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Crosstalk between EET and HO-1 downregulates Bach1 and adipogenic marker expression in mesenchymal stem cell derived adipocytes

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ABSTRACT

Epoxygenase activity and synthesis of epoxyeicosatrienoic acids (EETs) have emerged as important modulators of obesity and diabetes. We examined the effect of the EET-agonist 12-(3-hexylureido)dodec-8(2) enoic acid on mesenchymal stem cell (MSC) derived adipocytes proliferation and differentiation. MSCs expressed substantial levels of EETs and inhibition of soluble epoxide hydrolase (sEH) increased the level of EETs and decreased adipogenesis. EET agonist treatment increased HO-1 expression by inhibiting a negative regulator of HO-1 expression, Bach-1. EET treatment also increased βcatenin and pACC levels while decreasing PPARγ C/EBPα and fatty acid synthase levels. These changes were manifested by a decrease in the number of large inflammatory adipocytes, TNFα, IFNγ and IL-1α, but an increase in small adipocytes and in adiponectin levels. In summary, EET agonist treatment inhibits adipogenesis and decreases the levels of inflammatory cytokines suggesting the potential action of EETs as intracellular lipid signaling modulators of adipogenesis and adiponectin.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells which, under the appropriate culturing conditions, have the ability to differentiate into lineages of mesodermal tissue, that include skeletal muscle, adipocytes, bone, tendons and cartilage. MSCs are routinely isolated from several organs, including fetal liver, umbilical cord blood, and bone marrow [1–3]. Adipocytes differentiation plays a key role in the pathogenesis of diabetes. Adipogenesis begins with the commitment of MSCs to the adipocyte lineage, followed by terminal differentiation of preadipocytes to mature adipocytes. Cytokines and heme oxygenase (HO) activity have a regulatory role in mesenchymal stem cell microenvironment and hematopoiesis [4–6]. HO attenuates the overall production of reactive oxygen species (ROS) through its ability to degrade the pro-oxidant, heme, resulting in the production of carbon monoxide, biliverdin/bilirubin, and the release of free iron. These three products of heme degradation play an important role in signaling cascades, cell proliferation and differentiation [7–9]. HO exists as two isoenzymes, HO-1 (inducible) and HO-2 (constitutive) [10,11]. HO-1 is a stress response gene critical for bone marrow cell proliferation and differentiation [12]. Upregulation of HO-1 expression in obesity and type 2 diabetes results in a decrease in visceral and subcutaneous fat content, improved insulin sensitivity and increased insulin receptor phosphorylation [13–16]. MRI studies showed that up regulation of HO-1 decreased adiposity and adipocyte hypertrophy [13,15,17]. The decrease in HO-1 expression was associated with impairment in the MSCs production of adiponectin and increased adipogenesis [3,13,18]. In addition, HO-1 gene expression has a differential effect on osteoblasts and adipocyte cell proliferation and differentiation [2,3]. EETs administration decreased adiposity and insulin resistance in mice and rat models of obesity and diabetes via an increase in HO-1 gene expression and signaling cascades including the activation of AMP activated kinase (AMPK) and pAKT [19]. EETs induce HO-1 protein and HO activity [18,20,21]. Human stromal-MSCs express CYP450 monooxygenase and form EETs and 20-HETE [22]. MSCs have the ability to metabolize arachidonic acid to HETE at comparable levels to that seen in endothelial cells [23]. Additionally, the EET agonist, inhibited soluble epoxide

Abbreviations: MSCs, mesenchymal stem cells; EETs, epoxyeicosatrienoic acids; HO-1, heme oxygenase-1; PPARγ, peroxisome proliferator-activated receptor gamma; SREBP-1, sterol regulatory element-binding protein 1; FAS, fatty acid synthase.

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hydrolase (sEH), and reduced the rate of body weight gain in obese mice which was accompanied by an increase in HO-1 expression [19]. In the present study, we examined whether the EETs mediated decrease in adiposity is due to the direct effect of EETs on stem cell adipocytes via suppression of Bach-1 and subsequent increase in HO-1 expression and activity. EET also cause an increase in βcatenin and pACC and a decrease in the adipocyte differentiation markers PPARγ, C/EBPα and FAS.

2. Methods and procedures

2.1. Human bone marrow derived MSC differentiation into adipocytes

Frozen bone marrow mononuclear cells were purchased from Allcells (Emeryville, CA). After thawing, mononuclear cells were resuspended in an α-minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA). The cells were plated at a density of $1–5 \times 10^6$ cells per 100 cm^2 dish. The cultures were maintained at 37 °C in a 5% CO_2 incubator and the medium was changed after 48 h and every 3–4 days thereafter. When the MSCs were confluent, the cells were recovered by the addition of 0.25% trypsin/EDTA (Invitrogen, Carlsbad, CA). MSCs (Passage 2–3) were plated in a 75-cm^2 flask at a density of $1–2 \times 10^4$ cells and cultured in α-MEM with 10% FBS for 7 days. The medium was replaced with adipogenic medium, and the cells were cultured for an additional 21 days. The adipogenic media consisted of complete culture medium supplemented with DMEM-high glucose, 10% (v/v) FBS, 10 μg/ml insulin, 0.5 mM dexamethasone (Sigma–Aldrich, St. Louis, MO), 0.5 mM isobutylmethylxanthine (Sigma–Aldrich, St. Louis, MO) and 0.1 mM indomethacin (Sigma–Aldrich, St. Louis, MO). Media were changed every 2 days. MSC-derived adipocytes were cultured in adipogenic differentiation media and the EET-agonist (AKR-1-27-28) was added every 3 days at a dose of 1 μM.

