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PGC-1 alpha Regulates HO-1 Expression, Mitochondrial Dynamics and Biogenesis: Role of Epoxyeicosatrienoic Acid

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Abstract

Background/Objectives—Obesity is a risk factor in the development of type 2 diabetes mellitus (DM2), which is associated with increased morbidity and mortality, predominantly as a result of cardiovascular complications. Increased adiposity is a systemic condition characterized by increased oxidative stress (ROS), increased inflammation, inhibition of anti-oxidant genes such as HO-1 and increased degradation of epoxyeicosatrienoic acids (EETs). We previously demonstrated that EETs attenuate mitochondrial ROS. We postulate that EETs increase peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), which controls mitochondrial function, oxidative metabolism and induction of HO-1.

Subjects/Methods—Cultured murine adipocytes and mice fed a high fat (HF) diet were used to assess functional relationship between EETs, HO-1 and (PGC-1 α) using an EET analogue (EET-A) and lentivirus to knock down the PPARGC1A gene.

Results—EET-A increased PGC-1 α and HO-1 in cultured adipocytes and increased the expression of genes involved in thermogenesis and adipocyte browning (UCP1 and PRDM16, respectively). PGC-1 α knockdown prevented EET-A-induced HO-1 expression, suggesting that PGC-1 α is upstream of HO-1. MRI data obtained from fat tissues showed that EET-A administration to mice on a HF diet significantly reduced total body fat content, subcutaneous and visceral fat deposits and reduced the VAT: SAT ratio. Moreover EET-A normalized the VO₂ and RQ (VCO₂/VO₂) in mice fed a HF diet, an effect that was completely prevented in PGC-1 α

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Conflict of Interest

There are no conflicts of interest among the authors.

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deficient mice. In addition, EET-A increased mitochondrial biogenesis and function as measured by OPA1, MnSOD, Mfn1, Mfn2, and SIRT3, an effect that was inhibited by knockdown of PGC-1 α .

Conclusion—Taken together, our findings show that EET-A increased PGC-1 α thereby increasing mitochondrial viability, increased fusion potential thereby providing metabolic protection and increased VO₂ consumption in HF-induced obesity in mice, thus demonstrating that the EET-mediated increase in HO-1 levels require PGC-1 α expression.

Keywords

HO-1; metabolic dysfunction; epoxyeicosatrienoic acid

Introduction

Obesity is a global epidemic and a major risk factor in the development of metabolic syndrome and diabetes and associated complications that include cardiovascular disease, increased oxidized HDL levels, kidney disease, hypertension, and neuropathies (1–4). Epoxyeicosatrienoic acids (EETs) are arachidonic acid derived metabolites generated by a family of cytochrome P450 (CYP) monooxygenases and epoxigenases (5;6). Although rapidly subjected to hydrolysis by soluble epoxide hydrolase (sEH) to their respective dihydroxyepoxytriene acids as well as by esterification primarily to glycerophospholipids, their vasodilatory, anti-inflammatory, anti-adipogenic and antiapoptotic actions are well established and sEH-inhibition increases cellular and circulating EET levels (7–9). EET agonists prevent both adiposity and vascular complications *in vitro* and *in vivo* and obesity-induced adipose tissue expansion impairs the CYP epoxigenase pathway and the generation of EET *in vivo* (10–13).

Heme oxygenase (HO) consists of an inducible and a constitutive form (HO-1 and HO-2, respectively) that catalyzes the degradation of heme into equimolar amounts of biliverdin/bilirubin, carbon monoxide (CO) and iron (Fe²⁺ ion) (14;15). HO-2 maintains normal metabolic cellular functions, such as vascular tone, renal channel function and activity (15), as well as control of body weight, insulin sensitivity, and blood pressure (16). HO-2 deficiency is associated with oxidative stress, chronic inflammation, and reduced protection against diabetes-induced renal injury (17;18). HO-1, a stress responsive enzyme, offers increased protection against high fat diet (HF)-induced obesity (19–21) and regulates adipocyte stem cell differentiation (22;23), attributable to the potent antioxidant and anti-inflammatory properties of biliverdin/bilirubin.

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) is a major regulator of mitochondrial function, oxygen consumption and oxidative phosphorylation (24;25). PGC-1 α along with the transcriptional regulator PR Domain Containing 16 (PRDM16), are the major regulators of adipocyte browning and thermogenic activation of brown fat (26–28). Transgenic mice with mildly elevated muscle PGC-1 α levels are resistant to age-related obesity and diabetes and have a prolonged lifespan suggesting that PGC-1 α stimulates the secretion of factors from skeletal muscle that affect the function of other tissues (29). In fact, PGC-1 α and several of its responsive genes involved in oxidative

