Doctoral Dissertation

ROLE OF HEME OXYGENASE IN METABOLIC SYNDROME RELATED DISEASES

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ABSTRACT

Several hundred million people throughout the world have the metabolic syndrome (MetS), a cluster of metabolic and cardiovascular disorders. Obesity is one of the main underlying risk factors for the metabolic syndrome. The increasing prevalence of obesity and the metabolic syndrome is alarming because they increase the risk of type 2 diabetes, liver disease, cardiovascular disease and premature mortality. Adipose tissue is a key player in whole body metabolism and excess adipose tissue poses a major risk factor for the development of metabolic disorders. In response to nutritional overload, *de novo* adipocyte differentiation can serve as an adaptive mechanism by increasing the storage capacity of adipose tissue and maintaining normal adipocyte function. This in turn prevents systemic lipid overload, insulin resistance and low grade systemic inflammation, which are the major cause of Non alcoholic fatty liver disease. This PhD thesis is focused on Heme Oxygenase, an important antioxidant enzymatic system involved in many physiological and pathophysiological processes, and provides novel insights of its role into the regulation of adipogenesis, insulin resistance and hepatic lipid metabolism. **Chapter 2** describes the effect of N-acetylcysteine (NAC), a powerful antioxidant and precursor of glutathione, commonly used to treat chronic obstructive pulmonary disease, on triglycerides accumulation in bone marrow stromal cells-derived adipocytes. Adipocytes were treated with a fatty acids overload, miming obesity condition, and NAC that increased adiponectin, HO-1 and beta-oxidation markers, ameliorating the impaired adipocytes functions. **Chapter 3** investigate if the potentially protective effect of phenethyl ester of caffeic acid (CAPE), one of the main components of propolis with a flavonoid-like structure, and of a novel CAPE analogue, as heme oxygenase-1 (HO-1) inducers, could reduce pancreatic oxidative damage induced by excessive amount of glucose, affecting the nitric oxide synthase/dimethylarginine dimethylaminohydrolase (NOS/DDAH) pathway in streptozotocin-induced type 1 diabetic rats. The resulted showed as HO-1 inducers such as CAPE or its more potent derivatives may be useful in diabetes, counteracting the high glucose-derived oxidative stress. **Chapter 4** explores the effect of Olive leaf extract (OLE) from Sicilian cultivar in an *in vitro* model of hepatic steatosis to evaluate the protective effects again free fatty acids accumulation in hepatocytes. OLE treatment showed to induce HO-1 expression, decrease inflammation markers levels and increase mRNA levels of FABP-4 and SIRT1, two proteins involved in lipid metabolism, with a parallel decrease in number of lipid droplets. **Chapter 5** analyzed the effects of HO-1 activity inhibition on lipid metabolism and fibrosis process in two *in vitro* models of hepatic cells. The first model was create treating HepG2 cells with free fatty acids to induce steatosis, in presence or absence of the powerful HO-1 activity inhibitor SnMP alone or in combination with the HO-1 expression inducer CoPP. The OIL RED staining as well as the expression of lipid metabolism proteins showed that HO-1 activity inhibition exacerbates hepatocytes steatosis, while in the co-treatment with the CoPP these results were

reversed. A similar trend was obtained with the hepatic fibrosis model, where the TGF-B activated-LX2 cells showed to release more collagen in presence of SnMP alone, demonstrating that HO-1 activity have a pivotal role in both stesosis and fibrosis processes. **Chapter 6** explores how metabolic abnormalities in NASH are driven by decreases in hepatic HO-1 that is associated with an increase in the adipose-derived pro-inflammatory adipokine NOV in this obese mouse model of NASH. Concurrently, induction of HO-1 provides protection against insulin resistance as seen by the increases in insulin receptor phosphorylation. **Chapter 7** describes the central role of EET-A in attenuation of obesity-induced steatosis and hepatic fibrosis that leads to NAFLD. EET-A intervention diminishes fatty acid accumulation, fibrosis, and NAFLD associated with an increase in HO-1-PGC1α and increased insulin receptor phosphorylation. **Chapter 8** explores the effects of soluble epoxide hydrolase (sEH) deletion on various aspects of adipocyte-function, including programing for white vs. beige-like fat, and mitochondrial and thermogenic gene-expressions. sEH deletion results in a significant increase in EETs with a consequent decrease in adipocyte size, inflammatory adipokines NOV, TNFα, and an increase in adiponectin. Notably, activation of HO-1 gene expression further increased the levels of EETs, suggesting that the antioxidant HO-1 system protects EETs from degradation by ROS. **Chapter 9 i**nvestigate the Thymoquinone effects in obese mice model. The TQ attenuated the obesity-mediated decrease of oxygen consumption, improved mitochondrial biogenesis and through increased levels of HO-1 decreased the HF-induced OX-LDL. **Chapter 10** describes the preliminary data of a cross-talk effect between adipose tissue and liver in an obese mice model. The selective adipose tissue overexpression of HO-1, or HO-1+PGC-1a, showed to increase the key metabolism regulator proteins expression in the liver, while decreased inflammation cytokine NOV and fatty acid synthesis. **Chapter 11** summerize all the PhD thesis finding that taken together, provides new evidences that identifies the enzyme Heme Oxygenase as a key protein in metabolic homeostasis, preventing systemic lipid overload, insulin resistance and decreasing inflammation. Consequently, increase of HO-1 levels may provide a therapeutic approach to address the metabolic alterations associated with Diabetes type 2, obesity and NAFLD.

Chapter 1

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Obesity and Metabolic Syndrome: a growing epidemic

Obesity has emerged as one of the leading public-health issues in the past decades. According to the International Obesity Taskforce 1.1 billion people are overweight with a body mass index (BMI) over 25 kg/m2 and 312 million are classified as obese (BMI > 30 kg/m2) [1]. Whereas in the US the high prevalence of obesity (33.8% in 2007/08) [2] has attracted broad public attention, overnutrition has also become an increasing problem in Europe and in less developed countries (Fig. 1) [3]. According to a survey from 2008 the prevalence of obesity is 19% for men and 22% for women in Germany, whereas in Switzerland 15% of men and 10-11% of women are considered obese [4].

Though obesity itself is not a disease per se, it is a major risk factor for developing type II diabetes, cardiovascular disease [5-8]. In accordance with the increasing prevalence of obesity, the number of type II diabetes cases is expected to rise by 32% in Europe, 72% in the United States and over 100% in developing countries [3]. In 2006, the International Diabetes Federation published the latest definition of the metabolic syndrome describing a cluster of factors associated with an increased risk for atherosclerotic cardiovascular disease (CVD) and diabetes. For a person to be diagnosed with the metabolic syndrome the following criteria have been defined: central obesity measured by waist circumference plus two additional factors such as raised triglycerides (>150 mg/dl), reduced HDL cholesterol (<40-50 mg/dl), raised blood pressure (>130 mm Hg systolic or >85 mmHg diastolic) or raised fasting plasma glucose (>100mg/dl) [9, 10]. These defined criteria are important in order to diagnose people with metabolic syndrome early and to initiate lifestyle interventions and treatment before the development of diabetes and CVD.