2.2. Oil Red O staining

For Oil Red O staining, 0.21% Oil Red O in 100% isopropanol (Sigma–Aldrich, St. Louis, MO) was used. Briefly, adipocytes were fixed in 10% formaldehyde, washed in Oil-red O for 10 min, rinsed with 60% isopropanol (Sigma–Aldrich, St. Louis, MO), and the Oil red O eluted by adding 100% isopropanol for 10 min and OD measured at 490 nm.

2.3. Cytokines Measurement

Adiponectin (high molecular weight, HMW) and the inflammatory cytokines TNF-α, INF-γ, IL-1α, IL-8 were determined in the culture supernatant. Multiple assays were conducted for quantification of the proteins (AssayGate, Inc., Ijamsville, MD). All measurements were performed in triplicate and normalized by cell numbers as previously described [17].

2.4. SiRNA knockdown of sEH gene expression

Adipocyte stem cells were treated with predesigned siRNAs of the gene in adipocyte culture media using a N-TER kit (Sigma-Aldrich, St. Louis, MO) according to manufacture's protocol.

2.5. Western blot analysis

Cells were harvested using a cell lysis buffer as previously described [13,15,17]. The lysate was used to measure the protein levels of HO-1, CYP2J2, HO-2, PPARγ, FAS, pACC, βcatenin and C/EBPα. CYP2C23 antibodies has react with human CYP2C (antibodies were a gift from Dr. Jorge H. Capdevila, Nashville, TN). The phosphorylation of Acetyl-CoA carboxylase 1 (ACC1) was analyzed by immunoblotting with antibodies against phospho Ser79 ACC1 (Santa Cruz Biotechnology). Total ACC and β-actin were used as loading controls. Phosphorylation levels were quantified by scanning densitometry using an imaging densitometer normalized to the levels of total protein. The relative phosphorylation in each signaling molecule was calculated relative to the basal and/or control levels. Fatty acid synthase (FAS) antibodies, Wnt/βcatenin, PPARγ and C/EBPα were normalized to loading β-actin and presented as the ratio to the control. FAS, Wnt/βcatenin, PPARγ and C/EBPα (Santa Cruz Biotechnology), Bach 1, HO-1 and HO-2 were measured by immunoblotting with the corresponding antibodies and their levels were normalized to loading β-actin and the results are presented as relative to the basal or to the control levels as previously described [13,17].

2.6. Measurement of EETs and DHETs

MSCs were homogenized in 66% methanol containing a 500-pg mixture of internal standards [prostaglandin E2-d4, 8(9)-EET-d11, 11(12)-EET-d8, 12-hydroxyeicosatetraenoic acid-d8, 20-hydroxyeicosatetraenoic acid-d6, and 11,12-DHET-d11]. EETs and DHETs were extracted using solid phase C18-ODS AccuBond II 500-mg cartridges (Agilent Technologies, Santa Clara, CA).

All measurements were performed in triplicate and normalized by cell numbers as previously published [19].

2.7. mRNA isolation and real-time PCR quantification

Total RNA was isolated using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First strand cDNA was synthesized with Roche reverse transcription reagents. Total RNA (1 μg) was analyzed by real-time PCR.

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the TaqMan gene expression assay on an ABI Prism 7900 sequence analyzer according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA). Each reaction was run in triplicate. The comparative threshold cycle (CT) method was used to calculate the amplification fold as specified by the manufacturer. A value of 10 ng of reverse-transcribed RNA samples was amplified by using the TaqMan Universal PCR Master Mix and TaqMan gene expression assays (Applied Biosystems, Carlsbad, CA).

2.8. Statistical analyses

Statistical significance between experimental groups was determined by the Fisher method of analysis of multiple comparisons ($p < 0.05$ was regarded as significant). For comparison between treatment groups, the null hypothesis was tested by either a single-factor ANOVA for multiple groups or the unpaired *t*-test for two groups. Data are presented as mean ± SEM.

3. Results

3.1. The expression of adipogenic proteins marker during adipogenesis

We examined the levels of HO-1, CYP2J2, PPARγ and FAS during adipogenesis. As seen in Fig. 1A, PPARγ is increased ($p < 0.05$) at day 3, plateaued at day 6 and remained elevated at day 10. The increase in PPARγ was associated with an increase in FAS ($p < 0.05$) which