

Estradiol- 17 β , phytoestrogens and selective estrogen receptor modulators (SERM) induce reactive oxygen species production in human vascular smooth muscle and endothelial cells

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Introduction: Estrogens appear to confer multiple cardiovascular protective effects in vitro and in vivo in the experimental setting, but have thus far failed to positively affect cardiovascular outcome in prospective trials in post-menopausal women. In search for potential adverse vascular effects of estrogens we now attempt to determine whether or not estrogens affect ROS production in vascular cells.

Patients/ Methods: Vascular cells were grown as described before and the nitroblue tetrazolium (NBT) colorimetric method was used to determine ROS production in vascular cells.

Results: First, using the nitroblue tetrazolium (NBT) colorimetric method, estradiol-17 β (E2) induced 89 \pm 10% increase in ROS in cultured human vascular smooth muscle cells (VSMC). Second, ROS was also induced by 1) Phytoestrogens, including biochanin A (+92 \pm 13%) and carboxy BA (cBA, +76 \pm 25%), 2) Estrogen Receptor (ER) – specific agonists, including the ER α specific agonist PPT (+62 \pm 6%) and the ER β specific agonist DPN (+45 \pm 11%), 3) Two lipoxygenase products 12- and 15-hydroxyeicosatetraenoic acid (HETE), whose production is increased by estrogens, (+52 \pm 5 and +85 \pm 15% respectively). Third, several structurally unrelated inhibitors of ROS production such as N-acetyl cystein, DPI and curcumin inhibited both basal and estrogenic compounds-induced ROS formation. The mixed ER-agonist/ ER α selective antagonist raloxifene, which by itself stimulated ROS by +200 \pm 12%, reduced ROS production induced by E2, PPT, BA and cBA but not by DPN or 12 and 15 HETE. The general ER antagonist ICI 181840, which did not, by itself, induce ROS production, inhibited ROS induction by E2 and cBA. Fourth, baicalein, which inhibits the formation of 12 and 15 HETE through blockade of 12 and / or 15 lipoxygenase, inhibited ROS formation by all the compounds except 12 and 15 HETE. Fifth, in the endothelial cell line ECV (ECV340) the estrogenic agents E2, DPN, PPT and cBA all induced ROS production, which was inhibited by DPI.

Conclusions: These results indicate that estrogens may increase oxidative stress in the vasculature, in part via induction of lipoxygenase enzymes. This previously unrecognized feature of estrogenic compounds appears to be shared by multiple members of the estrogens family and is not exclusively linked to either ER α or ER β . The most serious potential hazard of these effects could be unwanted induction of inflammatory vascular reaction.

Effect of Testosterone Replacement Therapy on Artery Stiffness in older hypogonadal men

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Introduction: To assess arterial stiffness in a cohort of hypogonadal male and to investigate the effect of testosterone replacement therapy on arterial properties in this specific group.

Patients/ Methods: Eighteen male patients with untreated acquired hypogonadism due to either adult-onset idiopathic hypogonadotropic hypogonadism (n = 9) or pituitary tumor (n = 9) and twelve age-, sex, and weight-matched eugonadal healthy controls were recruited for the study. Arterial properties, plasma glucose, lipid profile, total and bioavailable testosterone levels were measured in fasting state. In the hypogonadal subjects, the effect of transdermal testosterone replacement therapy on arterial properties was studied by repeat non-invasive measurements at baseline, as well as 48 h and 90 days following the initiation of treatment. Arterial stiffness was evaluated using applanation tonometry and pulse wave analysis by three different standard devices which assess various measures of arterial stiffness: pulse wave velocity (PWV), augmentation index (AIx), and large / small artery compliance (C1 and C2).