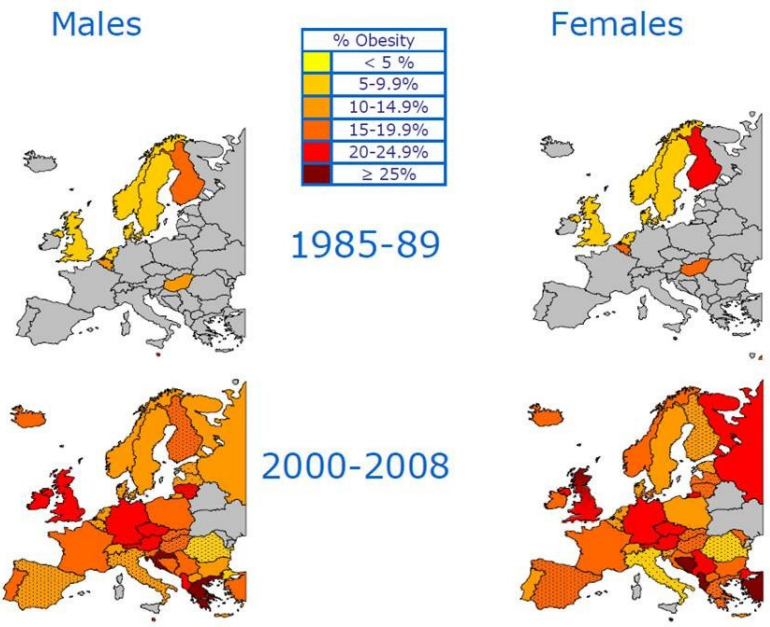

Figure 1 Trends in adult obesity prevalence in Europe, adapted from International Association of Obesity

Genetic predisposition

The causes of obesity and insulin resistance are diverse. Although our modern sedentary life style, with reduced physical activity and an unlimited offer of food, supports metabolic diseases, it cannot be neglected that genetic predisposition also plays a major role [11, 12]. Several monogenetic causes for obesity have been described, for example mutations in leptin and the leptin receptor or mutations in the melanocortin receptor 4 and pro-opiomelanocortin [13-16] leading to pronounced hyperphagia. Likewise, monogenetic disorders of glucose metabolism are known covering essential genes in β-cell development, insulin secretion and insulin signaling. One of the earliest examples is the discovery of mutations in the insulin receptor in 1988 [17]. Other monogenetic forms affect transcription factors such as HNF4α, HNF1α and β, PDX-1, NeuroD1 and KLF11 [18-24], which influence β-cell development and function. These mutations follow an autosomal-dominant inheritance and lead to a type of early-onset diabetes known as "maturity onset diabetes of the young" (MODY). Mutations in glucokinase (MODY2), the sulphonylurea receptor or the Kir6.2 potassium channel also compromise β-cell function [25-27], whereas mutations in insulin itself or AKT2 [28, 29] can disrupt insulin signaling. However, monogenetic cases are rare, most people prone to obesity or diabetes carry poly-genetic risk factors. Using new and fast sequencing techniques, much effort has been undertaken in the past several years to identify common

genetic variants associated with features of the metabolic syndrome. Genome wide association studies and meta analyses combining results from multiple studies [30-32] have led to the identification of numerous single nucleotide polymorphisms and loci, such as the fto gene, which is strongly associated with body mass index [33, 34]. Many of these identified variants lay within introns or even in non-coding regions. Therefore, one of the major challenges in the future will be to identify their function in the disease.

Drugs and treatments

The most effective treatment for people with metabolic syndrome is a drastic change in lifestyle, including weight loss, a healthy diet, regular physical activity and giving up smoking. A very effective option to reduce weight is bariatric surgery including gastroplasty, gastric banding, gastric bypass and biliopancreatic diversion [35, 36]. Interestingly, gastric surgery has beneficial effects on glucose control and insulin-resistance independent of weight loss within days of the surgery; the mechanisms for these effects are currently unclear [37, 38]. Other than invasive surgery, medical treatment for different features of the metabolic syndrome is common practice [39]. A widely used drug against hyperglycemia is metformin, which lowers hepatic glucose output, partially through AMPK activation [40]. Insulin analogues and drugs increasing insulin secretion such as sulphonylureas can be used to overcome insulin resistance in the early stages of the disease. Despite certain side effects, thiazolidinediones- PPARγ agonists- are also commonly prescribed in type II diabetes, as they indirectly enhance insulin sensitivity by modulating adipose tissue function [41]. Much hope has recently been raised by GLP-1 analogues, which stimulate insulin secretion, and DPP4-inhibtors that prolong GLP-1 action [42]. For the control of dyslipidemia, the most commonly used drugs are statins, which inhibit HMG-CoA reductase thereby blocking cholesterol synthesis [43]. Another popular class of drugs are the fibrates, a group of PPARα agonists that primarily enhance fatty acid oxidation [44]. Other possibilities are cholesterol-absorption blockers [45] that lower LDL serum levels.

For the treatment of obesity, most currently available drugs exert their effect on the central nervous system, for example inhibiting noradrenergic and serotonergic reuptake in the hypothalamus (sibutramine) [46] or antagonizing cannabinoid receptors [47]. Another strategy to reduce weight pharmacologically is the use of fat absorption blockers, e.g. through the inhibition of gastric and pancreatic lipases (orlistat) [48], which are most

effective in combination with a reduced-calorie diet. As adipose tissue growth is largely dependent on nutrient supply, it is not surprising that angiogenesis plays an important role in fat expansion [49, 50]. This may be a completely novel approach to inhibit excess adipose tissue accumulation [51]. Future studies are required to show whether this approach improves the metabolic status or on the contrary leads to adverse effects through ectopic lipid accumulation.

Overall, it has to be noted that the development of new drugs targeting features of the metabolic syndrome has had limited success over the past years. The multifactorial nature of the metabolic syndrome poses the problem of polypharmacy and finding suitable drugs targets that reduce multiple metabolic risk factors at a time has been proven difficult [39].

Adipose tissue function

Even though excess adipose tissue predisposes to metabolic complications, adipose tissue also has essential functions within the body. Adipocytes are the main storage site for excess energy in the form of triglycerides. An average man of 70 kg has about 15 kg of adipose tissue (21%) [52]. In the case of severe forms of lipodystrophy, such as Berardinelli-Seip congenital lipodystrophy [53], where the majority of normal adipose tissue depots are missing, fat is ectopically stored in extra-adipose tissue, leading to severe insulin resistance. Acquired lipodystrophy is also a side effect of antiretroviral drugs in HIV patients [54]. Besides its obvious storage function, adipose tissue has mechanical functions such as insulation and protection against mechanical forces. Moreover, adipose tissue functions as an endocrine organ. This was only discovered in 1994 with the description of leptin, which is secreted from adipose tissue and has systemic signaling capacity [55].

