Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2012 January ; 97(0): 1–16. doi:10.1016/j.prostaglandins.2011.10.002.

Epoxyeicosatrienoic acids and heme oxygenase-1 interaction attenuates diabetes and metabolic syndrome complications

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Abstract

MSCs are considered to be the natural precursors to adipocyte development through the process of adipogenesis. A link has been established between decreased protective effects of EETs or HO-1 and their interaction in metabolic syndrome. Decreases in HO-1 or EET were associated with an increase in adipocyte stem cell differentiation and increased levels of inflammatory cytokines. EET agonist (AKR-I-27-28) inhibited MSC-derived adipocytes and decreased the levels of inflammatory cytokines. We further describe the role of CYP-epoxygenase expression, HO expression, and circulating cytokine levels in an obese mouse, *ob/ob*^{-/-} mouse model. *Ex vivo* measurements of EET expression within MSCs derived from *ob/ob*^{-/-} showed decreased levels of EETs that were increased by HO induction. This review demonstrates that suppression of HO and EET systems exist in MSCs prior to the development of adipocyte dysfunction. Further, adipocyte dysfunction can be ameliorated by induction of HO-1 and CYP-epoxygenase, i.e. EET.

Keywords

MSC; EET-Agonist; HO-1; pAKT; adipocyte

INTRODUCTION

Metabolic syndrome is a constellation of metabolic abnormalities including central obesity, hypertriglyceridemia, low HDL levels, and hyperglycemia. Progressive development of insulin resistance is central to the pathogenesis of metabolic syndrome which in turn predisposes to development of type II diabetes mellitus along with increased incidence of cardiovascular disorders. A growing body of evidence suggests that increased oxidative stress to adipocytes is central to the pathogenesis of obesity associated metabolic syndrome. Increased oxidative stress to adipocytes causes dysregulation of inflammation-related adipocytokines, resulting in both vascular and cardiovascular complications [1–3].

Adipocytes are of mesodermal origin and bone marrow stromal cells serve as a reservoir for

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the recruitment and generation of preadipocytes. The heme-heme oxygenase (HO) system serves as an important cellular antioxidant defense system in obesity and diabetes [4, 5]. Epoxides have known anti-inflammatory [6] and antihypertensive actions [7, 8] and recent reports have demonstrated their suppression as contributory to hyperlipidemic states. However, nothing is known about whether deficits in the cytoprotective systems of HO and epoxides prior to adipocyte development govern adipocyte function.

Multipotent marrow stromal stem cells (now termed MSCs) were first described in 1974, in an attempt to provide direct evidence that the bone marrow included specific cells “that can generate connective tissue-forming cells” [9]. Determining mesenchymal stem cell fate and differentiation into specific lineages such as adipocytes, is highly dependent on the microenvironment of the bone marrow stroma. Therefore, differentiation and stem cell fate is determined by factors including soluble agents, spatial distribution of cells, extracellular matrix, and *in vitro* – number of cells plated *i.e.* cell density (Figure 1).

Cyto-protection of the stem cells microenvironment determine the stem cell fate *in vivo*. The microenvironment comprises of a multitude of factors and networks and a very complex system of regulation. Negative and positive regulators derived from stromal cells enhance other cell differentiation and its own proliferation and differentiation is controlled by heme levels [10–12]. Imbalances within these systems e.g., exposure to toxic substances, that increases ROS, inactivation and decrease heme levels, negatively modulate the environment, decreasing stromal cell longevity, pluripotency, and cell differentiation [13, 14]. Most recently, it has been shown that imbalances within this network of systems often occur during disease states such as Diabetes Mellitus [15] and osteoporosis [16, 17].

MSCs are recognized as a crucial component of the bone marrow microenvironment, and directly contribute to the development of hematopoietic stem cells, HSCs, readily distinguishable from MSCs by their cell surface markers. Early reports indicated that MSCs were either multipotent stem cells or a population of mixtures of committed progenitor cells, each with restricted potential. Further studies demonstrated MSCs to be a homogenous set of cells with specific cell markers, and pluripotent differentiation capabilities. MSCs are considered to express surface proteins CD29 (integrin beta 1), CD44 (hyaluronate receptor), CD73 (SH-3/SH-4), CD90 (Thy-1) and CD106 (vascular cell adhesion molecule-1) while they typically do not express hematopoietic cell markers, such as CD14 (monocyte surface protein), CD34 (mucosialin), and CD45 (common leukocyte antigen) [18].

Stromal/Mesenchymal Stem Cells and Hematopoiesis

To gain a more complete understanding of the complex network of cells, soluble factors, and extracellular matrix that exists within the bone marrow, it is important to first know how bone marrow develops. Marrow stromal cells are established in a developing marrow cavity after the formation of a bone collar, prior to hematopoiesis. The bone collar is established by osteoblasts that become eroded by osteoclast activity allowing for vascular invasion and formation of a marrow cavity. Slow blood flow and leakage through permeable endothelial cell walls into the cavity allow for the development of sinusoids and entrance of blood borne HSCs. Thus, creation of a space for the marrow stroma, involves a complex relationship and interaction of several cells *i.e.* stromal cells, HSCs, fibroblasts, immune cells, cells involved in bone formation/degradation, and the release of signaling diffusible molecules. Though many cells and interactions within the bone marrow are still unknown, a delicate balance must exist within this system to maintain homeostasis.

Heme synthesis and degradation within this microenvironment play pivotal roles in the regulation of growth and differentiation of erythroid and nonerythroid cells within the bone marrow. Heme affects both MSC and the bone marrow environment’s ability to generate

hematopoietic (CD34⁺) lineages [10, 11, 19]. Both of these processes are critical in regulating the supply of heme necessary for cell functions, transductional signal of growth factor network which regulates the hematopoietic microenvironment, as well as for the maintenance of adherent stromal cells/ MSCs [19].

Heme consists of an iron atom surrounded by a porphyrin ring structure and is actively involved in oxygen transport as the prosthetic group of hemoglobin, in prostaglandin synthesis of cyclooxygenase, and in the inactivation of oxygen molecules as the prosthetic group of mitochondrial and microsomal cytochrome P450 [5, 20, 21]. Heme synthesis begins with the synthesis of D-Aminolevulinic acid (δ -ALA) from amino acid glycine and succinyl-CoA and is catalyzed by ALA synthase, the rate limiting enzyme in the heme biosynthetic pathway. ALA-synthase is strictly regulated by intracellular iron and heme concentrations (Figure 2).

