealed that PKCβII, PKCδ and PKCε mediate ERK2 activation by GnRH, while, PKCα, PKCβII, PKCδ and PKCε mediate ERK2 activation by PMA. Also, PKCα, PKCβII, PKCδ and PKCε are involved in GnRH- and PMA-stimulation of JNK-1 in a cell-context dependent manner.

Conclusions: Thus, the contribution of selective PKCs to ERK and JNK activation is ligand- and cell-context dependent. We present preliminary evidence that persistent vs. transient redistribution of selected PKCs, or re-distribution of a given PKC to the perinuclear zone vs. the plasma membrane may dictate it's selective role in ERK, or JNK activation.

PPARα regulates systemic blood pressure by modulating the central Renin-Angiotensin sytem (RAS) predominantly through renin expression

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Introduction: We have previously shown that the absence of PPARα abolishes hypertension in the Tsukuba Hypertensive Mouse (THM), an animal doubly transgenic for human renin and angiotensinogen. Most of the protection appeared to stem from downregulation of the humoral renin-angiotensin system (RAS) as a result of a profound suppression of the human renin gene expression. However, in C57/Bl6 mice subjected to the aldo-salt model, the absence of PPARα seemed to affect thirst and salt appetite. As under this paradigm of mineralocorticoid-hypertension, the peripheral RAS is shut down yet the central RAS is typically activated, this suggested a potential role for PPARα a in the regulation of the central RAS. The goal of the present study was to specifically assess the contribution of PPARα to the expression of the central RAS system under various conditions of peripheral RAS activation.

Patients/ Methods: 21 C57/Bl6 mice, and 23 PPARalpha null (PPARKO) mice previously subjected to unilateral nephrectomy, underwent the aldo-salt protocol for 4 weeks (continuous SC infusion of a pressor dose of aldosterone via an osmotic minipump, and access to 1% NaCl drinking water), at the end of which they were studied in metabolic cages for 24 h. Similarly, 16 adult THM mice, and 20 THM lacking PPARalpha (THMKO) that had ad lib access to tap water were studied in metabolic cages. Blood pressure was recorded noninvasively. At the end of the experiments, animals were sacrificed, RNA was extracted from the hypothalami, and RAS component expression was assessed by real-time PCR. Immunohistochemistry of the RAS was performed on THM/THMKO brains.

Results: In both models absence of PPARα protected the animals from hypertension, and significantly affected drinking pattern. In the aldo-salt model, PPARKO mice drank significantly less salt water and ate less than the C57/Bl6 control mice (Table 1). In the THM model, absence of PPARα reduced drinking by 43% ($P=0.0001$), and urine output by 66% ($P<0.0001$). In both models, hypothalamic mouse renin expression was significantly lower in the absence of PPARα. In THMKO animals, human renin expression was 2 orders of magnitude lower than in THM. Additionally, human angiotensinogen was also significantly reduced. In contrast, in neither model did the absence of PPARα affect the level of the angiotensin II type 1 receptor or that of the angiotensin converting enzyme mRNA. Immunohistochemistry studies of THM/THMKO mice generally concurred with the mRNA results.

Conclusions: These studies highlight the role of PPARα in the expression of the central RAS, both when the peripheral RAS is overexpressed (THM model), or shut down (aldo-salt model), primarily by its effect on the expression of renin, the rate-limiting step of the system. The results underscore the importance of the central RAS in the generation and maintenance of systemic blood pressure, at least in part through its impact on drinking behavior and salt appetite.