Molecular mechanisms of nutrient management in the adipocyte

As mentioned earlier, adipose tissue stores and manages fatty acid pools within the body. Fatty acids reach the adipocyte via the circulation in three major forms: non-esterified fatty acids (FFA) are associated with albumin in the serum [56], lipoprotein lipase (LPL) is associated with the outer membrane of the adipocyte via heparane sulphate proteoglycans [57] and can hydrolyse FFA from triglyceride-rich chylomicrons [58] and very low density lipoprotein particles (VLDL) can be taken up completely by

the adipocyte via the VLDL receptor (VLDL-R) or low density lipoprotein receptor-related protein (LRP) [59, 60]. Although FFAs can enter the cell via passive diffusion, several membrane proteins are also implicated in their uptake such as fatty acid transport protein 1 (FATP1) and CD36, especially for long-chain fatty acids [61]. Intracellularly, fatty acids are transported by fatty acid binding proteins (FABPs), especially A-FABP and to a minor extent E-FABP [62]. After entry into the cell fatty acids are esterified by acyl-coA-synthetase and used for triacylglyceride (TAG) synthesis on the endoplasmatic reticulum (Figure. 2) [63]. Apart from fatty acids, adipocytes take up glucose via GLUT4 transporters, which are translocated to the membrane upon insulin stimulation [64]. An intermediate metabolite of glycolysis is also needed for TAG synthesis, as dihydroxyacetone-phosphate is converted to glycerol-3-phosphate, the backbone of triglycerides, by glycerol-3-phosphate dehydrogenase (GPDH). Additionally, adipocytes use acetyl-CoA for *de novo* fatty acid synthesis via acetyl-CoA carboxylase (ACC) and the multifunctional enzyme fatty acid synthase (FAS) [65]. For storage, TAG are incorporated into lipid droplets, which form at the ER and are covered with PAT family proteins such as perilipin [66]. These anabolic pathways are mainly driven by insulin in the fed state, which in turn stimulates the major transcription factor for fatty acid and triglyceride synthesis, sterol regulatory element binding protein 1c (SREBP-1c) [67]. During fasting and exercise, when stored nutrients need to be mobilized, lipolysis is stimulated in adipocytes and leads to the release of free fatty acids and glycerol, which can be metabolized by other tissues [68]. Triglyceride hydrolysis is a three-step process driven by adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL). The major signaling pathways activating lipolysis are β-adrenergic stimulation and the fasting hormone glucagon. Both lead to activation of adenylate cylase and the production of cAMP, which in turn activates protein kinase A (PKA). Subsequently, PKA phosphorylates perilipin and HSL for the mobilization of fatty acids (Figure. 2) [69]. Insulin suppresses lipolysis via phosphorylation of phosphodiesterase 3B (PDE3B) by Akt leading to hydrolysis of cAMP. Additionally, α2-adrenergic stimulation can activate an inhibitory G-protein thereby suppressing lipolysis [70]. Thus, circulating hormones and adrenergic innervation in the adipocyte reciprocally regulate anabolic and catabolic pathways.

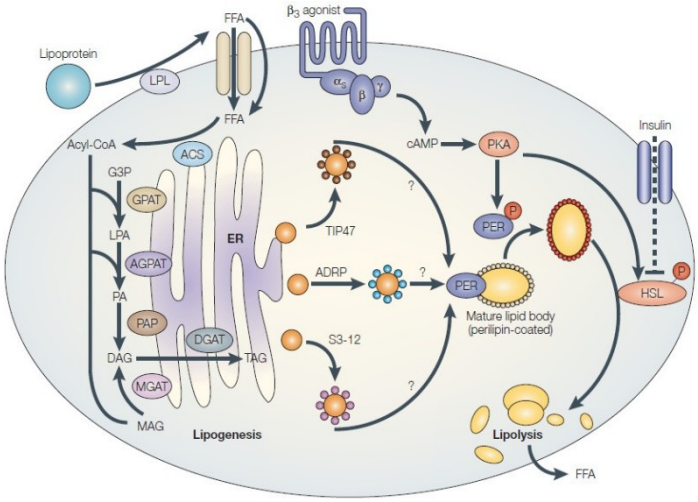

Figure 2. Lipid management in the adipocyte. Free fatty acids are released by lipoprotein lipase (LPL), taken up by the cell and incorporated into triglycerides. Lipid droplets coated with PAT family proteins (TIP47, ADRP, S3-12, perilipin) emerge from the ER. After β-adrenergic stimulation, activation of proteinkinase A (PKA) leads to phosphorylation of perilipin (PER) and hormone sensitive lipase (HSL) and free fatty acids are released by lipolysis. ACS: acyl-CoA synthetase, GPAT: glycerol-3-phosphate acyltransferase, AGPAT: sn-1-acylglycerol-3-phosphate acyltransferase, PAP: phosphatidic-acid phosphohydrolase, MGAT: monoacylglycerolacyltransferase, DGAT: diacylglycerol acyltransferase *from Shi et al [71].*

Fat depots within the body

There are many different adipose tissue depots within the body. One special subclass is brown adipose tissue, which is especially developed in infants and small rodents, where it can be induced by cold exposure [72]. Brown adipocytes have many small lipid droplets, in contrast to white adipocytes, which have one large lipid core. The main function of brown adipocytes is the production of heat through expression of uncoupling-protein 1 (UCP1). It has long been believed that only children and rodents have brown adipose tissue, which is located in distinct pads adjacent to the shoulder blades. However, it has recently been shown that brown adipocytes exist in adult humans and are interspersed into the common white fat pads and might also be inducible by cold [73, 74]. Inducing brown adipocytes by specific agents could therefore be an interesting novel therapeutic intervention to increase energy expenditure and treat obesity.

On the other hand, white adipose tissue represents the majority of body fat. Based on the localization adipose tissue depots can be classified as subcutaneous adipose tissue

underneath the dermis or visceral adipose tissue within the cavities of the body. Most visceral adipose tissue lies within the abdominal cavity with the gonadal, the mesenteric and the retroperitoneal fat compartments being the most prominent (Fig. 3) [52]. This distinction is not only of anatomical interest but also has functional implications. It has been shown that visceral and subcutaneous adipose tissue show distinct gene expression patterns [75]. Furthermore, visceral adipose tissue poses a greater risk for metabolic complications than subcutaneous adipose tissue [76]. The reasons for this are currently not clear. Potential explanations include that factors secreted from the visceral adipose tissue drain directly into the portal vein or that adipocyte function itself is altered in different depots, which will be discussed further below. Due to hormonal differences, overweight women tend to have more subcutaneous adipose tissue, whereas overweight men show increased central adiposity and are thus more prone to metabolic complications [77]. Moreover, treatment with glitazones leads, amongst other things, to redistribution of fat to the subcutaneous compartment, thereby ameliorating the metabolic profile without causing weight loss [78].

Adipose tissue composition

Cells within the adipose tissue secrete many important signaling molecules, which control whole body metabolism. The first adiponekine to be discovered was leptin. Leptin is an 18 kDa protein primarily secreted from adipocytes and signals through different isoforms of the leptin receptor [79, 80]. Leptin is a major satiety signal and suppresses food intake through direct hypothalamic repression of the orexigenic peptides NPY and AgRP [81]. Leptin also enhances fatty acid oxidation and improves peripheral insulin sensitivity via indirect signaling over the central nervous system, as well as directly through peripheral activation of AMP kinase (AMPK) [82]. Another important adipokine is adiponectin, which signals as a multimer through its receptors adipoR1 and adipoR2 and has a strong insulin-sensitizing effect [83]. Two adipokines primarily secreted by visceral adipose tissue are visfatin and omentin [84]. Whereas visfatin activates glucose uptake via the insulin-receptor, omentin has insulin-sensitizing effects. Resistin, on the other hand, promotes glucose output from the liver, thereby increasing plasma glucose levels. Another adipocytokine secreted from adipocytes is retinol-binding protein 4 (RBP4), which primarily transports retinol in the blood but also strongly impairs insulin

signaling in muscle and liver [85]. Finally, adipocytes and macrophages residing within the adipose tissue can also secrete many inflammatory cytokines such as TNFα and IL-6 [86].