Heme synthesis is essential for bone marrow-adherent cells to produce substances that promote hematopoiesis and for long term bone marrow culture (LTBMC) proliferation, progenitor cell production, and maintenance [11]. Furthermore, supplementation with hemin in adherent stromal/mesenchymal stem cells significantly increases the myeloid progenitor compartment and the longevity of culture without altering the erythroid compartment [22]. Therefore, heme provides an excellent environment for hematopoiesis and the subsequent increase in the number of red blood cells. Although, heme is necessary for cellular hemoglobin synthesis by erythroblasts, increased levels of free heme are toxic, resulting in an array of oxidative tissue damage [23]. Excessive heme within the bone marrow is responsible for cellular toxicity and activation of reactive oxygen species (ROS). Heme degradation by HO, a microsomal protein, is a multistep process, which is initiated with the binding of HO apoprotein to heme. The reaction initiates with the NADPH cytochrome P450 reductase-dependent reduction of the ferric heme-iron in the HO-heme complex, which binds O₂ to form an oxyferrous intermediate that in turn accepts a second electron from NADPH. Two forms of HO have been discovered thus far; the original inducible isoenzyme was designated HO-1 and the second constitutive isoenzyme was designated HO-2. HO-2 contributes to normal physiological functions such as renal channel activity, transport, and vascular tone; whereas HO-1 is inducible by heavy metals, cytokines, UV light, oxidative stress, inflammatory cytokines and many drugs (Figure 3) [4]. They catalyze the degradation of heme in an identical manner.

Bone marrow derived MSCs are particularly susceptible to oxidative stress caused by bone marrow toxicity due to environmental pollutants, such as benzene [24], and azidothymine (AZT) treatment for HIV infected patients [25]. Additionally, radiation and chemotherapeutic drugs affect MSCs and the bone marrow microenvironment [26, 27]. These toxins also cause severe bone marrow suppression, manifested as anemia and leucopenia. Several reports showed anemia and neutropenia, occurring as a result of AZT treatment, could be reversed by the induction of HO-1 by hemin [14, 28]. Heme alone and in combination with erythropoietin promoted effective erythropoiesis during AZT therapy [14, 28]. IL-10 regulates inflammatory and immunosuppressive responses in a HO-1 dependent manner [29, 30]. These studies illustrate an important immunoprotective role of increased HO-1 expression within the bone microenvironment. HO-1 is thus crucial in protecting against oxidative stress caused by the generation of free radicals and bone marrow toxicity, by providing a rich environment for MSC growth and stem cell differentiation. Thus a number of questions arise: 1) Is HO-1 required for the maintenance of a healthy marrow stromal environment and MSC, 2) What would happen if there is a deficit of HO-1 within the bone marrow? How would such a defect affect stem cell differentiation and growth? 3) Are there any disease states that are related to both low HO-1 expression and HO activity within the bone marrow? 4) Can upregulation of HO-1 rescue these deficits?

A decrease in HO-1 expression is frequently associated with increased superoxide production and oxidative stress leading to impairment of MSC function and fat content in bone marrow [3]. Additionally, a decrease in HO-1 gene expression levels in Type 2 Diabetic patients has been reported [31–33]. Based on these findings, using a combination of MSCs transplant with an HO-1 inducer prevents Type 2 Diabetes in obese mice [34], suggesting that adipogenesis may result from changes in MSC phenotype as a result of genetic modulation.

It has been suggested that diseases such as Diabetes Type 1 and 2, and other autoimmune diseases are actually disorders in stromal MSCs and their microenvironment [35–37]. A series of experiments using bone marrow transplantation of the entire bone marrow (including both MSCs and HSCs) has been used successfully for the treatment of diabetes [38, 39]. To demonstrate autoimmune disease such as diabetes are originally stem cell disorders, autoimmune-prone mice which show thymic abnormalities, were transplanted with either thymus or bone marrow from normal mice, and vice versa [40, 41].

Oxidative stress has long been implicated in the pathogenicity of insulin resistance in Type 2 Diabetes and of cardiovascular complications [42, 43]. Oxidative stress is one of the underlying mechanisms in the pathogenesis of hyperglycemia induced tissue damage, β cell dysfunction, and endothelial dysfunction. Hyperglycemia increases O_2^- production within the mitochondria leading to several downstream cellular events culminating in oxidative tissue damage within multiple organ systems. Chronic oxidative stress has also been linked to glucose toxicity and cellular destruction of β cells in Type 2 Diabetes [43, 44]. Thus induction of β cell death, whether induced by oxidants administered exogenously elicited by cytokines, occurs through an imbalance between apoptotic and anti-apoptotic processes. O_2^- reacts readily with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). Peroxynitrite formation reduces NO bioavailability and induces oxidative damage to proteins (tyrosine residues), nucleotides and cellular membranes. It may also diffuse to subendothelial layers and induce LDL oxidation in turn accelerating foam cell formation and atherosclerosis. Thus, without an increase in the antioxidant system to compensate for the increases in ROS, increased oxidative stress leads to the activation of stress-sensitive intracellular pathways and the formation of gene products that cause cellular damage and contribute to the late complications of diabetes [45].

The levels of HO-1 expression, HO activity and its products, CO and bilirubin, are decreased in humans and in animal models of Type 2 Diabetes [32, 46]. HO-1 is a stress response, chaperone protein and one of the mechanisms by which it reduces ROS is by increasing glutathione and extracellular superoxide dismutase levels and by simultaneously decreasing O_2^- production [47]. Therefore, the decrease in HO-1 expression which is associated with increased superoxide production and oxidative stress may lead to impairments of MSC function and become a major contributing factor to the pathogenesis of metabolic syndrome. Increased levels of HO activity also attenuate the production of ROS through the extra soluble dismutase [48, 49] biliverdin and CO. Both biliverdin and bilirubin are natural antioxidants and the levels of bilirubin are inversely related to the atherogenic risk. In addition, bilirubin inhibits oxidation of LDL and acts as a potent scavenger of oxygen radicals [50].

Thus there are a number of contributing factors involved in the pathogenesis of diabetes. However, the role of HO appears to be central to elucidating the pathogenesis of diabetes. Thus it is proposed to study a model in which the only defect is HO deletion, so that the effects of HO-1 can be studied in isolation. Under these experimental conditions, the contributions of HO deletion to MSC function and development of a depository insulin resistance can be determined. This is the central theme of this review.

Epoxyeicosatrienoic Acids (EETs)

There is a secondary cytoprotective system that is perturbed during the diabetic state and in HO-2 deletion: i.e., the CYP450 system. CYP450 derived metabolites have a broad spectrum of biological activities including regulation of vascular tone, cellular proliferation, inflammation and a number of other regulatory functions [51, 52]. It has been known for four decades that the heme HO system and CYP450 systems are interlinked. Elevated levels of HO activity lead to a concomitant decrease in CYP450 levels and CYP450 dependent functions [53]. Early studies established the CYP450 enzyme system as an endogenous arachidonic acid (AA) metabolic pathway with potential functions in cell and organ physiology [52, 54]. AA is present *in vivo* esterified to cell membrane glycerophospholipids and in response to hormonal stimulation, phospholipases are activated to release AA from membrane phospholipids [55].

In the presence of NADPH and molecular oxygen, CYP-450 metabolizes AA to several oxygenated metabolites, including four regioisomeric epoxides [(1) 5,6; 8,9; 11,12; 14,15 epoxyeicosatrienoic acids (EETs)], (2) six regioisomeric cis-trans-conjugated mono-hydroxyeicosatrienoic acids (HETEs); (3) ω and ω -1 alcohols [52, 56] (Figure 4).