phosphorylation display reduced protein and mRNA expression levels in muscle and adipose tissue of patients with chronic heart failure and diabetes mellitus type 2 (DM2) (30–34). Furthermore mice lacking PGC-1 α in adipose tissue fed a HF diet develop insulin resistance and increased circulating lipid levels (35). Additionally, the uncoupling proteins are intramembranous of the mitochondrial proteins that play a key role in thermogenesis. UCP1 is present exclusively in brown adipose tissue (36) and myokines drive UCP1 in a PGC-1-dependent manner to increase energy expenditure (36) and possibly oxygen consumption. These studies suggest that PGC-1 α is a key moderator of energy metabolism and in preventing the development of metabolic syndrome and DM2. As a transcriptional co-activator, PGC-1 α enhances the expression of many transcription factors and can bind to members of the nuclear receptor family, including nuclear respiratory factor (NRF)-1 and -2 (35;37).

Adipocyte differentiation is associated with an increase in ROS and inflammatory cytokine levels and a concomitant decrease in mitochondrial function. Although ROS are being generated in several cellular compartments, the bulk of ROS (about 90%) contribute to mitochondrial energy metabolism (38). Sirtuin 3 (SIRT3), one of the seven mammalian sirtuins, is a major mitochondrial deacetylase and has recently been discovered to be the target of PGC-1 α and is important in mitochondrial processes, such as suppression of ROS, mitochondrial biogenesis, and energy metabolism (38), including mitochondrial fatty-acid oxidation (39). The mitochondrial network morphology is tightly linked to energy and metabolic demands as well as viability and depends greatly on quality control, involving mitochondrial fusion (the merge of dysfunctional to functional) and fission (budding and isolation of dysfunctional mitochondria, orchestrated by the dynamin-related protein 1 (DRP1) and the fission, mitochondrial 1 (Fis1) proteins (40;41)). The autosomal dominant optic atrophy 1 (OPA1) protein is situated on the mitochondrial inner membrane that, along with the mitochondrial fusion proteins, Mitofusin 1 and 2 (Mfn 1 and 2), located on the mitochondrial outer membrane, facilitate the mitochondrial fusion process (42;43). That the dynamics of the mitochondrial fusion/fission processes need to be tightly regulated is exemplified by the link between the reduction of mitochondrial fusion and the development of obesity and insulin resistance (41;44;45). Conversely, reduced hepatic steatosis, insulin resistance, and increased mitochondrial function and fusion potential is present in hepatocyte mitochondria of rats fed high-fish-oil diet as compared to rats fed a high-lard diet (41).

We recently reported that EETs increase HO-1 expression and mitochondrial viability, and exert their function on adipocyte differentiation via activation of PGC-1 α *in vitro* (46). The present report examines whether the EET-mediated regulation of adiposity is due to activation of PGC-1 α and an increase in HO-1 levels in cultured adipocytes *in vitro* and in mice fed a high fat diet *in vivo* and investigates the mechanistic EET-mediated actions on mitochondrial function in reference to mitochondrial viability and fusion mediators mitochondrial superoxide dismutase (MnSOD/SOD2), SIRT3, Mfn1, Mfn2, and OPA1. We further examined the impact of HF, EETs, and lack of PGC-1 α on metabolic alterations and on VO₂ and respiratory quotient (RQ). We demonstrate that EET is upstream of the PGC-1 α signaling pathway responsible for increased levels of HO-1 and insulin phosphorylation and further that the EET-mediated induction of HO-1 is dependent on PGC-1 α . Our results

indicate that the EET-PGC1 α -mediated reduction in adiposity in mice fed a HF diet involves an increase in VO₂. This study highlights the existence of an EET-PGC-1 α axis that is associated with increased levels of HO-1 that, concomitantly, upregulates mitochondrial function, MnSOD and SIRT3, and fusion mediators, Mfn1 and Mfn2, and OPA1, which, together, serve to regulate adipocyte cell differentiation and obesity-induced hypertension and improve metabolic homeostasis.

Materials and Methods

Cell culture

3T3-L1 murine pre-adipocytes were purchased from ATCC (ATCC, Manassas, VA). After thawing, 3T3-L1 cells were cultured in 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 cultures were maintained at 37°C in a 5% CO₂ incubator and the medium was changed after 48h and every 3–4 days thereafter as described previously (10). For adipogenesis studies the medium was replaced with adipogenic medium (Dulbecco's modified Eagle Medium (DMEM) with high glucose (Invitrogen), supplemented with 10% (v/v) FBS, 10 μ g/ml insulin (Sigma-Aldrich, St. Louis, MO), 0.5 mM dexamethasone (Sigma-Aldrich), and 0.1mM indomethacin (Sigma-Aldrich) and the cells were cultured for an additional 7 days. Cells were cultured in the absence and presence of the EET analog EET-A ((S)-2-(11-(nonyloxy) undec-8(Z)-enamido) succinic acid) in a dose of 10 μ M. To examine the effect of inhibition of HO activity, SnMP (5 μ M, Tin mesoporphyrin, Frontier Scientific) was added for the duration of EET-A treatment. At the experimental endpoints, cells were collected by trypsinization, washed once with PBS and then lysed for protein measurements and for RNA extraction.