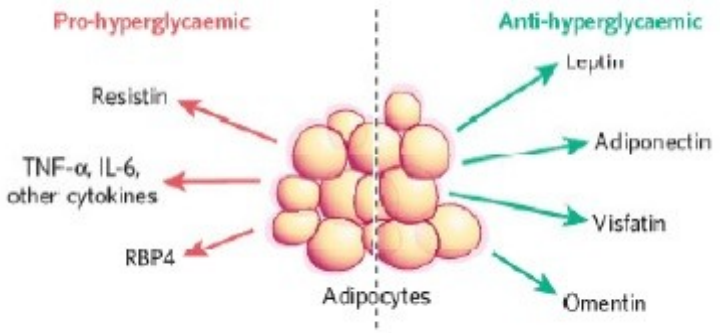

Figure 3 Secreted adipocytokines from adipose tissue and their influence on glucose metabolism. *Rosen et al [80].*

Adipocyte differentiation and adipose tissue plasticity

Adipose tissue has the remarkable capacity to remodel and adapt to the nutritional status of the body. As adipocytes continue storing lipids, they can change in size several fold [87]; this mechanism of growth is commonly referred to as hypertrophy. In addition, adipose tissue is able to regenerate fat cells by *de novo* adipogenesis from adipocyte precursor cells. This alternative mechanism of adipose tissue growth is called hyperplasia. In fact, adipocytes undergo a constant turn-over during life, making fat a highly dynamic tissue. Many of the genetic processes involved in adipocyte differentiation have been revealed by cell culture experiments either using preadipocyte cell lines, such as 3T3-L1 or 3T3-F442A, primary stromal-vascular fraction, embryonic fibroblasts or bone marrow stromal cells, all of which can be differentiated into adipocytes *in vitro*. One of the major regulators of adipogenesis is the transcription factor peroxisome proliferator-activated receptor PPARγ2 [88], which is mandatory for the adipocyte lineage as well as for the maintenance of the adipocyte phenotype. CCAAT-enhancer binding proteins (C/EBPs) also play an important role in adipogenesis, with C/EBPβ and C/EBPδ being expressed during early differentiation and inducing PPARγ and C/EBPα expression [89]. Not surprisingly, the family of Krüppel-like factors (KLFs), which generally control cell proliferation and differentiation, also have a large impact on adipocyte differentiation. Whereas KLF5, KLF6 and KLF16 promote differentiation, other family members like KLF2 and 7 inhibit the process[80]. In contrast to

proadipogenic factors, many factors are expressed in the preadipocyte to maintain the undifferentiated state; these factors are generally downregulated upon induction of differentiation. Examples include the surface protein preadipocyte factor1 (PREF1) [90, 91] and members of the GATA family, e.g. GATA2 and GATA3 [92]. Another general principle of regulation is the recruitment of co-activators and co-repressors, which often mediate histone acyltransferase (HAT) or histone deacetylase (HDAC) activity, respectively. Examples include the proadipogenic HATs CREB-binding protein (CBP) and p300 or the anti- adipogenic nuclear receptor co-repressor NcoR [93]. In multipotential cells such as mouse embryonic fibroblasts, differentiation into different myogenic, osteogenic or adipogenic lineages reciprocally inhibit each other. One well-known case is the reciprocal inhibition of PPARγ and RUNX2 deciding between an adipogenic versus an osteogenic fate [94, 95]. Apart from transcriptional regulation, extracellular signaling is also an important factor for adipogenesis. Examples for secreted proteins that trigger inhibitory signaling pathways are Wnt10b [96], sonic hedgehog (SHH) or TGFβ [97, 98]. On the other hand, extracellular proteins promoting adipogenesis include several members of the bone morphogenic protein family such as BMP2 and 4 [99] and different fibroblast growth factors (Fig. 6) [100, 101].

Another important step in adipogenesis is extracellular matrix (ECM) remodelling [102, 103]. During differentiation, the fibronectin-rich matrix of a preadipocyte needs to be converted to the typical basement membrane of a mature adipocyte, which includes laminin, nidogen/entacin, type-IV and -VI collagens [104]. Two main systems involved in reshaping the extracellular matrix are the plasminogen/plasmin cascade as well as the matrix metalloproteinase and tissue inhibitor of matrix metalloproteinases (MMP/TIMP) proteins. During adipogenesis plasmin is activated by kallikrein and degrades the fibronectin-rich preadipocyte stromal matrix [104]. The matrix metalloproteinases and their inhibitors have diverse effects on adipogenesis and their expression is differentially regulated during the differentiation process. Whereas several MMPs such as MMP2, MMP9 and MT1-MMP promote adipogenesis (the effect of MT1-MMP being only evident in 3D culture [105]), others like MMP3, MMP11 and MMP19 have the inverse effect [102]. The TIMPs, binding and inhibiting a variety of different MMPs, add another complex layer of regulation to this system. Whether different components of the ECM modulate differentiation by physical force, by sequestering important growth factors or even mediating signals themselves needs to be further investigated. For

extracellular matrix proteins, as well as for many other proteins involved in adipogenesis, it will be crucial not only to look at the process of forced *in vitro* differentiation in a 2D culture but also to develop novel *in vivo* techniques in order to shed light on the mechanisms of adipogenesis *in vivo* in a natural 3D environment.

Adipose tissue in obesity: how free fatty acids impair metabolic homeostasis

Excess adipose tissue increases the risk for a number of diverse conditions such as atherosclerosis, hypertension, insulin resistance and cancer. Much effort has been undertaken to understand the molecular changes in adipose tissue function in the context of obesity that lead to these secondary complications. One important malfunction of adipose tissue during obesity is a detrimental increase in lipolysis concomitant with an excess release of non-esterifed fatty acids. Free fatty acids are thought to be one of the major culprits for insulin resistance; they acutely inhibit insulin stimulated glucose uptake in muscle, as well as insulin-mediated suppression of hepatic glucose output [106, 107]. The mechanisms involved in these processes are diverse. Primarily, elevated FFA levels lead to intracellular accumulation of fatty acid based signaling molecules such as diacylglycerol (DAG) or long-chain acyl-CoA. DAG activates protein kinase C (PKC), which can in turn inhibit insulin signaling by serine-phosphorylation of insulin receptor substrate 1 and 2 (IRS 1/2)[7]. PKC also activates NADH oxidase, thus increasing reactive oxygen species and inhibiting NO production [108]. This is especially harmful in endothelial cells leading to decreased vasodilation and hypertension. Additionally, intracellular fatty acids lead to activation of inflammatory pathways, mainly via activation of nuclear factor κB (NFκB) and c-jun N-terminal kinase (JNK) [109]. Moreover, increased serum FFAs also provoke cardiomyopathy, increasing the risk for myocardial infarction [110] and promote atherosclerosis, at least indirectly, via increased inflammation as well as elevated hepatic VLDL output [111]. In β-cells, long term exposure with high FFA concentrations causes impaired glucose-stimulated insulin secretion, thus blocking compensatory insulin release [112].