The four regioisomers can be hydrolyzed enzymatically by soluble epoxide hydrolase (sEH) to the corresponding diols (dihydroxyeicosatrienoic acids [DHTs]) (Figure 5). Although rapid conversion of EETs to their corresponding diols has been viewed as rendering them biologically inactive, there is evidence that DHETs do have some activity, albeit reduced activity and cellular function [57–62].

Human MSCs and kidney CYP450 metabolizes AA to EET and 20-HETE [63, 64]. Multiple CYP450 families contribute to EET biosynthesis in a given tissue and/or cell type, in humans CYP2C and CYP2J are primarily responsible. EET biosynthesis can be influenced by a number of factors including those that affect P450 expression and or activity, nutritional state, and genetic variations. ROS, specifically H₂O₂, decreases EET synthesis [65]. CYP450 production of DHETs and EETs are reduced in renal tubular sites during treatment of rats with a high fat diet [66]. Genetic polymorphisms in CYP450 genes have resulted in reduced AA epoxygenase activity [67]. These results, when taken together, underscore the importance of environmental and genetic factors in altering epoxide synthesis and biological function.

EETs are both autocrine and paracrine mediators and function primarily in the cardiovascular and renal systems. EETs exhibit well defined vasodilatory, anti-inflammatory, and antiapoptotic actions. Due to their strong vasodilatory actions within renal arterioles and the endothelium, EETs remain a principal candidate for the role of endothelium derived hyperpolarizing factor (EDHF). EETs dilate the preglomerular arterioles by activating the renal smooth muscle cell Ca(2+)-activated K(+) channels and hyperpolarizing smooth muscle cells [68]. CYP450 metabolites also act as second messengers for many paracrine and hormonal agents, including a number of vasodilatory substances, namely endothelin, angiotensin, NO, and PGI₂. These results and others are consistent with identifying EETs as EDHFs.

Recently, it has been reported that EETs have important non-vasodilatory roles, specifically in diseases such as diabetes and obesity [69, 70]. Under obese conditions, such as the Zucker obese rat, CYP-450 expression is decreased and sEH activity is increased [71]. Total adipose sEH activity was higher in obese mice. PPAR γ agonists increased the expression of sEH in mature 3T3-L1 adipocytes *in vitro* and in adipose tissue *in vivo* [72]. Additionally, EET stimulation directly affects the endocrine functions of the pancreas in regulating blood glucose via insulin and glucagon release. Arachidonic epoxygenase CYP2J is expressed and

highly localized in the islets of Langerhans cells and especially the glucagon producing alpha cells in both humans and rats [73]. EETs stimulate the release of both glucagon and insulin from isolated rat pancreatic islets [74]. There is regioisomer specificity associated with this release e.g.; 5,6-EET directly stimulated the release of insulin but has no effect on glucagon release, whereas 8,9-, 11,12-, and 14,15-EETs increased glucagon release without affecting insulin secretion [74].

The metabolic actions of EETs become more complex as metabolic products. DHET have been shown to be involved in lipid metabolism. Both 14,15 EET and 14,15 DHET activate and bind to PPAR α and PPAR δ which regulate the β -oxidation of fatty acids [75]. Thus, DHETS may have potential biological effects [76]. Though these particular effects of EETs and DHETS are poorly understood, these findings suggest that both EETs and DHETS play a role in both lipid metabolism and inflammation and therefore may play a critical role in signaling processes involved in diabetes and obesity.

The existence of a CYP-450 dependent system within the bone marrow has been hypothesized since 1981 [77]. Human bone marrow adherent stromal cells (MSCs) possess a CYP450 system that metabolized AA [63]. Furthermore, CYP450-arachidonic metabolites are released from adherent cells in the bone marrow and are present in cellular incubation mixture [63]. However, the role of these arachidonic metabolites including EETs within the bone marrow remains unclear.

Since both the heme-HO and CYP450 systems expression are diminished within both the diabetic and obese states and are both involved within the hematopoietic system, the low levels of these two systems could influence MSC differentiation in bone marrow leading to a specific lineage, i.e., an increase in adipogenesis. Further studies are warranted to examine the relationship of EET and HO-1 within development of adipocyte function.

Process of Adipogenesis- Adipocyte Development

The development of adipocytes in mice and humans follows a defined pathway that begins with a common MSC that is pluripotent and is referred to as adipogenesis [78]. The early steps of the pathway leading to the generation and the commitment of MSCs to the adipocyte lineage is unknown. Hypothetically, the determination of the fate of MSCs occurs early in the stages of cell differentiation ("commitment") and involves the interplay of intrinsic (genetic) and environmental (local and systemic) conditions to ultimately define cell fate. Factors such as those discussed earlier that determine MSC proliferation and differentiation also govern early adipocyte development and function. Currently, however, little is known about this process- from MSC to preadipocyte differentiation. The steps governing the process from preadipocyte to adipocyte differentiation however, have been well defined.

During adipogenesis MSC-derived or preadipocytes differentiate into lipid-laden and insulin-sensitive adipocytes [79]. An overview of the stages of adipogenesis is presented in Figure 6. The acquisition of adipocyte phenotype and development of adipocyte function is characterized by chronological changes in the expression of multiple genes. This process is highly regulated by the appearance of early, intermediate and late mRNA/protein markers and triglyceride accumulation (Figures 1 and 7).

Briefly, the stages of adipocyte differentiation are: (1) MSC-derived adipocyte growth arrest; (2) clonal expansion, (3) a second stage of growth arrest or early differentiation and (4) terminal differentiation- development of mature adipocyte phenotype [80, 81]. Concurrently with the development of a mature adipocyte, there is an increase in transcription or *de novo* expression of several genes including- Glut 4, insulin receptor, fatty

acid synthase (FAS), [80]. In the early phase of differentiation, preadipocyte cells are morphologically similar to fibroblasts. After clonal expansion, continued induction of adipogenesis leads to a drastic change in cell shape. Preadipocytes convert to a spherical shape, lipid droplets accumulate and the preadipocyte progressively acquires the morphological and biochemical characteristics of a mature adipocyte followed by triglyceride accumulation [81].

PPAR γ and c/EBP α are central components of this network and are critical factors that initiate a cascade of other factors that enhance differentiation of adipocytes. A secondary level of control exists, in which recruited coactivators and corepressors ultimately define the phenotype and metabolic fate of differentiated adipocytes.

PPARs are part of the steroid/retinoid nuclear hormone receptor superfamily and include isoforms α , γ , and δ [78]. PPARs play a role in cellular development, differentiation, and metabolism. PPAR γ , specifically, is crucial for adipogenesis and is necessary [82], along with C/EBP α , for adipocyte differentiation. PPAR γ for differentiation, survival and metabolism. PPAR γ is required for the maintenance of adipocyte differentiation. In differentiated 3T3L-1 cells, dominant-negative PPAR γ expression led to dedifferentiation with a resultant loss of lipid accumulation and a decreased expression of adipocyte markers [83]. Additionally, the deletion of PPAR γ within the germline renders these cells non-viable.