Results: Age- and blood pressure-adjusted PWV was significantly higher in hypogonadal men (8.90 ± 2.29 vs. 6.78 ± 1.16 m/s in the control group, $p = 0.025$). Testosterone therapy increased bioavailable testosterone level from 0.58 ± 0.31 to 1.35 ± 0.74 and 2.26 ± 1.79 ng/dl after 48h and 3 months, respectively ($p = 0.001$). PWV decreased from 8.9 ± 0.31 m/s at baseline to 8.24 ± 0.3 m/s 48 hours after initiation of treatment ($p = 0.03$), a decline which persisted after 3 months of treatment (8.25 ± 0.27 m/s, $p = 0.03$). C1 significantly increased after 3 months of treatment from 22.5 ± 1.22 ml/mmHg \times 10 at the baseline to 28.8 ± 1.46 ml/mmHg \times 10 ($p = 0.03$).

Conclusions: Male hypogonadism is associated with increased PWV, which is rapidly but incompletely ameliorated by normalization of circulating testosterone levels.

Insulin counteracts glucotoxic effects by suppressing thioredoxin-interacting protein expression in pancreatic beta-cells.

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Introduction: In type 2 diabetes (T2DM) glucose toxicity leads to beta-cell apoptosis with decreased beta-cell mass as a consequence. Thioredoxin-interacting protein (TXNIP) is a critical mediator of glucose-induced beta-cell apoptosis. Since hyperglycaemia leads to elevated serum insulin, we hypothesized that insulin is involved in the regulation of TXNIP expression in beta-cells.

Patients/ Methods: We studied the expression of TXNIP in INS-1E beta-cells and in islets of *Psammomys obesus* (*P. obesus*), an animal model of T2DM, in response to glucose and different modulators of insulin secretion and its effects on beta-cell apoptosis under conditions of glucotoxicity. TXNIP expression was analyzed by quantitative real-time PCR and Western blot. Apoptosis was analyzed by Western blot for cleaved caspase 3 and using the cell death ELISA PLUS assay (Roche Diagnostics, Mannheim, Germany). *P. obesus* and rat (INS-1E) insulin immunoreactivity was determined using commercial human and rat RIA kits (Linco Research, St. Charles, MI, USA).

Results: TXNIP expression was markedly augmented in islets from diabetic *P. obesus* and in beta-cells exposed to high glucose. In contrast, adding insulin to the culture medium or stimulating insulin secretion with different secretagogues suppressed TXNIP. Moreover, inhibition of glucose and fatty acid-stimulated insulin secretion with diazoxide increased TXNIP expression in beta-cells. Nitric oxide (NO), a repressor of TXNIP, enhanced insulin signal transduction, whereas inhibition of NO synthase abolished its activation, suggesting that TXNIP inhibition by NO is mediated via stimulation of insulin signaling. Treatment of beta-cells chronically exposed to high glucose with insulin reduced beta-cell apoptosis. Finally, we found that RNAi knock-down of TXNIP mimicking the effect of insulin prevented glucose-induced beta-cell apoptosis.

Conclusions: Insulin is a potent repressor of TXNIP, operating a negative feedback loop that restrains the stimulation of TXNIP by chronic hyperglycaemia. Repression of TXNIP by insulin is probably an important compensatory mechanism protecting the beta-cells from oxidative damage and apoptosis in T2DM.

Conditioned medium from TNF α -pre-treated adipocytes induces insulin resistance in hepatic cells via increased IL-1 β secretion

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Introduction: Liver insulin resistance is a hallmark of diabetes and obesity. It is tightly associated with accumulation of visceral adipose tissue that was shown to exhibit chronic inflammation in obesity. The aim of this study was to identify secreted factors from adipocytes in response to an inflammatory stimulus that can impair insulin signaling in hepatocytes.

Patients: for 18 hours in the absence and α Methods: 3T3-L1 adipocytes were exposed to TNF presence of Rosiglitazone or YVAD (caspase I inhibitor II). Conditioned medium (CM) was collected during the subsequent 24h, and IL-1 levels were analyzed by ELISA. FAO hepatoma cells, treated with or without IL-1RA were exposed to control-CM or to CM from TNF-treated adipocytes (TNF-CM), after which insulin signaling and glycogen synthesis were assessed.