The role of changing adipocytokine profiles in obesity

Apart from increased lipolysis, the adipocytokine profile of adipose tissue also undergoes major changes in obesity. The release of most adipokines is elevated in the state of obesity. This is also true for the metabolically beneficial hormone leptin. However, leptin signaling is concomitantly impaired, making it an unattractive target for the treatment of obesity [113]. One exception from generally elevated adipokine levels is adiponectin, whose secretion is diminished in obesity [114]. Inflammatory cytokines are highly upregulated in adipose tissue of obese subjects. For example, monocyte chemoattractant protein 1 (MCP-1) is secreted in high amounts from adipocytes leading to massive macrophage infiltration [86]. Other examples for proinflammatory cytokines either released from macrophages or from adipocytes directly are TNFα, IL-1β, IL-6 and C-reactive protein (CRP). Interestingly, many of the cytokines are secreted to a greater extent from visceral adipose tissue [115, 116], adding to the theory that visceral fat leads to more severe metabolic effects than subcutaneous fat. Cytokines released from adipose tissue induce a systemic low-grade inflammation promoting insulin resistance [117]. Moreover, TNFα inhibits triglyceride synthesis through downregulation of PPARγ and its target gene LPL as well as the glucose transporter GLUT4. At the same time, TNFα inhibits insulin-mediated attenuation of lipolysis, increases the cAMP pool and downregulates the lipid droplet protein perilipin, all of which further enhances FFA release [118].

Mitochondrial dysfunction in adipocytes

Though more discussed in the context of muscle function, mitochondrial dysfunction has also gotten some attention with regard to the adipocyte. Metabolizing fatty acids via β-oxidation takes place in the mitochondria; impaired mitochondrial function therefore promotes systemic lipid overload and ectopic lipid accumulation. In muscle cells as well as in adipocytes reduced mitochondrial numbers and function are associated with obesity [119, 120]. On the other hand, the insulin-sensitizing agent rosiglitazone promotes mitochondrial biogenesis [121, 122]. However, whether reduced mitochondrial function primarily leads to accelerated accumulation of lipids or whether intracellular lipid overload and an increased rate of β-oxidation ultimately damage

mitochondria, as it has been suggested in muscle cells [123], needs to be further analyzed in adipose tissue.

Adipose tissue fibrosis

Another aspect of adipose tissue dysfunction in the obese state that has been largely neglected concerns the adipose tissue matrix. It has been shown that the expression of matrix proteins is highly upregulated in adipose tissue of obese subjects, rendering the adipose tissue scaffold more rigid [124]. Surprisingly, ablation of collagen VI, a highly abundant extra-cellular matrix protein in the adipose tissue, leads to improvement of whole-body energy homeostasis in obese animals [125]. Additionally, a variety of matrix metalloproteinases, which shape the extra-cellular matrix, are deregulated in adipose tissue in the state of obesity [126, 127]. Whereas to date the functional impact of an altered extracellular matrix on mature adipocytes remains elusive, many studies have established a link between extracellular matrix remodeling and adipogenesis, thus modulating adipose tissue plasticity.

Adipocyte cell size and adipogenesis

Several studies have made the observation that subgroups of obese but otherwise metabolically healthy subjects exist [128-130]. This is accompanied by high insulin sensitivity, low levels of inflammation markers and decreased visceral obesity. The underlying mechanisms are not fully understood. One possible explanation for the difference in metabolic phenotypes can be the fact that total fat mass itself describes the functional status of adipose tissue insufficiently. This is true for differences in fat depots, i.e. subcutaneous versus visceral, but also for single cells within the adipose tissue. Over 40 years ago Salans *et al.* showed that insulin-responsiveness in isolated human and rat adipose tissue is related to adipocyte cell size, with larger cells being increasingly insulin-resistant [131, 132]. Since then many studies have supported this theory: large cells show a distinct gene expression profile with elevated expression of inflammatory markers [133], the lipolytic rate is higher in hypertrophied cells and further stimulated by TNFα , and insulin-stimulated GLUT4 [134, 135] translocation for glucose uptake depends on cell size [136]. A recent cohort study supports the idea that adipocyte size, especially in the omental fat compartment, is a critical parameter describing metabolic health in obese subjects [137].

Adipocyte size in the adipose tissue is mainly determined by the ability of the mature adipocyte to grow in size (hypertrophy) versus the ability to build new fat cells through adipogenesis (hyperplasia). Thus, increased adipocyte differentiation in response to excess nutrient supply will ultimately lead to a larger number of smaller cells, which remain insulin sensitive and secrete less inflammatory signals (Fig. 7). Recently, it has been shown that visceral adipose tissue mainly grows via hypertrophy, whereas for subcutaneous adipose tissue a hyperplastic response is predominant in mice fed a high fat diet [138]. This observation could be another important explanation for the more detrimental effect of excess visceral adipose tissue. In human adipose tissue this effect is less evident. Judging by gene expression of differentiation markers in obese women, hyperplasia is predominant in the subcutaneous fat depot [139]. Van Harmelen et al. describe increased proliferation of human subcutaneous SVF but no difference in differentiation capacity between fat depots [140]. On the contrary, Tchkonia *et al.* report increased differentiation in subcutaneous compared to visceral SVF [141]. Age- and sex-dependent differences could play an important role here, as preadipocyte pools might exhaust with age and increasing adiposity and sex steroids have opposing effects on adipocyte differentiation [142, 143].

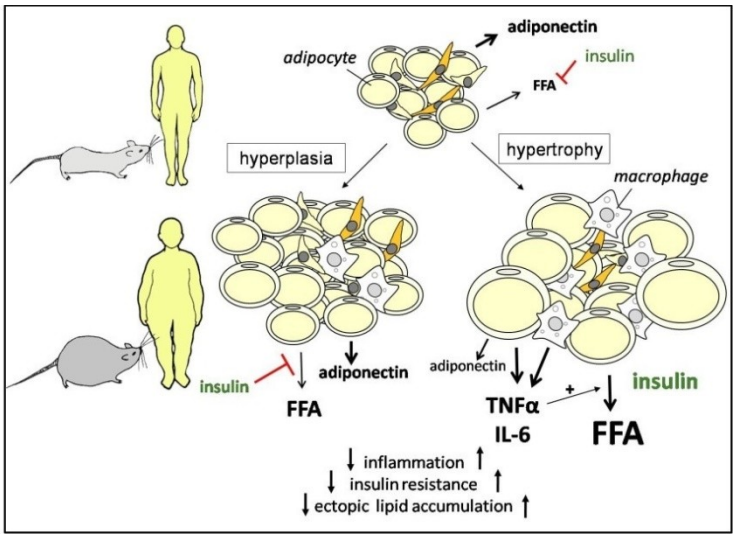

Figure 4 Adipose tissue growth via hyperplasia (adipocyte differentiation) or hypertrophy (increasing volume of existing adipocytes) and the resulting metabolic consequences.

In conclusion, adipocyte differentiation is an important adaptive response to excess nutrition and influences the metabolic outcome of obesity.

NAFLD: Definition and pathogenesis

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive fat accumulation on the liver as defined by the presence of steatosis > 5% of liver weight according to histological analysis or by a proton density fat fraction (PDFF) > 5.6% in proton magnetic resonance spectroscopy (^{1}H-MRS) or magnetic resonance imaging (MRI) [144]. Moreover, the definite diagnosis of NAFLD requires the exclusion of excess alcohol consumption (≥ 30g a day in men or ≥ 20g a day in women) [144, 145]. Notably, the methods used to exclude excess alcohol consumption (anamnesis, biochemical markers) are not fully reliable. Additionally, there is no threshold for harmful alcohol consumption as the alcohol-related hepatic injuries appear to increase uniformly with the amount of alcohol consumed [145, 146]. Thus, the diagnostic thresholds are more or less arbitrary [145]. The secondary causes to NAFLD, such as hepatotoxic medical history during the past six months, viral hepatitis, hemochromatosis and chronic autoimmune liver diseases, also need to be excluded [145]. Of pharmacological agents, methotrexate, glucocorticoids, isoniazid, amiodarone and tamoxifen are known inducers of fatty liver [147].