PPAR γ can be activated by both endogenous and exogenous synthetic ligands. Endogenous ligands of PPAR γ are largely unknown, but several lipid metabolites have been implicated including polyunsaturated fatty acids and eicosanoids [79]. Synthetic ligands include thiazolidinediones (TZDs), a class of widely used drugs that are known PPAR γ agonists, that act specifically to increase insulin sensitivity. TZDs increase insulin sensitivity by stimulating muscle glucose disposal and inhibiting hepatic glucose output. There are three plausible mechanisms that could explain how the activation of PPAR γ acts as a systemic regulator of insulin sensitization. Firstly, pharmacological activation of PPAR γ leads to adipose expansion through adipocyte hyperplasia, increasing the number of new preadipocytes. These adipocytes are small in size and able to effectively store lipids, thereby reducing lipotoxicity in the muscle and liver and release of adiponectin [84, 85]. This process involves activation of the genes encoding molecules that promote lipid storage and lipogenesis, *i.e.* aP2 (fatty-acid binding protein), CD36 (receptor for lipoproteins), and FATP-1 (fatty acid transporter) [85]. Activation of these genes leads to repartitioning of lipids resulting in increased triglyceride content of adipose tissue, lowered free fatty acid content in circulation and availability for liver and muscle use, thereby improving insulin sensitivity. Secondly, PPAR γ agonists act by inhibiting the expression of inflammatory cytokines including TNF- α which promote insulin resistance [86]. Thirdly, PPAR γ agonists stimulate the production of adiponectin, which promotes fatty acid oxidation and insulin sensitivity in muscle and liver [85], thereby decreasing glucose output by the liver and increasing glucose usage by the muscle. Thus, PPAR γ is necessary for induction of adipogenesis, maintenance of the adipocyte lineage, and to act as a systemic regulator of insulin sensitivity and adipokine production.

The CCAAT enhancer binding proteins (C/EBPs) belong to the basic-leucine zipper class of transcription factors which are all expressed during adipogenesis [80]. C/EBP β and C/EBP δ are expressed early during adipogenesis and promote the main regulators of terminal adipocyte differentiation, PPAR γ and C/EBP α . The interaction between PPAR γ and C/EBPs establishes the mature state of adipocytes with all of their appropriate and necessary functions. The sequence of events is as follows: an increase in C/EBP- β above a threshold level induces expression of PPAR- γ . Upon ligand activation, PPAR- γ , in concert with C/

EBP- α , leads to full adipocyte differentiation [81]. After PPAR γ stimulation, fat cells lacking C/EBP α , are able to accumulate lipid, express most adipogenic markers, but have poor insulin sensitivity due to lower levels of insulin receptor and low insulin receptor substrate-1 (IRS-1) production [80]. In addition, insulin-dependent glucose uptake is also completely absent, demonstrating a crucial role for C/EBP α in regulation of insulin sensitivity. Taken together, current data demonstrates the necessity of both C/EBP α and PPAR γ in not only inducing development of adipocytes but also their importance in establishing adipocyte function; i.e. glucose uptake and insulin sensitivity (Figure 7).

The physiological stimuli that control MSC differentiation *in vivo* are largely unknown. However, induction of adipogenesis *in vitro* requires a “differentiation cocktail” containing high glucose, insulin, glucocorticoids, and additional factors such as dexamethasone, methylisobutylxanthine and/or indomethacin. These induce the specific signaling cascades necessary to switch the genetic programming from preadipocytes (or stem cell fate) to mature adipocyte mode. Insulin is a required regulator of both early and late adipogenesis. In the early stages of adipogenesis, insulin mediates its actions through insulin growth factor-1 receptor signaling, and later through insulin receptor as insulin receptors increase during the late stages of adipogenesis. Further down the insulin cascade, inhibition of phosphatidylinositol-3-kinase (PI3K) as well as loss of *Akt1* or 2/protein kinase B (PKB) represses adipogenesis [87]. The insulin signaling cascade eventually leads to activation, directly and indirectly through blockade of repressors of C/EBP α and PPAR γ . Insulin also promotes glucose uptake in both muscle and adipose tissue as well as triglyceride catabolism [85], regulating the normal metabolic functions of the mature adipocyte. Glucocorticoids are part of the nuclear hormone family, and their actions are not well characterized in adipocyte differentiation. However, *in vitro* studies have shown that glucocorticoid treatment is either required for differentiation or acts to accelerate the process. In 3T3L-1 cells, this action is activated through induction of C/EBP- δ [82]. Methylisobutylxanthine (MIX), an inhibitor of phosphodiesterases, increases intracellular cAMP, activating adipocyte differentiation in a PKA- independent manner [88]. MIX has also been shown to increase the expression of C/EBP- β which is required for the subsequent expression of PPAR- γ [81]. Similarly, indomethacin acts as an upstream regulator of PPAR γ 2, by increasing the expression of C/EBP- β [89].

Although factors controlling adipogenesis *in vivo* are largely unknown, there are a number of candidates that mediate adipocyte differentiation in culture (*in vitro*) and are thought to control adipocyte accumulation and function *in vivo*. Two main factors fit this criterion: (1) high glucose and (2) ROS. They have been implicated as the link between adipogenesis and metabolic diseases such as T2DM. As described earlier, recent studies have demonstrated induction of oxidative stress by high glucose (through three mechanisms, NAD(P)H oxidase, xanthine oxidase, and mitochondrial respiratory chain) is associated with diabetic complications [90]. Therefore, these factors may lead to adipocyte differentiation associated with adipocyte dysfunction and formation of adipocytes external to normal adipocyte depots i.e. muscle, liver, and pancreas leading to advanced diabetic complications.

How do ROS and high glucose lead to adipocyte dysfunction and insulin resistance? Firstly, we must define what adipocyte function is. Adipose tissue is a key endocrine organ whose functions include (1) lipid uptake, storage, and synthesis; (2) secretion of endocrine, paracrine, and autocrine factors that regulate insulin sensitivity and glucose uptake; and (3) secretion of anti-inflammatory molecules such as adiponectin, IL-1, and IL-10 (Figure 8). Critically, increases in adipocyte size result in decreased secretion of adiponectin, and increased secretion of inflammatory molecules such as MCP-1, IL-6, and TNF- α that have systemic effects on other tissues including, vasculature, liver, muscle, and β cells.

Adipokines, are highly diverse in terms of structure and physiologic function and are involved in the regulation of blood pressure (angiotensinogen), vascular haemostasis (PAI-1), lipid metabolism e.g. (retinol binding protein, cholesteryl ester transfer and angiogenesis (VEGF)). The release of these cytokines by adipocytes has led to the conclusion that adipose tissue has many systemic effects which are altered during adipocyte hypertrophy and obesity. The most studied adipokines include adiponectin, acrp30, which is exclusively secreted from adipose tissue and is known for its role in insulin sensitivity, and the pro-inflammatory cytokines TNF- α , IL-6, IL-1 β , IL-8, IL-10, and MCP-1 that are released during adipocyte hypertrophy and obesity [91, 92].

In summary, overnutrition (excessive amounts of FAs) and high glucose produces a hypertrophied adipocyte resulting in detrimental perturbations in both mitochondrial and ER function, both of which undergo cellular changes that result in the increased generation of ROS and activation of an inflammatory cascade. This culminates in the release of inflammatory mediators that are associated with insulin resistance and negative downstream effects (Figure 8).