Results: Incubation of FAO cells with control CM had no effect on insulin signaling. However, insulin-stimulated IR ($p < 0.05$) and IRS-1 tyrosine phosphorylation ($p < 0.05$), PKB ($p < 0.01$) and GSK3 serine phosphorylation ($p = 0.01$) were diminished following exposure of FAO cells to TNF-CM, compared to control-CM treated cells. Moreover insulin-stimulated glycogen synthesis was also decreased. IL-1 β levels in TNF-CM were increased 10-fold compared to control-CM, but significantly decreased when CM medium was collected from adipocytes pre-treated with TNF in the presence of rosiglitazone or YVAD. Supporting a role for IL-1 β in mediating dys-regulated adipocyte-hepatocyte crosstalk, hepatocyte insulin resistance was partly prevented ($p < 0.05$) at both the signaling and metabolic levels by both rosiglitazone and YVAD. Finally, the role of secreted IL-1 in the induction of hepatocyte insulin resistance was verified by demonstrating that IL-1 receptor antagonist (IL1-Ra) partly prevented hepatocyte insulin resistance induced by TNF-CM ($p < 0.05$). Furthermore, utilizing YVAD on adipocytes and/or hepatocytes confirmed that the major source of IL-1 β in this system is the adipocytes.

Conclusions: We conclude that IL-1 β release from adipocytes pre-exposed to an inflammatory stimulus participates in mediating impaired insulin action in hepatocytes.

The lipid peroxidation product 4-hydroxynonenal uncouples insulin-stimulated Akt phosphorylation from its activation, resulting in adipocyte insulin resistance

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Introduction: Although it is accepted that diabetes and obesity are associated with increased oxidative stress, and oxidative stress as an isolated factor can cause insulin resistance, the exact oxidative mediators and molecular mechanisms are poorly understood. 4-hydroxynonenal (HNE) is a major peroxidation end-product of unsaturated fatty acids, which has been shown to be elevated in patients with diabetes and obesity. Moreover, its conjugation to proteins was suggested to constitute the major carbonyl modification in adipose tissue in these conditions. The aim of the present study was to elucidate the signaling defect induced in adipocytes by exposure HNE.

Methods: Differentiated 3T3-L1 adipocytes were exposed to 0-100microM HNE for 4h, rinsed, and insulin signaling and action were studied after stimulation with 100 nM insulin. GLUT4 translocation and fusion was studied by introducing by electroporation a GFP-GLUT4myc plasmid, and assessing externalization of the myc epitope in un-permeabilized cells using anti-myc Ab.

Results: As previously reported, HNE impaired insulin actions including the stimulation of glucose uptake in 3T3-L1 adipocytes in a time and dose dependent manner. This was mediated by impaired capacity of the hormone to stimulate GLUT4 translocation and fusion with the plasma membrane. Intriguingly, insulin-stimulated phosphorylation of Akt on both Ser473 and Thr308 residues was increased 3-4 fold following HNE pre-treatment, despite a gradual decrease in total Akt content. HNE notably increased Akt phosphorylation also in the absence of insulin. To determine whether insulin resistance induced by HNE occurred downstream of normal or even exaggerated Akt activation by the hormone, phosphorylation of Akt substrates was assessed. Although phosphorylation of GSK3 on residues 9/21 mirrored Akt phosphorylation at shorter incubation times and at lower doses of HNE, it suggested a discrepancy between Akt phosphorylation and activation at higher doses. We next utilized the anti-phospho-Akt substrate (PAS) antibody generated against the phosphorylated Akt consensus sequence on target proteins. The notable increase in basal Akt phosphorylation was associated with a mild elevation in phosphorylation of various Akt substrates. Yet, despite the robust elevation in insulin-stimulated Akt phosphorylation induced by HNE, Akt-mediated phosphorylation of its various target proteins, including AS160, was markedly diminished.

Conclusions: These results suggest that the lipid peroxidation 4-HNE can cause adipocyte insulin resistance by uncoupling Akt phosphorylation from the enzyme's activity in response to insulin stimulation.

Pegylated leptin antagonist is a potent orexigenic agent – preparation and mechanism of action.