NAFLD is an umbrella term covering simple non-alcoholic fatty liver (NAFL), in which there is pure hepatosteatosis only (or steatosis with either mild inflammation or ballooning but not both), and non-alcoholic steatohepatitis (NASH). The co-existence of all these three histopathological features, i.e., steatosis, inflammation and ballooning, is required for NASH, which also covers the most progressive forms of NAFLD: fibrosis, cirrhosis and HCC [144]. The spectrum of NAFLD is illustrated in Figure 5.

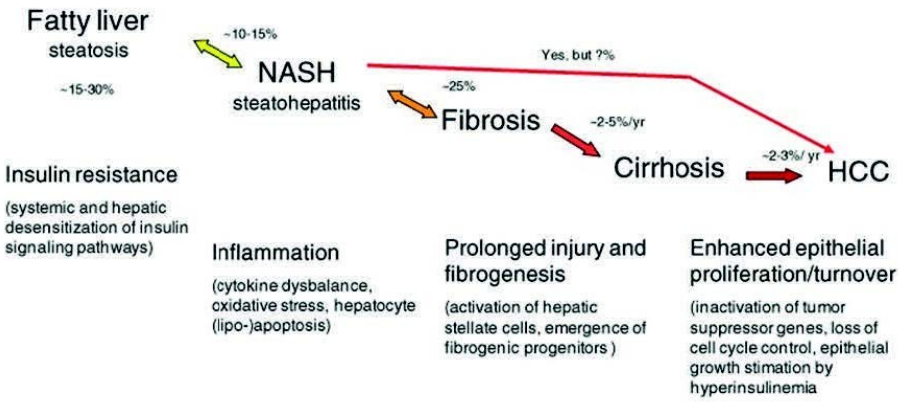

Figure 5. The spectrum of NAFLD. NAFLD comprises different stages with stage-specific risk factors and pathomechanisms. Estimated risks of progression are displayed. HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis (Schuppan & Schattenberg, 2013) [148].

NAFLD progresses slowly [144]. Traditionally, NAFL has been thought to be a benign disease, but an accumulating body of evidence has shown that NAFL may progress to NASH [148-150]. It is estimated that over time about 30–40% of subjects with NAFL and elevated liver enzymes will develop NASH and 40–50% of those with NASH will have fibrosis [151]; in all NAFLD subjects, these numbers are thought to be about 10–15% and 25%, respectively [148]. NAFL may even leap directly to fibrosis [152], in which there may still exist mild inflammation but without other mandatory NASH criteria [153], or prior NASH may has been missed. In addition, NASH and even NAFL may progress to non-cirrhotic HCC [154] and, thus, cirrhosis is not a mandatory step in the malignant disease progression as was previously thought. Especially male diabetics are at risk of non-cirrhotic HCC [150].

According to a meta-analysis by Singh *et al.*, 34% of all NAFLD subjects show NAFLD progression, 43% have stable disease and in 22% of NAFLD subjects, the disease improves over time. Moreover, the rate of fibrosis progression in subjects with NASH is doubled in comparison to the rate in NAFL subjects (the mean annual fibrosis progression rate is 0.07 in NAFL versus 0.14 in NASH, corresponding to an average progression by at least one fibrosis stage (from F0 up to F4) over 14.3 years in NAFL versus 7.1 years in NASH, respectively), although it should be noted that the variability in the rate is broad (Singh *et al.*, 2015). Indeed, the rate of the disease progression is characteristically slow but very varying depending on, for example, age (reflecting the cumulative sum of metabolic exposures and longer disease

duration), genetic variants, hormonal factors (males and postmenopausal women at risk of fibrosis), ethnicity (Asians and Hispanics at the greatest risk), presence of diabetes and obesity and the degree of steatosis [150]. The overall risk of cirrhosis in subjects with simple steatosis is under 4% in 20 years of follow-up whereas in NASH subjects it is 25% in nine years [150].

The pathogenesis of simple steatosis and the consequences it has for the lipid metabolism are depicted in detail in chapter Lipid metabolism in NAFLD. Briefly, sedentary lifestyle accompanied by excess caloric intake leads to accumulating visceral adipose tissue, insulin resistance and the release of proinflammatory factors. These promote the lipolysis of free fatty acids (FFA) from visceral adipose tissue to the liver [155]. Simple hepatosteatosis is formed when hepatic FFA input is greater than output, both of which are altered in subjects with NAFLD [156, 157].

The progression of NAFL to NASH and fibrosis requires a complex and multifactorial interplay of genetic, epigenetic, environmental, inflammatory, adipose tissue-derived factors and intrinsic microbial factors [158, 159]. The high levels of FFAs and many other lipid metabolites in a hepatocyte are lipotoxic. As a consequence, mitochondrial dysfunction with oxidative stress and production of reactive oxygen species and endoplasmic reticulum stress associated mechanisms are activated. Moreover, the proinflammatory factors from visceral adipose tissue are present in the liver [160]. The inflamed environment leads to chronic hepatic inflammation, which is further amplified by unfavorable genetic and epigenetic modifications and alterations in the gut flora [149]. The alterations in the gut flora are derived from an unbalanced diet (high fat, high sugar/fructose, low fiber, nutrient/vitamin deficiency), which causes gut microbiota dysbiosis, mild inflammation and alteration in gut barrier function leading to increased translocation of microbial components into splanchnic veins which takes part in the formation of NAFLD progression [161].

Once NASH and the chronic inflammation have developed, hepatocyte necrosis and apoptosis is promoted. Apoptotic bodies from the damaged hepatocytes can activate hepatic stellate cells and Kuppfer cells, which drive the formation of liver fibrosis by inflammatory and fibrogenic responses. Thereby, transformation of hepatic stellate cells into myofibroblasts takes place [162]. This results in the accumulation of collagen, proteoglycans and glycoproteins and, thereby, changes in the extracellular

matrix composition [163, 164]. Activated hepatic stellate cells also enhance the pro-inflammatory responses and the formation of the vicious circle between inflammation and the profibrotic processes [162]. The transition between hepatic stellate cells to myofibroblasts involves signaling pathways, which in NASH-driven fibrosis seems to be dominated by Hedgehog signalling [162].

This theory of the progression of NAFLD is called the multiple-hits theory and it has replaced the outdated two-hits theory [149] and distinct hit theory [165]. However, it is known that the development of progressive NAFLD occurs over such a long time course that progressive histological follow- up is difficult. To date, no biochemical marker specific for NASH has been found that could point to the distinct pathophysiological routes.