Systemically, increases in ROS are related to the production of inflammatory cytokines, obesity and insulin resistance. As discussed earlier, induction of HO-1 decreases superoxide and ROS generation, resulting in an increase in adiponectin and a subsequent elevation in tolerance to ROS in the heart [93]. An inverse relationship between levels of ROS and adiponectin exists and was demonstrated in an elegant study that encompassed patients, cultured adipocytes and obese mouse models. Fat accumulation was closely correlated with markers of systemic oxidative stress [94]. In addition, plasma adiponectin levels correlated inversely with systemic oxidative stress. In cultured adipocytes, the oxidative stress suppressed mRNA expression, secretion of adiponectin and increased IL-6 and MCP-1 mRNA expression [34]. Furthermore, treatment with the NADPH oxidase inhibitor, apocynin, reduced oxidative stress in WAT and increased plasma adiponectin levels in KKA γ mice [94]. These results indicate that a local increase in oxidative stress in accumulated fat caused dysregulated production of adipokines. The down regulation of adiponectin expression was partially attributed to reduction of nuclear PPAR γ gene expression under conditions of oxidative stress [94].

Induction of HO-1 modulates metabolic syndrome, obesity, and insulin resistance [1, 3, 95–99]. The changes that are observed after increased expression of HO-1 in obese and diabetic animal models include: (1) prevention of weight gain (2) reduction of inflammatory cytokines levels (3) restoration of normal insulin sensitivity and (4) improved vascular reactivity. Taken together, these are the major components of metabolic syndrome and possible mechanisms by which each of the parameters are modified by HO.

Heme Oxygenase and Prevention of Weight Gain

A number of studies have demonstrated that HO-1 inducers, CoPP and/or L-4F, an apomimetic peptide, when administered to animal models of obesity result in a reduction of weight gain [3, 97] without a change in food intake. However, the mechanism(s) governing metabolic adaptation to food intake and fat loss during HO-1 induced weight loss are unknown. A number of potential mechanisms include the direct effect of increased levels of HO activity increasing serum adiponectin levels [3, 96]. Adiponectin acts as a “starvation signal”, therefore indicating that adipocyte cell size is small and available for triglyceride accumulation. This allows for the movement of ectopic fat deposition from muscle and liver to subcutaneous depots [84]. This is supported by the demonstration that HO-1 induction in the Zucker Diabetic Rats (ZDF) led to a decrease in both subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) with a greater decrease in VAT than in SAT as examined

by Magnetic Resonance Imaging (MRI) [96]. This is a possible indication of both adipose tissue remodeling and a shifting from visceral to subcutaneous depots, within SAT itself. Loss of visceral and subcutaneous fat could also be due to the use of fatty acids as an energy source during weight reduction [97, 100]. Another possible mechanism for the reduction in weight gain observed post HO-1 induction is a remodeling of adipose tissue to a more immature state. Since HO-1 induction does not result in a change in food intake, or other metabolic changes such as energy expenditure and O₂ consumption, this is indicative of a local effect of increased HO-1 expression on adipose tissue itself and not of changes within the central nervous system. Adipose tissue can be maintained in a relatively immature state compared with other organs that are weight stable. This is supported by studies showing in vitro induction of HO-1 leads to decreased adipogenesis, indicating an increase in the number of preadipocytes that have not yet undergone adipocyte differentiation [3, 101]. 8-week old obese male mice were injected intraperitoneally with 200ug/100mg of L-4F daily for 6 weeks. L-4F was discontinued at week 10 with obese mice gaining weight at a faster rate than obese vehicle mice. Recommencement of L-4F administration at 12 weeks decreased body weight to levels similar to those seen in obese animals continuously treated with L-4F. The changes during intermittent L-4F treatment are suggestive of continuous adipose remodeling [1]. Lastly, increased HO-1 expression resulting in the prevention of weight gain could be associated with AMP- dependent protein kinase (AMPK) phosphorylation. AMPK is considered a whole body energy sensor because it plays an important role in the regulation of energy expenditure and appetite [102]. Activation of AMPK via phosphorylation increases β -oxidation and decreases lipogenesis facilitating a reduction in formation of adipose mass. The neuronal control of food uptake is regulated via a complex interplay between anorexigenic and orexigenic signals converging on distinct neuronal populations within the hypothalamus [102]. Activation of AMPK within the hypothalamus results in an increase in food intake and weight gain. Although, HO-1 induction results in AMPK activation within adipose tissue resulting in reduced lipogenesis, there is no change in food intake, thus HO-1 appears to act locally within the adipose tissue and not centrally within the hypothalamus.

Heme Oxygenase and Reduction of Inflammatory Cytokines

Chronic low grade inflammation is a critical link between obesity and pathological complications stemming from obesity including insulin resistance and T2DM. The prevention of inflammation may be key to limiting disease progression from 'healthy' nondiabetic obese to diabetic obese. Therefore, the role of HO-1 in limiting inflammation becomes central to potential therapeutic applications. CO contributes to the anti-inflammatory properties of HO-1. A low concentration of CO inhibited the LPS-dependent production of macrophage-derived pro-inflammatory cytokines (i.e. TNF- α , IL-1 β , and macrophage inflammatory protein-1 β) whereas it increased the LPS-induced expression of IL-10 [4, 103]. Activation of both sGC and p38 MAPK has been implicated in the suppression of inflammatory cytokines by HO-1/CO activation [103, 104]. There may be additional, currently unknown, mechanisms by which HO-1 induction reduces inflammatory markers. Several recent studies have revealed that some well-known and commonly used drugs including aspirin, upregulate HO-1 expression and HO activity to attenuate cardiovascular inflammation [105] (PMID: 17101869). However, by which mechanism(s) this is accomplished remains unknown.

Heme Oxygenase: Restoration of Insulin Sensitivity

Induction of HO-1 has long-lasting anti-diabetic actions. The underlying mechanisms include the potentiation of agents implicated in insulin-sensitization and glucose metabolism such as GLUT4, adiponectin, AMPK, cAMP, and cGMP, along with elevation of

antioxidants such as SOD, catalase, ferritin, and enhancement of the total antioxidant capacity [4]. Up-regulation of HO-1 by a number of inducers increases plasma insulin release while concomitantly improving glucose tolerance (IPGT) and insulin tolerance (IPTT) [106]. The HO system is known to regulate insulin release, with CO playing a central role. Under normal physiological conditions, islets of Langerhans produce both CO and NO to regulate insulin and glucagon secretion. NO is a negative modulator of glucose stimulated insulin release, while CO stimulates insulin secretion. Additionally, oxidative stress destroys β -cells [107] and depletes adiponectin mRNA and, therefore, adiponectin production [108]. Another mechanism by which HO may increase insulin release is by the rescue of ROS-mediated pancreatic β cell destruction [109].

Adiponectin is well known for its insulin sensitizing actions via stimulation of 5'-AMP-activated protein kinase in muscle and liver [110] and by facilitating an increase in glucose uptake within the muscle as discussed earlier. We and others have also demonstrated increased expression of HO-1 enhances expression of GLUT-4. Reduced glucose uptake has been implicated as a cause of insulin resistance and glucose intolerance [111]. The mechanism by which HO enhances GLUT4 is still unclear.