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Introduction: Leptin, a pleotropic adipokine, is a central regulator of appetite and weight and a key immunomodulatory protein. While leptin deficiency and associated weight gain can be achieved by ablation of the leptin (ob) or leptin-receptor (db), to our knowledge it has not been successfully induced in adult animals as so far no efficient method for leptin antagonism in the adult setting has been previously developed.

Patients/ Methods: Increasing the molecular size of our recently-constructed mouse leptin antagonist (MLA) by pegylation, was achieved using various polyethylene (PEG) reagents, attached covalently to leptin antagonist by alpha-amino group.

Results: The in vivo half-life of mono-pegylated (with 20 kDa PEG) leptin antagonist was increased respectively by 13- fold to 22.7 hours. Despite lower in vitro activity the activity in vivo was dramatically higher. Administration of the pegylated mouse leptin antagonist (20 kDa PEG-MLA) for 8-10 days to mice produced a rapid, significant and reversible weight gain of up to 30%, due to increased food consumption. The weight gain was secondary to fat accumulation that was confined mainly to the mesenteric region without fat accumulation in the liver. The mechanism of severe central leptin deficiency resulted mainly from inhibition of leptin transport across the blood-brain barrier as evidenced by follow-up after the distribution of PEG-MLA conjugated to Alexa Fluor® 680 in the body, which was continuously tracked using the IVIS in-vivo imaging system.

Conclusions: Our results introduce a novel tool inducing reversible leptin deficiency. It enables the study of leptin's involvement in metabolic and immune processes, blocking undesired leptin activity affecting the immune system and may serve as a potent and reversible modality inducing weight gain in states of cachexia.

Novel Adiponectin receptor 1 isoform – a potential new player in liver and brain metabolism regulation

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Introduction: Adiponectin, an adipocyte-derived abundant plasma protein, with insulin-sensitizing, anti-inflammatory and anti-atherogenic properties, is suggested to be involved in the control of energy homeostasis through its actions in peripheral tissues and in the brain. The biological activities of adiponectin are mediated by two distinct receptors, AdipoR1 and AdipoR2. Using bioinformatics analysis we identified novel alternatively splice variants of human AdipoR1 with a distinct expression pattern in adult and fetal tissues. Whereas most of these transcripts encode the wild-type AdipoR1 (WT-R1) protein, one transcript encodes a truncated receptor isoform, Trunc-R1, possessing a distinct C-terminus sequence. The aim of the current study was to characterize this novel AdipoR1 isoform.

Patients/ Methods: Employing a combination of bioinformatics, molecular biology, and biochemical techniques, Trunc-R1 was characterized in various tissues and cell lines.

Results: We found by quantitative RT-PCR analysis that the Trunc-R1 encoding transcript, is expressed primarily in the human brain and liver. Protein dot blot and Western blot analysis with isoform specific antibodies revealed that Trunc-R1 is indeed translated into functional protein and is abundant in human brain tissues, with lower expression in the liver, while WT-R1 and WT-R2 are highly expressed in the muscle and liver, respectively. Notably, Trunc-R1 expression was significantly higher in liver and brain of obese/diabetic ob/ob mice and of mice fed a high-fat diet compared with wild-type mice. Immunohistochemistry experiments demonstrated that Trunc-R1 is highly expressed in different brain areas like pituitary and basal ganglia. Similar to WT-R1, Trunc-R1 is localized mainly at the cell membrane, in different human tissues, while in basal ganglia it is localized mainly in the cytoplasm of neuronal cells. In addition, Trunc-R1 forms hetero-multimers with WT-R2 in human liver tissues that may be important for regulating adiponectin biological activity.

Conclusions: A novel AdipoR1 isoform, Trunc-R1, was identified. Our findings indicate a putative function for Trunc-R1 in the brain. Furthermore, its altered expression in liver and brain in obesity/diabetes may point to a role of Trunc-R1 in regulating adiponectin and insulin sensitivity. The function of Trunc-R1 and its interplay with the different AdipoRs is currently under investigation.