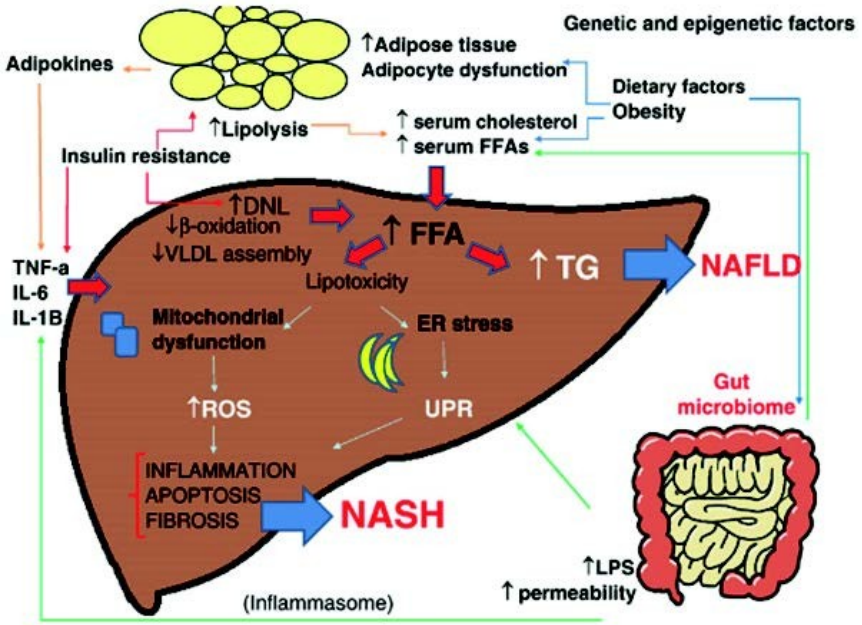

Figure 6. Multiple hit hypothesis for the development of NAFLD [165].

Epidemiology of NAFLD

According to a meta-analysis of 86 studies with more than 8,500,000 adults from 22 countries in the years 1989–2015, the global prevalence of NAFLD is about 25% [166]. By continent, the prevalence was 13.5% in Africa, 23.7% in Europe, 24.1% in North America, 27.4% in Asia, 30.5% in South America and 31.8% in the Middle East [167]. In Finland, the prevalence of NAFLD is reported to be around 20% with male

gender, physical inactivity, MetS and its components as risk factors [168, 169]. Today, the annual population-based NAFLD incidence is thought to be around 3–5% [144]. The pooled overall NASH prevalence estimate among biopsied NAFLD patients was 59%, and 7–30% among all NAFLD patients without indication for liver biopsy [144]. The estimated population-based prevalence of NASH in the USA and Finland is about 3–5% [167, 170] while obesity, type 2 diabetes (T2D) and male gender are risk factors for NASH [170]. However, due to the requirement of histological analysis to confirm NASH, the true prevalence of NASH in different populations is unknown. It is also noteworthy that the progression of NAFLD is very slow and may take decades [171]. Thus, noting the mean age of the NAFLD subjects at the time of diagnosis, progression to cirrhosis would in many cases take well beyond the life expectancy [166]. In NAFLD subjects, the estimates for the cirrhosis- related deaths over time are 1–4% [172]. HCC occurs at the frequency of 0.44/1,000 person- years [166].

The global prevalence of obesity among adult population is about 37% [173]. Obesity is a strong risk factor for NAFLD [174-176], but from the epidemiological point of view, it is not the only predictor of NAFLD as the pooled overall obesity prevalence estimates among NAFLD patients and among NASH patients were 51% and 82%, respectively [144]. Indeed, in addition to obesity and excess caloric intake, genetic susceptibility, cultural phenomena and environmental factors such as dietary composition, physical exercise, environmental chemicals and intestinal microbiota are supposed to affect the NAFLD prevalence and severity [166, 177]. Also MetS and separately, all components of MetS, parallel the prevalence of NAFLD and increase the risk of NASH, especially with advancing age [178, 179]. Noteworthy, about 70–90% of T2D patients have NAFLD, 20% have NASH, and 5–7% have advanced fibrosis (≥ F3 by the Kleiner classification, see chapter Diagnosis of NAFLD) [149]. Due to the silent nature of the disease, the exact prevalence trends are not known. However, given the pandemics of obesity and T2D and the increasing proportional number of liver transplantations for the NAFLD/NASH subjects, the prevalence of NAFLD and NASH is thought to have increased during the recent decades and is still expected to grow [144, 178].

The histological diagnosis of NAFLD

The histologic characterization of NAFLD spectrum by definition includes the description of hepatosteatosis and cell injury in addition to inflammation and fibrosis [180]. Hepatosteatosis is graded on its severity: normal (grade 0) < 5%, mild (grade 1) 5–33%, moderate (grade 2) 34–66% and severe (grade 3) > 66% [178, 181]. It should be noted, however, that these percentages are different than the percentages used in the qualification of steatosis by magnet resonance-based imaging modalities [178]. As stated above, the NAFLD spectrum consists of two clinically and histologically different conditions with different prognosis: NAFL and NASH, the latter covering the more progressive forms including fibrosis, cirrhosis and HCC [144]. In NAFL, there is pure steatosis only or steatosis with mild lobular/portal inflammation at maximum but without hepatocellular ballooning or steatosis with hepatocellular ballooning but without inflammation, whereas in NASH there is presence of steatosis, ballooning and lobular inflammation [144, 180]. Ballooning predicts more progressive disease and is also associated with fibrosis [182]. NASH is called early NASH if there is no fibrosis (F0) or mild fibrosis (F1), fibrotic NASH when significant fibrosis (≥ F2) or advanced fibrosis (≥ F3) is present, or cirrhotic NASH when cirrhotic-stage fibrosis (F4) exists, as originally classified by Kleiner [181]. Other features that can be seen in NASH but are not necessary for the diagnosis are, for instance, megamitochondria, microvacuolar steatosis, portal inflammation, Mallory-Denk bodies, apoptotic bodies and polymorphonuclear infiltrates [144]. The histological activity and severity of NAFLD and NASH can be graded with NASH Activity Score (NAS), which consists of 14 histological features, each giving scores, thus being an unweighted sum of steatosis, lobular inflammation and hepatocellular ballooning scores. Score < 3 correlates with non-NASH and score ≥ 5 correlates with NASH [180, 181]. However, in the long-term follow-up NAS seems to have low prognostic value [173]. It has been criticized for the fact that steatosis has a disproportionate impact on the score. As a consequence, NAS does not adequately detect NASH and thus does not predict prognosis, either [149]. The Steatosis, Activity and Fibrotic score is an alternative NAFLD and NAS although it does not seem to correlate with long-term prognosis either [183]. Fibrosis staging is based on the Kleiner classification, as explained above.

Although histology is the golden standard in the diagnosis of NAFLD in its all forms and many other acute or chronic liver diseases, there are some weaknesses and limitations in this diagnostic method. First, the risk of major complications in percutaneous liver biopsy requiring hospital admission (i.e., haemorrhagic complications, pneumothorax, biliary peritonitis) is around 4–8% in blind biopsies and 0.5–2% in ultrasound-guided biopsy, of which about two-thirds are discovered within two hours and nearly all cases within 24 hours after the procedure [184, 185]. The death rate has been reported to be 0.009–0.11% with malignancy as a risk factor [186]. Second, the size of the liver biopsy specimen which should contain at least 6–8 portal triads, represents about 1:50,000 of the total liver mass [185]; as a result, only local analysis of fat accumulation, inflammation, ballooning and fibrosis is available, none of which are always evenly distributed. Moreover, only semiquantitative grading of steatosis is possible [187, 188]. Third, the determination of the steatosis grade is highly subjective and dependent on each pathologist's individual opinion and fourth, due to NAFLD and NASH being so common diseases, it is clear that not everybody with suspected NAFLD or NASH can undergo a liver biopsy [188].