Development of PGC-1 α deficient adipocytes

SMART vector lentiviral shRNA-PPARGC1A or scrambled RNA (Dharmacon, Lafayette, CO) was applied to 3T3-L1 cells to establish a stably transduced cell line. Briefly, 1×10^6 cells were seeded in 6-well plates 1 day prior to transduction. On the day of transduction, the transduction medium was made by 1×10^6 transducing units (TU) of lentiviral particles with 0.5ml α -MEM growth medium, applied to each well and incubated for 3h to maximize the contact between each cell and lentiviral particles. Cells were also treated with the transduction medium without lentiviral particles which served as un-transduced control. Growth Medium (1.5 ml) was then added to each well in the presence of 8 μ g/ml polybrene (final concentration). After 48 h incubation, antibiotic selection medium (α -MEM growth medium with 10 μ g/ml puromycin) was used to kill all the un-transduced cells. Cells were then cultured and maintained as outlined above.

Animal experimentation

All animal experiments followed an NYMC IACUC institutionally approved protocol in accordance with the NIH Guidelines. C57bl6 mice were used in the studies. Mice were divided into 4 groups: (1) lean, (2) HFD, (3) HFD + EET-A, (4) HFD+EET-A+PGC-1 α lentivirus. Lean mice (group 1) were fed ad libitum a normal chow diet containing 11 % fat,

62 % carbohydrate, and 27.0 % protein with a total calories of 12.6 KJ/g. The remaining animals (groups 2, 3, and 4) were fed a high-fat diet containing 58 % fat (from lard), 25.6 % carbohydrate, and 16.4 % protein with total calories of 23.4 KJ/g (Harlan, Teklad Lab Animal Diets, US) for 8 Weeks. Mice were treated as following, group (1) normal chow diet, group (2) HF diet, group (3) EET-A was injected, intraperitoneally, for 2 weeks daily at a dose of 1.5 mg/100 gm of body weight as previously described (1), group (4) EET-A was injected, intraperitoneally, for 2 weeks daily at a dose of 1.5 mg/100 gm of body weight and each animal received a 2 bolus injection of PGC-1 α Lentivirus was injected into the retro orbital vein at a concentration of 40–70 *10⁶ TU/mouse in 80–100 μ l (Dharmacon, Lafayette, CO, US), as previously described (1). At the end of the 8-week period, mice were anesthetized with sodium pentobarbital (65 mg/Kg, i.p.) and, at the time of sacrifice, body weight and fat content were measured.

Real-time qPCR and Western Blot Analysis

Total RNA was extracted from 3T3 cells using TRIzol® (Ambion, Austin, TX) and from frozen adipose tissue by RNeasy® Lipid Tissue (Qiagen), as indicated by the manufacturer. RNA was determined by measuring the absorbance at 260 nm (A260) with a Biotek™ plate reader and the Take3™ plate (Biotek, Winooski, VT), and assessed by the A260/A280 ratio. cDNA was synthesized from total RNA (Applied Bio systems) using a High Capacity cDNA Reverse Transcription Kit (Applied Bio systems). Real-time PCR was performed using TaqMan® Fast Universal Master Mix (2x), on a 7500 HT Fast Real-Time PCR System (Applied Bio systems). Specific TaqMan® Gene Expression Assays probes for mouse PGC1 α , PRDM16, UCP1, adiponectin, SIRT3, Mfn1, Mfn2 and GAPDH were used as described previously (4). For *in vitro* western blot analysis pelleted cells were lysed with lysis buffer supplemented with protease and phosphatase inhibitors (cOmplete™ Mini and PhosSTOP™, Roche Diagnostics, Indianapolis, IA). Frozen adipose tissues were ground under liquid nitrogen and suspended in homogenization buffer (mmol/l: 10phosphate buffer, 250 sucrose, 1.0 EDTA, 0.1 PMSF, and 0.1% v/v tergitol, pH 7.5). Immunoblotting for HO-1, UCP1, PEG1/MEST, MnSOD, Mfn-2, OPA-1, PGC-1 α , COX-IV (Cytochrome c oxidase subunit-IV), adiponectin, β -actin and GAPDH was performed on cell and mouse adipose tissue lysates as previously described (2; 5).

Magnetic Resonance Imaging

Subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) volumes were determined by MRI. Total fat, SAT, and VAT volumes, VAT/SAT ratio, and fat/body volume ratio were calculated as described earlier (7).