Heme Oxygenase and Vascular Reactivity

The role of the HO system in vascular protection is well documented [112–115]. There are a number of mechanisms by which HO mediates the improvement in vascular function, and some specifically involve the release of CO. NO and CO share many properties, both behave as gaseous messengers and signaling molecules, suggesting that CO may have a similar physiological role of NO [116]. Like NO, HO-derived CO influences the sGC and cGMP pathways, both of which serve to regulate both blood pressure and vascular contractility [117]. CO is capable of acting via multiple mechanisms, including the direct modulation of cGMP levels and K^+ channels in smooth muscle cells and the indirect modulation of endothelium-dependent vasoconstrictors and myogenic factors [118]. However, the physiological relevance of CO as a vasodilator is controversial. CO is more chemically stable than NO but is much less potent than NO as a relaxing agent [119]. Additionally, since both biliverdin and bilirubin are efficient scavengers of ROS, and bilirubin is the most abundant antioxidant in most tissues; these offer cytoprotection of vascular cells against oxidative stress.

Heme Oxygenase and Adipogenesis

There is little information available on the relationship between HO and adipogenesis. It is known that as cells undergo adipogenesis, ROS production increases thus fully differentiated adipocytes produce significantly more ROS than preadipocytes [94]. Morphologically, there is an increase in the adiposity in cells differentiated under oxidizing conditions as determined by increased Oil Red O detection of lipid accumulation [120]. In terms of obesity, adipocytes, when compared to preadipocytes, are metabolically distinct due to the production of higher basal levels of intracellular ROS. Cobalt protoporphyrin (CoPP), reduced superoxide levels in MSCs derived from obese Zucker rats [3]. Therefore, administration of CoPP during adipogenesis would lead, it was proposed, to decreased adipogenesis. Human MSCs treated with adipogenic media over a two-week period and challenged with an additional 10 mM of glucose (hyperglycemic conditions) resulted in an increase in adipogenic differentiation. With the addition of CoPP, adipogenic differentiation decreased, indicating a preponderance of preadipocytes [101]. These results indicate that HO-1 may be influential in the regulation of genes controlling adipocyte differentiation. However, whether HO-1 upregulation can modulate adipogenesis and the potential effects

on further adipocyte function within a highly oxidative environment such as in an HO-2 deletion animal model remains to be elucidated.

EETs and Adipogenesis

The effect of EETs on adipogenesis and signaling cascades involving adipogenesis such as generation of HO-1, adiponectin, AMPK, and pAkt have recently come under intense scrutiny. Human MSC-derived adipocytes have been recently reported to express CYP-epoxygenase 2J2 and its products, EETs. EETs decreased adipocyte differentiation via an increase in HO-1 expression and a decrease in PPAR γ , C/EBP α and FAS levels. These results suggest that EETs act to inhibit and/or delay adipocyte differentiation and to regulate lipid metabolism in developing preadipocytes [121].

Since both HO-1 and EET levels play a role in immunological responses, and vascular diseases, our hypothesis is that MSC-derived adipocytes are not only a product of environmental stimuli and the right balance in ROS, but also HO and EET interactions contribute to adipogenesis and adipocyte function. Therefore, a decrease in either HO-1 or HO-2 may shift the balance in favor of increase in ROS, decreasing EETs, leading to adipocyte dysfunction and the release of inflammatory mediators.

Role of HO-1, HO-2 and EETs: Recent Observations

We have demonstrated that HO-2 plays a vital role in determining adipogenesis and visceral adipose function. We have defined a role for HO-1, in the regulation of body weight, adipocyte differentiation, and the secretion of inflammatory cytokines. The HO-1 rescue of HO-2 deficiency resulted in restoration of adipocyte function via cellular composition changes of lipid droplets, a decrease in secretion of inflammatory cytokines, and up-regulation of adiponectin. siRNA inhibition of either HO-2 or HO-1 lead to an increase in adipogenesis (Figure 9) and laid the foundation for examining the contribution of HO-1 in adipogenesis and in the rescue of metabolic syndrome. Previously, *in vivo* studies have demonstrated that HO-2 deficiency leads to manifestations of metabolic syndrome, including obesity, hypertension, and insulin resistance [122]. HO-2 deletion has also resulted in enhanced diabetes-induced renal dysfunction and morphological injury, and with induction of HO-1 these morphological changes are prevented [123].

Bone marrow derived MSCs from HO-2^{-/-} mice demonstrated an increase in adipogenesis and accumulation of lipid droplets resulting in adipocyte hypertrophy in HO-2^{-/-} mice. HO-2 deletion resulted in decreases in HO-1 protein expression within MSC-derived adipocytes compared to cells derived from WT mice. However, this is not surprising because HO-2 is critical for HO-1 expression and failure to up-regulate the HO system has been linked to several metabolic consequences i.e. oxidative stress [124, 125], insulin resistance [97], and renal damage secondary to hypertension [126, 127]. Therefore, lack of HO may cause an imbalance within the system contributing to the ROS-mediated generation of inflammatory products and associated systemic complications. Bilirubin and CO act as powerful antioxidants and at normal concentrations, unconjugated bilirubin is an efficient scavenger of singlet oxygen and acts as a reducing agent. Bilirubin plays a protective role by acting as a reducing agent in cells with high oxidative stress generated by hydrogen peroxidase. Bilirubin can act also by inhibiting the generation of oxidants via inhibition of protein kinase C and NADPH oxidase. This may be one of the mechanisms by which increased HO-1 expression attenuates the diabetes-mediated generation of oxidants. CO, though not an antioxidant, causes induction of antioxidant genes. Lack of HO activity or insufficient production of heme degradation products also contribute to inflammation directly. Direct anti-inflammatory effects of CO have been observed both *in vitro* and *in vivo* in experimental model of sepsis [128]. Pretreatment with CO, inhibits

lipopolysaccharide-mediated expression of proinflammatory cytokines such as TNF- α , IL-1 β , and macrophage inflammatory protein-1 β , while simultaneously increasing the expression of anti-inflammatory cytokine IL-10 in both endothelial cells and macrophages. In addition, the activation of sGC and p38 in an mitogen-activated protein kinase (MAPK) have been implicated in the suppression of inflammatory cytokines by HO-1/CO activation [97]. Hence, it is likely the diminished activity of the HO system may contribute to increased oxidative stress and inflammatory conditions and subsequent derangements in adipocyte and metabolic function.

These findings indicate that induction of HO-1 caused a decrease in droplet formation and adipogenesis in HO-2^{-/-} bone marrow-derived adipocyte stem cells but increased secretion of adiponectin in the culture media. This novel observation was accompanied by the demonstration that induction of HO-1 resulted in a reciprocal decrease in secretion of TNF- α , IL-1 α , and MCP-1. These results were reversed with SnMP mediated non selective inhibition of HO activity. The decrease in lipid droplet size indicative of smaller adipocytes, decreased inflammatory cytokine levels accompanied by increases in adiponectin levels *in vitro* are suggestive of changes in the preadipocyte environment similar to changes seen in treatment of metabolic syndrome.