Oxygen consumption

Animals were acclimatized in the oxygen consumption chambers for 2 hours three times a week for 3 weeks. Oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were measured using the Oxylet gas analyzer and air flow unit (Oxylet; Panlab-Bioseb, Vitrolles, France). Individual mice were placed in the chambers and had the flow rate set to maintain the dCO₂ between 0.4 and 0.8 as specified by the manufacturer's instructions. Hourly respiratory quotients were measured using the VCO₂ and VO₂ obtained by the gas analyzer.

This process was performed twice on each mouse. The data for VO_2 are expressed as the consumed volume of oxygen per kilogram body weight per minute (ml/kg/min). The respiratory quota is expressed as CO_2 eliminated/ O_2 consumed.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. Significance of difference in mean values was determined using one-way analysis of variance followed by Bonferroni's post-test for comparison between groups. $P < 0.05$ was considered to be significant.

Results

EET mediated induction of PGC1- α , UCP1, PRDM16 and adiponectin

Western blot analysis and real-time PCR demonstrated a significant ($p < 0.05$) increase in PGC-1 α protein and mRNA expression after treatment of adipocyte cells with EET-A (Figure 1 A–C). Concomitant treatment with EET-A and SnMP decreased PGC-1 α protein and mRNA expression when compared to EET-A treated cells (Figure 1 A–C). Uncoupling protein 1 (UCP1) was significantly upregulated in cells treated with EET-A ($p < 0.05$), an increase that was abrogated in cells treated with SnMP along with EET-A (Figure 1 A and D). We examined the effect of EET-A on the brown fat specific gene PRMD16 and found that PRMD16 mRNA levels were increased ($p < 0.05$) in adipocyte cells treated with EET-A (Figure 1 E). SnMP attenuated the EET-A mediated increase in PRMD16 mRNA expression. The adiponectin mRNA expression level in the adipocyte cells treated with EET-A was significantly higher ($p < 0.05$) compared to untreated adipocytes and was attenuated by SnMP administration ($P < 0.05$) (Figure 1 F).

PGC-1 α knockdown prevents EET-mediated HO-1 induction, decreases UCP1, adiponectin, and COX-IV, while increasing PEG1/MEST expression

To examine whether EET induction of HO-1 is mediated through PGC-1 α we knocked down PGC-1 α in adipocyte cells using PGC-1 α shRNA. As seen in Figure 2 A and B, the EET-mediated increase in HO-1 protein was completely abolished in PGC-1 α deficient cells. Adiponectin protein levels were significantly lower in PGC-1 α deficient cells as compared to WT cells (Figure 2 A and C). While treatment with EET-A increased adiponectin protein levels in WT cells ($p < 0.05$), it did not affect the adiponectin levels in PGC-1 α deficient cells (Figure 2 A and C). UCP1 mRNA expression and protein levels were lower ($p < 0.05$) in PGC-1 α deficient cells as compared to WT cells (Figure 2 D and E, respectively). Treatment of adipocyte cells with EET-A significantly ($p < 0.05$) reduced PEG1/MEST protein expression as compared to vehicle control cells (Figure 2 A and F). Moreover, the expression of the COX-IV protein was increased in control cells treated with EET-A ($p < 0.05$). Importantly, the protein level of COX-IV in PGC-1 α deficient cells was lower ($p < 0.05$) as compared to WT control cells (Figure 2 G and H).

Magnetic Resonance Imaging of adipose tissues

Total body fat volumes of subcutaneous adipose and visceral adipose tissues (SAT and VAT, respectively) were determined by MRI in mice fed a HF diet with and without treatment with EET-A (Figure 3 A–D). As seen in Figure 3 C and D, the total, SAT, and VAT volume

of obese mice was significantly reduced in mice treated with EET-A. More importantly, the SAT-to-VAT ratio was markedly reduced in the obese mice treated with EET-A.

Effect of EET on oxygen consumption

We examined the effect of EET-A on mice fed a HF diet on both O_2 consumption and the ratio of CO_2/O_2 . As expected, mice on a HF diet displayed a significant ($p<0.05$) decrease in VO_2 consumption. However, HF-diet fed mice treated with EET-A, exhibited a significant ($p<0.05$) increase in oxygen consumption with a concomitant lowering of VCO_2/VO_2 . PGC-1 α deficient animals on a HF-diet treated with EET-A also displayed a significant ($p<0.05$) decrease in VO_2 consumption and an elevated VCO_2/VO_2 . (Figure 3 E and F)

Effect of EET-A administration on HO-1, PGC-1 α , Mfn1, Mfn2, MnSOD, OPA-1, and SIRT3 levels in adipose tissues of high fat diet fed control and PGC-1 α deficient mice

The levels of MnSOD and HO-1 in adipose tissue were not affected by a HF diet (Figure 4 A, B, and E). Interestingly, EET-A treatment of mice on a HF diet significantly ($p<0.05$) increased the adipose tissue levels of both MnSOD and HO-1 as compared to both untreated lean and obese mice (Figure 4 A, B, and E). The observed increase was PGC-1 α -dependent as the levels of both MnSOD and HO-1 in adipose tissue from PGC1 α -deficient mice were significantly ($p<0.05$) reduced as compared to HFD mice treated with EET-A alone (Figure 4 A, B, and E). The levels of PGC-1 α , and the mitochondrial fusion-associated proteins Mfn2 and OPA1, in visceral adipose tissue of mice fed a HF diet were significantly ($p<0.05$) reduced as compared to control mice fed regular chow (Figure 4 A, C, D and F). Importantly, the levels of these proteins were normalized in HF diet mice treated with EET-A, an effect that was dependent on PGC-1 α (Figure 4 A, C, D and F).

Analysis of mRNA levels confirmed the EET-A mediated increase of Mfn2 in adipose tissue (Figure 4 G). Furthermore, the Mfn1 mRNA levels were also significantly ($p<0.05$) increased in HF diet fed mice treated with EET-A as compared to mice fed a HF diet alone (Figure 4 H). Importantly, knockdown of PGC-1 α completely prevented the EET-A mediated effect on Mfn1 mRNA levels (Figure 4 H).

Finally, the mRNA levels of the mitochondria associated histone deacetylase, SIRT3, were significantly ($p<0.05$) elevated in adipose tissues of mice fed a HF diet and treated with EET-A (Figure 4 I), meanwhile the EET-A mediated increase of SIRT3 mRNA levels were significantly ($p<0.05$) impaired in PGC1 α -deficient mice fed a HF diet (Figure 4 I).

Discussion

This study demonstrates that EET is located upstream of PGC-1 α in its signaling cascade and, in turn, that PGC-1 α is crucial for the induction of HO-1. Together these factors play an important role in the regulation of the adipocyte stage of differentiation. We show that EET is a powerful inducer of PGC-1 α -mediated downstream signaling and mitochondrial viability and function, including the processes of oxidative phosphorylation, fusion, and mitochondrial quality control, resulting in improved metabolic parameters in mice fed a HF diet, highlighting that the EET and PGC-1 α interplay has a significant role in adiposity, diabetes and obesity. Four key findings substantiate this conclusion.

The first key finding is that EET-A-treatment increases PGC-1 α , HO-1 and adiponectin levels in cultured murine adipocyte cells (*in vitro*) and, more importantly, that these EET-mediated actions do not take place in PGC-1 α deficient cells, a strong indication that they are located downstream of PGC-1 α in the signaling pathway. The transcriptional coactivator, PGC-1 α , is a nuclear protein that was first identified as a regulator of peroxisome proliferator-activated receptor gamma (PPAR γ) in brown adipose tissue rich with mitochondria and which has a special role in thermogenesis (24) and is a potent transcriptional coactivator of nuclear receptors and other transcription factors (47;48). PGC-1 α is highly expressed in tissues with high energy demands, such as heart, skeletal muscle, and brown adipose tissue wherein it plays a critical role in the maintenance of mitochondrial function as well as in the cellular energy metabolism (49;50) and its induction regulates mitochondrial oxidative metabolism as well as mitochondrial biogenesis and the recruitment of chromatin modifying enzymes (51–54). The importance of PGC-1 α in adipose tissue has been established in mice lacking PGC-1 α , specifically in adipocytes, which develop insulin resistance and have increased circulating lipid levels when fed a HF diet (35). Thus, the role of PGC-1 α in energy homeostasis suggests it to be a target for both anti-obesity and diabetes drugs (48).

This is supported by our observation that in *in vitro* cultures of murine adipocytes EET-A treatment increases the expression of UCP1 and PRDM16 that play an essential role in thermogenesis and in the browning of adipocytes, which is associated with an increased number of mitochondria (26–28). This EET-mediated increase was also shown, at least in part, to be dependent on HO-1 as both UCP1 and PRDM16 levels were decreased in cells treated with the HO-activity inhibitor SnMP. Furthermore, the EET-A mediated increase of PGC-1 α expression was prevented by SnMP, indicating that EET is working through the HO system that controls cellular levels of heme and the potential of heme-mediated terminal adipocyte stem cell differentiation and enlargement. Our results are corroborated by other studies that have shown that EET, together with SIRT1, regulates PGC-1 α expression (55–57), suggesting that they are upstream regulators of PGC-1 α . Although EET downregulates the BTB And CNC Homology 1, Basic Leucine Zipper Transcription Factor 1 (BACH1), an inhibitory transcriptional regulator of HO-1, in favor of increased HO-1 levels (58) we now show that the EET-mediated action on HO-1 is also dependent on PGC-1 α expression. These results identify PGC-1 α as a crucial component of the EET-augmented regulatory pathway involved in the control of HO-1 and adiponectin production as well as mitochondrial viability and function.