Although, these findings clearly indicate limiting adipocyte size is a prerequisite for healthy metabolic function adipose expansion also leads to a complete normalization of metabolic parameters despite morbid obesity [84]. Though, at first this may seem contradictory to the findings presented here; the data is in partial agreement to these findings. This phenomenon is described in the treatment with thiazolidiones and PPAR γ agonists in a transgenic *ob/ob*^{-/-} mouse model overexpressing adiponectin. In this animal model, the mice had massive adipose tissue accumulation along with normal glucose levels, insulin, triglyceride, and FFAs were significantly lowered even after exposure to glucose and lipid challenges. In addition, TNF- α and IL-6 were significantly reduced suggesting that macrophage infiltration and inflammation were minimal due to the fact that these mice displayed hyperplasia, increasing the number of small adipocytes that are more insulin sensitive without hypertrophy. This may be one reason that a reduction in adipogenesis and adipocyte size results in improved metabolic function. In contrast, the induction of HO-1 leads to an increase in preadipocytes, the precursors of new mature healthy adipocytes while lowering the number of hypertrophic adipocytes. Further experiments are required to determine whether one or both of these mechanisms are pertinent.

A second concern is with the use of pharmacological inducers and inhibitors, as there is always a concern of sensitivity and specificity. Inducers and inhibitors such as CoPP and SnMP have some non-selective activity and may act differently under certain experimental conditions. However, these discrepancies remain minimal and future use of multiple inducers of HO-1 can give further proof, therefore more evidence that results are specifically due to HO-1 induction.

A third consideration within this study was whether HO is the only system that is affected and contributes to adipocyte hypertrophy and adipocyte dysfunction. As stated earlier, previous studies have indicated that HO-2 deficiency leads to a metabolic syndrome phenotype. *In vivo* treatment of these mice with an EET agonist ameliorated the apparent metabolic syndrome phenotype via increases in adiponectin and with associated increases in pAMPK signaling. Additionally, aortic CYP2C11 was markedly reduced in HO-2^{-/-} mice. Decreased CYP2C11 protein expression paralleled decreases in the levels of renal and vascular EETs and estimated epoxygenase activity [122]. We examined whether decreased EET expression occurred in MSCs prior to adipogenesis in HO-2 null mice and then determined the contributions of EET to EET levels are decreased in bone marrow derived

cells prior to adipocyte generation from HO-2 null mice; and this deficit can be restored with HO-1 induction via CoPP. This is also a decrease in activity of the arachidonic acid metabolic pathway that yields EETs. Treatment with an EET agonist (AKR-I-27-28) was effective in suppressing adipogenesis in a dose dependent manner (Figure 10). EETs behaved in a similar manner to CoPP in the reduction of inflammatory cytokine MCP-1, TNF- α , and IL-1 α with an accompanying increase in adiponectin secretion *in vitro* (Figure 10).

Together, these results demonstrate an interplay between the HO and EET systems both prior to and during adipocyte development. Also, HO is a requirement for EET production. In turn, EETs can act to induce HO-1 and therefore rescue adipocyte function *in vitro*. The specific mechanisms by which HO-1 and EET regulate adipogenesis and adipocyte metabolism require further study, but a number of mechanisms are likely to participate. In addition to the vasodilatory effects of EETs, EETs also produce an anti-inflammatory effect on the endothelium via the inhibition of cytokine induced NF κ B transcription and activation of PPARs [70, 129–131]. In addition to their beneficial effects in the vasculature and their anti-inflammatory actions, EETs also affect lipid metabolism and insulin sensitivity. Also, CYP expression is decreased and sEH expression is increased in both obese Zucker rats [71] and human MSC derived adipocytes [121]; indicating deficiency in EET production and increased EET degradation occurs early in developing adipocyte hypertrophy and dysfunction. Previously, EETs were shown to be potent inducers of HO-1 and HO activity, suggesting one of the possible mechanisms involved in their actions on inflammation may be through up-regulation of the HO system [132]. The results described here confirm a positive feedback loop in which HO-1 induction increases EET levels, and vice versa [121]. Another possible mechanism by which HO increases EETs may be related to the well known antioxidant actions of the HO system. Oxidative stress is an integral component of metabolic syndrome that plays a role in both endothelial dysfunction and insulin resistance becoming a causal factor in disease progression and future risk outcomes [94, 133, 134]. Additionally, oxidative stress and O₂⁻ reduce EET levels [66, 130]. A lack of HO-2 creates an environment that promotes oxidative stress-related disturbances reflected in increased levels of O₂⁻ and decreases in EC-SOD [48, 49]. Therefore, suggesting the highly pro-oxidant environment in HO-2^{-/-} mice allows for reduced production of EET levels and thereby possible activation of the ER stress response initiating the inflammatory signaling cascade [121, 135]. Our results strongly suggest this cascade can be prevented via HO-1 induction. Induction of HO-1 in obese and diabetic models, increases HO activity resulting in decreases in oxidative stress and ROS production and a reestablishment of the antioxidant and ROS balance [101], restoration of EET levels, attenuation of adipogenesis, and prevention of inflammatory cytokine recruitment *in vitro*.

In vitro studies provide much information about early cellular events that take place during adipose development, but are limited in terms of determining depot specific differences. In particular, triglyceride and fat accumulation within the visceral adipose depot has been associated with multiple abnormalities in insulin signaling, glucose intolerance, hypertension, and dyslipidemia that characterize metabolic syndrome and contribute to an increased risk of atherosclerosis and coronary syndrome in these patients. Additionally, subcutaneous fat tissue which is considered a more healthy fat tissue differs from visceral adipose tissue in three main ways (1) expression of specific receptors; (2) secretion of a specific adipokine pattern; and (3) location [136–138]. Therefore, we examined whether HO expression is diminished in the visceral adipose tissue and whether upregulation of HO gene expression within two models of obesity: (1) HO-2 null mice and (2) *ob/ob* leptin deficient mice would attenuate adiposity and shift the adipokine secretion pattern *in vivo*. In vivo, weekly treatments of CoPP resulted in the prevention of weight gain in HO-2 deficient mice. Gene expression levels of HO-1, adiponectin, and CYP2J5, a CYP450 epoxygenase that

metabolizes arachidonic acid into EETS, were significantly lower in visceral adipose tissue of HO-2^{-/-} mice compared to WT mice. Induction of HO-1 resulted in restoration of HO-1, CYP2J5, and adiponectin mRNA to levels similar to that of WT mice, indicating EET and adiponectin production are reduced within the visceral adipose tissue of HO-2^{-/-} mice but can be rescued. Several reports have determined visceral adipose tissue in obese individuals and reported that they have a different gene expression profile compared to lean individuals [139]. As to whether upregulation or downregulation of certain genes will be curative or therapeutic is a complex matter and still unknown. Much information obtained is still unclear within this area. However, findings described above provide definitive evidence for the involvement of the HO-Adiponectin-EET axis in adipogenesis and adipocyte signaling both *in vitro* and *in vivo*.