We have previously shown that heme levels are increased with, while also promoting, adipocyte differentiation, while the levels of HO-1 are reduced (1;46). Additionally, increased heme levels, either by exogenous addition of hemin or by the inhibition of HO-activity induce or enhance adipogenesis of pre-adipocytes (59), and mesenchymal stem cells alongside the development of oxidative stress and attenuation of SIRT1 expression (22;60). Although heme is critical for increased adipocyte differentiation and hypertrophy it is an essential substrate for numerous enzymes and its levels must be tightly regulated to minimize toxicity while HO-1/heme is essential to the maintenance of homeostatic regulation of mitochondrial respiration. Recently, the nuclear heme receptor, Rev-erba, was identified as a negative regulator of heme levels via its ligand activated repression of

PGC-1 α expression that, in turn, leads to inhibition of 5'-Aminolevulinate Synthase 1 (ALAS1), the rate limiting enzyme in heme biosynthesis (61;62). Not only does HO-activity inhibition increase heme levels, but also results in lower levels of its metabolic products biliverdin and CO. Whether these products themselves play a role in PGC-1 α regulation remains to be clarified. Simultaneously, we show that PGC-1 α regulates HO-1 expression, thus putting PGC-1 α in a central position with a capacity to both increase heme levels in favor of terminal adipogenic differentiation, and to reduce heme levels by increasing HO-1 expression, as a result of EET-treatment, that favors prevention of terminal adipocyte differentiation. Therefore, there is a balance of heme levels and stage of adipocyte differentiation and is regulated by the rate of heme degradation (HO-1 induction) and heme synthesis (PGC-1 α -ALAS1 induction) necessary for increase of heme and terminal differentiation and hypertrophy (46).

HO-2 and HO-1 play a regulatory role on mitochondrial function and apoptosis(63–65). HO-2 deficiency has a negative impact on superoxide anion levels, EC-SOD and the mitochondrial signaling pathway (17;64), but its definite role in preservation of mitochondrial function is not fully understood. We have shown that HO-1 is present in the mitochondrial inner membrane and cortex (64) and we and others have in several reports shown that HO-1 plays a role in mitochondrial biogenesis, cytochrome oxidase levels as well as in mitochondrial quality control (66–68). Additionally, HO-1 induction increased mitochondrial function in ischemic cardiac damage (69) and experimental diabetes (66). In contrast, drug-mediated disruption of mitochondrial membranes and mitochondrial structure also had adverse effects on heme metabolism (70).

The second key finding is that EET-A treatment of obese mice decreases both VAT and SAT. More importantly, the VAT/SAT ratio was reduced in mice treated with EET-A. EET decreases adiposity and insulin resistance in an animal model of obesity and diabetes involving an increase in HO-1 gene expression and in the AMPK/AKT signaling cascade (16). In addition, EET inhibits BACH-1 expression, a negative transcriptional regulator of HO-1 expression (58). While EET has been shown to increase HO-1 expression, increased HO-1 levels lead to a recovery in EET levels in HO-2 null mice (71) as well as in EC-SOD null mice (72), thus completing a positive feed-back loop.

We have previously reported HO-1-EET interactions and their beneficial role in regulating adipogenesis. Specifically, EETs decrease mesenchymal stem cell-derived adipocyte differentiation *in vitro* via upregulation of HO-1-adiponectin-AKT signaling and a decrease in PPAR γ expression (11). Moreover, endothelial overexpression of CYP2J2 in mice fed a HF diet normalized adipocyte function, insulin levels, blood glucose, body weight and inflammatory markers (9). Our results have been confirmed by others attesting to the regulation of adipocyte differentiation by EETs both *in vivo* and *in vitro* (13). These results clearly point to a regulation of PGC-1 α that is directly dependent on EET and indirectly, via increased EET levels, on HO-1 (Figure 5).

The third novel finding is that EET-A is associated with a normalization of both VO₂ and the RQ in mice fed a HF diet as compared animals on regular chow (lean mice). This increase may be related to an alternate metabolic strategy of HF-fed mice to EET i.e., to increase the

metabolic rate in response to increased EET-A-mediated PGC-1 α expression and mitochondrial thermogenesis and function as indicated by the increased levels of UCP1 *in vitro* and SIRT3 *in vivo* leading to a reprogramming of adipose stem cells to brown-like cells thus allowing the maintaining of normal mitochondria and energy consumption and a subsequent decrease in body fat. Meanwhile, the EET-A-mediated normalization of VO₂ and RQ was completely prevented in PGC-1 α deficient mice on a HF diet.

Cytochrome c Oxidase Subunit IV (COX-IV) is a crucial component of the cytochrome c oxidase complex, which catalyzes the final step in the electron transfer chain and regulates oxidative phosphorylation and total respiration. Impaired activity of the cytochrome c oxidase complex has been linked to a wide range of disorders including stroke, hepatic failure, fatal lactic acidosis, and exercise intolerance, and is a major cause of subsequent mitochondrial abnormalities (73–75). A reduced total oxygen consumption in mice deficient in lung specific COX-IV subunit 2 has been reported (76). We measured the protein levels of COX-IV *in vitro* in WT and PGC-1 α deficient adipocyte cells treated with EET-A and found that the COX-IV levels were elevated in WT cells treated with EET-A. However, the COX-IV levels of PGC-1 α -deficient cells were significantly reduced.

The fourth key finding is that visceral fat from mice fed a HF diet showed impaired mitochondrial viability-, quality control- and fusion-associated parameters as compared to lean mice. While treated with EET-A normalized or enhanced these mitochondrial parameters, PGC-1 α -deficiency prevented the EET-A-mediated effects, thus indicating that the EET-A-mediated normalization of diet-induced mitochondrial dysfunction is PGC-1 α -dependent. Our results linking EETs to mitochondrial viability are supported by a report showing that EETs promote mitochondrial biogenesis and protect neurons from oxidative damage (77). However, to our knowledge our study is the first to show that EETs stimulate mitochondrial fusion associated processes as indicated by the increased expression of Mfn1 and 2, and OPA1. Furthermore, the EET-PGC1 α axis increases MnSOD, which is a critical component of mitochondrial resistance to superoxide induced damage. Even though a HF diet itself did not reduce MnSOD levels in visceral fat, the EET-A mediated increase was effectively reduced in PGC-1 α -deficient mice corroborating our recent report showing that MnSOD levels are impaired in PGC-1 α deficient 3T3-L1-derived adipocytes, contributing to increased mitochondrial derived superoxide formation (46). Similarly, the levels of SIRT3 were reduced in PGC-1 α -deficient mice on high fat diet treated with EET-A as compared to mice on a HF diet treated with EET-A alone. About 90% of ROS contribute to the mitochondrial energy metabolism (38). Furthermore, SIRT3 is the target of PGC-1 α and important in mitochondrial suppression of ROS, mitochondrial biogenesis and metabolic homeostasis (38;78), including mitochondrial fatty-acid oxidation (39). The visceral adipose tissue levels of the mitochondrial fusion mediating proteins, Mfn1, Mfn2 and OPA1, were significantly downregulated with a HF diet. While, EET-A-treatment normalized, or enhanced the levels of these proteins in animals on high fat diet, PGC-1 α -deficiency completely prevented the EET-A-mediated effects. Several reports have shown the importance of mitochondrial function and dynamics, health, and aging, for review see (43). Knockout mice Mfn1, Mfn2 and OPA1 are all embryonic lethal (78). In humans, mutations in OPA1 are linked to hereditary blindness, and Mfn2 mutations are known to cause Charcot-Marie-Tooth disease (42;78;79). In mice, a liver-specific knockout of Mfn2 was

linked to impaired glucose metabolism and insulin signaling (80). Our results linking PGC-1 α -dependent increase of HO-1, thus potentiating the beneficial effect of PGC-1 α itself are supported by a recent report in which doxorubicin-induced oxidative stress increased mitochondrial fragmentation and Fis1 expression, an effect that was prevented by HO-1 induction favoring expression of Mfn1 and Mfn2 (68).

These results support our hypotheses linking EET-A to PGC-1 α mediated regulation of HO-1 expression and levels of heme and potentially biliverdin and CO, to mitochondrial health as well as to metabolic adipocyte reprogramming that may influence adipocyte hemostasis and delay terminal differentiation. The reported findings suggest that induction of HO-1, possibly via its metabolic products biliverdin and CO, leads to an increase in PGC-1 α through an increase in EET levels thereby providing metabolic protection as a mechanism to regulate HF diet induced obesity in mice. The present study is of considerable interest from both a basic and a clinical perspective clearly defining the existence of an EET- PGC-1 α regulatory module that can be manipulated to ameliorate the deleterious effect of HF diet-induced insulin resistance, diabetes and metabolic syndrome via induction of HO-1 leading to control of mitochondrial dynamics and biogenesis. HO-1 expression is instrumental for mitochondrial integrity (68). A deeper understanding of the mechanism of EET- PGC-1 α necessary to increase insulin sensitivity, HO-1 expression and HO activity will provide new approaches to the treatment of obesity-diabetes and improve the effectiveness of EET- PGC-1 α downstream signaling in the treatment of vascular disease.

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Author contributions are as follows: S.P.S performed experiments; L.B. performed experiments, collected literature citations, and edited manuscript; J.S. conducted ELISA assay and edited manuscript; N.G.A. planned and designed the experimental protocol, and wrote the first draft of the manuscript; J.C. designed the PGC-1 α sequence.