To determine further whether targeting the HO system *in vivo* would be useful as a potential therapeutic strategy in metabolic syndrome, HO expression and induction was studied in *ob/ob*^{-/-} mice. Induction of HO-1 regulated adiposity in *ob/ob*^{-/-} via an increase in adipocyte HO-1 protein levels. EET levels were decreased within bone marrow cells isolated from *ob/ob*^{-/-} mice and up-regulated with CoPP treatment, confirming results in the HO-2^{-/-} mice that both HO and EET are deficient during obesity, and CoPP rescues both systems. A third novel finding is that induction of HO-1 was associated not only with a decrease in adipocyte cell size but with an increase in adipocyte cell number *in vivo*. Upregulation of HO-1 was also associated with increased levels of adipocyte pAKT, pAMPK and PPAR γ . Previous studies have indicated that insulin resistance and impaired PI3K/pAKT signaling can lead to the development of endothelial dysfunction [140]. Increased HO-1 expression was associated with increases in both AKT and AMPK phosphorylation; these actions may protect renal arterioles from insulin mediated endothelial damage. By this mechanism, increased levels of HO-1 limit oxidative stress and facilitate activation of an adiponectin-pAMPK-pAKT pathway and increased insulin sensitivity. Induction of adiponectin and activation of the pAMPK-AKT pathway provides vascular protection [141, 142]. A reduction in AMPK and AKT levels may also explain why inhibition of HO activity in CoPP-treated *ob/ob*^{-/-} mice increased inflammatory cytokine levels while decreasing adiponectin. The action of CoPP in increasing pAKT, pAMPK and PPAR γ is associated with improved glucose tolerance. Insulin resistance is an independent factor for the development of both endothelial [140] and vascular dysfunction [143, 144]. CoPP improved vascular function as manifest by increased insulin sensitivity and pAKT and pAMPK levels.

Others have shown that increased phosphorylation of insulin receptors and vascular function may be a response to the increase in pAMPK and pAKT crosstalk [145–147]. Further activation of pAMPK and pAKT increase glucose transport, fatty acid oxidation and mitochondrial function [148–150]. pAKT and AMPK act as fuel sensors in the regulation of energy balance and the resultant the crosstalk of AMPK-AKT has been shown to regulate NO bioavailability and vascular function [146, 151, 152]. Furthermore, activated AMPK alone has been suggested as therapeutic target to ameliorate endothelial dysfunction [153–155]. The novel effect of CoPP on the HO-1-adiponectin-pAKT-pAMPK-module i.e., an increase in HO-1, increases in adiponectin and the subsequent increase in AKT-AMPK crosstalk and signaling pathway provide a beneficial mechanistic basis for CoPP mediated vascular protection. Thus CoPP appears capable of reprogramming adipocytes resulting in the expression of a new phenotype containing adipocytes of reduced cell size, increased number and restored insulin sensitivity. This approach, based on the induction of HO-1 in adipocytes, offers potential as a therapeutic means of addressing obesity mediated metabolic derangements and restoring vascular function.

HIGHLIGHTS

>HO-1 expression diminished during adipogenic differentiation of MSCs. > EET agonist increase HO-1 expression. > EET agonist may suppress the recruitment of adipocyte signaling pathways in adipose tissue. > HO-1 or EET essential for suppression of PPAR-gamma and aP2. > EET and HO-1 increases adiponectin-pAMPK signaling.

Abbreviations

AA	Arachidonic acid
Acrp30	Adiponectin
5-ALA	5-Aminolevulinic acid
AMPK	AMP dependent protein kinase
AZT	Zidovudine
C/EBP	CCAAT enhancer binding protein
cGMP	cyclic guanosine monophosphate
CO	Carbon monoxide
CoPP	Cobalt protoporphyrin
EC-SOD	Extracellular superoxide dismutase
EDHF	Endothelium derived hyperpolarizing factor
EETs	Epoxyeicosatrienoic acids
ER	Endoplasmatic reticulum
Glut4	Glucose transporter 4
HIV	Human immunodeficiency virus
HO	Heme oxygenase
H₂O₂	Hydrogen peroxide
HSCs	Hematopoietic stem cells
IL-1	Interleukin 1
IRS-1	Insulin receptor substrate 1
KKAy	Yellow Kuo Kondo obese mouse
LDL	Low density lipoprotein
MAPK	Mitogen activated protein kinase
MCP-1	Macrophage chemoattractant protein 1
MIX	Methyl isobutylxanthine
MSCs	Mesenchymal stem cells
NO	Nitric oxide
pAKT	Phosphorylated 5' - Adenosine monophosphate (AMP) protein kinase
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A

PPAR	Peroxisome proliferator activated receptor
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
sGC	Soluble guanylate cyclase
SnMP	Tin (Sn ⁴⁺) mesoporphyrin
T2DM	Type 2 diabetes mellitus
TNF-alpha	Tumor necrosis factor alpha
TZDs	Thiazolidinediones
VAT	Visceral adipose tissue
WAT	White adipose tissue
WT	Wild type

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Figure 1.

A schematic diagram of conditioning of MSCs by microenvironmental niches.

Differentiation potential is based on the position of a given cell within the colony. Clonally derived MSCs behave differently in response to soluble factors because of microenvironmental preconditioning.

Figure 2. Enzyme intermediates of heme biosynthetic and degradative pathways. Modified from [20, 21, 156].

Figure 3.
Schematic representation of the heme degradative pathway.

Figure 4.
The three metabolic pathways of arachidonic acid (AA). HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid.

Epoxyeicosatrienoic Acids

Figure 5.
Schematic diagram of epoxyeicosatrienoic acid synthesis from arachidonic acid and conversion into corresponding diols by epoxide hydrolase.

Figure 6.

Molecular mechanisms underlying adipocyte dysfunction in a hypertrophied adipocyte. Hyperglycemia results in the increase of ROS production within the mitochondria via a number of mechanisms including a reduction in the glutathione/glutathione disulfide ratio. Overloading the endoplasmic reticulum (ER) with FFA results in impaired protein folding and an unfolded protein response (UPR). UPR results in an increase of reduced molecular oxygen which acts as cellular toxic ROS. ROS generation mediates a pro-inflammatory cascade resulting in insulin resistance.

Figure 7.

The effect of HO-1 and HO-2 siRNA on adipogenesis. Lipid droplets area was determined by Oil red O staining after 14 days. * $p < 0.01$ vs. control and # $p < 0.001$ vs. HO-1 siRNA. Each bar represents means $\pm$ SE of 5 independent experiments. SnMP, inhibitor of HO activity was used as a control.

Figure 8.
Overview of stages of adipocyte differentiation. PPAR-γ, peroxisome proliferator-activated receptor-γ; C/EBP,CCAAT/enhancer binding protein).

Figure 9.

Dose response effect of EET agonist AKR-I-27-28 on HO-2 $\square$ /- derived MSC adipocyte differentiation. EET agonist AKR-I-27-28 attenuates adipocyte differentiation in a dose dependent manner.

FIG 10.
HO-1 and EET effects on adipogenesis and adipose tissue.