ABBREVIATIONS

BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1
CO	Carbon monoxide
COX-IV	Cytochrome C oxidase subunit IV
DM2	Diabetes mellitus type 2
EET	Epoxyeicosatrienoic acid
EET-A	EET agonist
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HO	Heme oxygenase

Mfn	Mitofusin
MnSOD/SOD2	Manganese-containing superoxide dismutase/superoxide dismutase 2, mitochondrial
OPA1	Optic atrophy 1 (autosomal dominant)
PEG1/MEST	Paternally-expressed gene 1 protein/Mesoderm specific transcript
PGC-1α	Peroxisome proliferator-activated receptor gamma, coactivator 1
PPARγ	Peroxisome proliferator-activated receptor gamma
PRDM16	PR domain containing 16
ROS	Reactive oxygen species
SAT	subcutaneous adipose tissue
sEH	Soluble epoxide hydrolase
SIRT	Sirtuin
SnMP	Tin mesoporphyrin
UCP1	Uncoupling protein 1
VAT	visceral adipose tissue

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Figure 1.

Western blot and densitometry analysis for PGC-1α protein (A and B) and mRNA (C) levels in differentiated adipocyte cells in the presence of EET-A, or EET-A+SnMP. Western blot and densitometry analysis for the expression of the UCP1 protein levels in differentiated adipocyte cells in the presence of EET-A, or EET-A+SnMP (D). PRDM16 (E), and adiponectin (F) levels in differentiated adipocyte cells in the presence of EET-A, or EET-A+SnMP. *p<0.05 vs. control, #p < 0.05 vs. EET-A alone, n=3.

Figure 2.

Protein and mRNA expression analysis in WT and PGC-1 α deficient differentiated adipocyte cells adipocytes in the presence and absence of EET-A (10 μ M) treatment. Representative blots of heme oxygenase-1 (HO-1), adiponectin, uncoupling protein 1 (UCP1), paternally-Expressed Gene 1/mesoderm specific transcript (PEG1/MEST), glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), and β -Actin (A), Densitometric analysis of HO-1 (B), adiponectin (C), and UCP1 protein levels (D), normalized to β -Actin. Analysis of UCP1 mRNA expression levels (E). Densitometric analysis PEG1/MEST protein normalized to GAPDH (F). Protein expression analysis of COX-IV in WT and PGC-1 α deficient differentiated adipocyte cells in the presence and absence of EET-A (10 μ M) treatment. Representative blots of COX-IV and β -Actin (G), and densitometric analysis (H). n=3, *p<0.05 vs control. *p<0.05 vs. WT, #p< 0.05 vs. WT, ##p < 0.05 vs. WT-EET-A. White bars WT, black bars PGC-1 α deficient cells.

Figure 3.

Sagittal magnetic resonance imaging (MRI) and representative axial cross-sections of a mouse fed high fat diet alone (A), and a mouse fed a HF diet and treated with EET-A (B). Subcutaneous and visceral adipose tissues (SAT and VAT, respectively) are highlighted with red arrows. Analysis of total body, and fat volumes in HF diet fed mouse (C), and a HF diet fed mouse treated with EET-A (D). VO_2 oxygen consumption (ml/kg/min) (E) and RQ (CO_2 eliminated/ O_2 consumed) (F) in mice fed regular chow (lean), mice fed a HF diet, mice fed a HF diet treated with EET-A (HF+EET), and PGC-1 α -deficient mice fed a HF diet treated with EET-A (HF+EET+PGC1 α KD). n=4, *p<0.05 vs lean, #p<0.05 vs HF, ##p<0.05 vs HF+EET.

Figure 4.

Effect EET-A treatment of HF diet fed control and PGC-1α-deficient on protein and mRNA expression of genes involved in mitochondrial biogenesis and dynamics. Representative blots (A) and densitometric analysis of manganese superoxide dismutase (MnSOD) (B), optic atrophy 1 (OPA1) (C), mitofusin 2 (Mfn2) (D), heme oxygenase 1 (HO-1) (E), and Peroxisome proliferator-activated receptor gamma, coactivator 1 (PGC-1α) (F). mRNA expression of Mfn2 (G), Mfn1 (H), and sirtuin 3 (SIRT3) (I) in adipose tissues of lean, a HF diet fed (HF), a HF diet EET-A-treated (HF+EET), and PGC-1α-deficient fed a HF diet mice (HF+EET+PGC1α KD). Results are means ±SE, n = 4, *p < 0.05 vs. lean, #p < 0.05 vs HF, ##p < 0.05 vs HF+EET.

Figure 5.

Schematic description of the EET-PGC-1 α -HO-1 interplay, increased insulin sensitivity, mitochondrial function, and reduced inflammation. Epoxygenase-derived EETs increase of HO-1 expression in a process that is dependent on PGC-1 α expression. The increase in PGC-1 α – HO-1 interplay subsequently increases insulin sensitivity, mitochondrial biogenesis and function and expression of energy dependent enzymes, including Mfn-1, Mfn-2, SIRT-3, MnSOD, and OPA-1, thus attenuating the development of obesity, metabolic syndrome and ultimately diabetes mellitus type 2.