Lipid metabolism in NAFLD

Under physiological conditions, dietary fatty acids are absorbed from the small intestine, aggregated into triglycerides and merged into chylomicrons. After entering the lymphatics, about two-thirds are delivered to adipose tissue and one-third to the liver. There is considerable traffic between adipose tissue and the liver during eating and fasting. For instance, under fasting conditions fatty acids are released from adipose tissue to the liver where they are oxidized *in situ* by mitochondria or aggregated to triglycerides and VLDL particles and secreted to the plasma [189]. Moreover, excess carbohydrates are also synthetized to fatty acids in hepatic *de novo* lipogenesis [190]. Thus, under physiological conditions the liver is more a processor of fatty acids than a storage depot [189]. It is also notable that while in physical activity, the uptake of FFAs (and carbohydrates) to the skeletal muscles is enhanced resulting in reduced hepatic influx and, thus, accumulation of triglycerides.

When the hepatic FFA availability (influx and *de novo* synthesis with esterification to triglycerides) is greater than FFA disposal (oxidation and secretion),

hepatosteatosis develops [156, 157]. In the Western world, the most common background for this is higher energy intake as compared to energy expenditure: lipids (mainly free fatty acids from adipose tissue) begin to accumulate in tissues and organs not designed to store fat, such as the liver or the omentum. This phenomenon is called ectopic fat accumulation and hepatosteatosis is thus an example of it. Each of the variables (influx, *de novo* synthesis, oxidation and secretion) is altered in NAFLD [157]. In hepatosteatosis, the lipids are mainly triglycerides (Alkhouri, Dixon, & Feldstein, 2009), the primary source of energy storage and transport (Browning & Horton, 2004), but other lipid metabolites such as different FFAs, diacylglycerols, free cholesterol, cholesterol esters, ceramides, and phospholipids are also present [191]. There are two types of NAFLD: obese/metabolic NAFLD, resulting from energy surplus, and genetic NAFLD due to genetic alterations in lipid metabolism in the liver – although combinations of these are also commonly met [178, 179].

Lipid metabolism in obese/metabolic NAFLD

Higher energy intake than energy expenditure either due to sedentary lifestyle, excess energy intake or both causes energy imbalance that leads to the storage of excess energy as fat in white adipose tissue [155]. The accumulating visceral adipose tissue leads to local hypoxia, predisposes to the death of adipocytes, the infiltration of macrophages surrounding the dead adipocytes and the release of proinflammatory cytokines such as NK-κB, tumor necrosis factor α (TNF-α), IL-1β, IL-6, IL-8 and transforming growth factor-β [155, 192]. The inflamed adipose tissue is insulin resistant, that promotes the release of FFAs from visceral adipose tissue to the liver [155]. Visceral fat has been suggested to be more harmful compared to subcutaneous fat baceause it may release more pro-inflammatory cytokines than subcutaneous fat, the rate of lipolysis is higher in visceral fat and FFAs from visceral fat are released directly into the portal vein [193]. All these are amplified by the decrease of plasma adiponectin secretion from the inflamed visceral adipose tissue [176]. Thus, increased release of FFAs from visceral adipose tissue results in increased hepatic FFA influx [156]. The FFA influx is further increased and redirected to the liver by the increased transcription of FFA translocase CD36 in hepatocellular cell membranes and skeletal muscle whereas the expression is decreased in adipose

tissue [156]. Other fatty acid translocases in the hepatocellular membrane are fatty acid transport protein 2 and 5 [189]. Soon after entering the hepatocellular cytoplasm, FFAs are rapidly converted to fatty Acyl-CoAs. The molecular cascade behind this process is not yet fully understood. However, it may be that fatty acid transport protein possesses fatty acyl-CoA synthetase activity or that it may activate the long chain acyl-CoA synthetases resulting in the formation of Acyl-CoAs [189]. Acyl-CoAs are the key players in hepatic fatty acid metabolism. They can be a) oxidized in mitochondria, which results in the formation of Acetyl-CoAs and energy, b) synthesized to triglycerides and stored in the liver [194] or, c) secreted as VLDL particles to the Disse space outside the hepatic cells, or d) are precursors in *de novo* lipogenesis [189]. In *de novo* lipogenesis, Acetyl-CoA is converted through various cycles of metabolic reactions to palmitic acid. Acetyl-CoA itself is derived either from Acyl- CoA (oxidation in mitochondria is described above) or glucose (after glycolysis and the oxidation of pyruvate, which is then converted to Acetyl-CoA) [156, 189]. Palmitic acid is elongated and desaturated by long chain fatty acid elongase 6 and stearoyl-CoA desaturase 1 to monounsaturated fatty acids. Next, glycerol-3-phosphate from glycolysis is esterified with newly synthesized fatty acid to generate lysophosphatidic acids by glycerol-3-phosphate acyltransferase [195]. Finally, after the catalyzing reactions by 1-acylglycerol-3-phosphate acyltransferase, lipin 1 and acyl-CoA:diacylglycerol acyltransferase, triglycerides are formed [189].

Hepatic *de novo* lipogenesis is primarily controlled by sterol regulatory element binding proteins 1c (SREBP-1c) [196] and carbohydrate responsive element binding protein [197]. SREBP-1c is controlled by insulin and carbohydrate responsive element binding protein by glucose, and these genes activate the expression of several lipogenic genes active in *de novo* lipogenesis [156]. Once the liver gets fatty, the ability of insulin to suppress glucose production in the liver is repressed but somehow it preserves its key regulator role in SREBP-1c induction [198].

Thus, in hyperinsulinemia, i.e., insulin resistance, SREBP-1c is induced and lipogenesis is upregulated [198]. The mechanisms behind this called ‘selective insulin resistance’ are not yet completely elucidated, but it may be explained by the simultaneous regulation of gluconeogenesis (through FoxO1) and *de novo* lipogenesis (through SREBP-1c). However, this selective insulin resistance is a key player in the pathophysiology of type 2 diabetes development in a subject with NAFLD [189, 198].

Moreover, an *in vivo* study of postprandial glucose metabolism revealed that increased *de novo* lipogenesis is a predictor of the development of NAFLD [199]. Altogether, *de novo* lipogenesis is increased by 2- to 3-fold in individuals with NAFLD as compared to indviduals without NAFLD, indicating a substantial role in the NAFLD pathogenesis [200]. FFA oxidation occurs primarily in the mitochondria and to a lesser extent in peroxisomes and endoplasmic reticulum [201]. Carnitine palmitoyltransferase (CPT) 1 and 2 translocate fatty Acyl-CoAs from cytosol across the mitochondrial membrane. Within the mitochondria, Acyl- CoAs are consumed step by step by the β-oxidation cycle into acetyl-CoAs, which are further oxidized by the mitochondrial tricarboxylic acid cycle to generate energy [189]. Deficiencies in mitochondrial FFA oxidation lead to hepatosteatosis (Fabbrini *et al.*, 2010). Because there are no reliable methods to measure hepatic FFA oxidation rate directly, it is controversial to which direction fatty acid oxidation is altered in NAFLD [156]. It is evident, however, that fatty acid oxidation is not increased to the same extent as *de novo* lipogenesis and/or FFA influx are increased, and, thus, the FFA input and output are not in balance [189]. It is of interest that mitochondrial dysfunction in FA oxidation may amplify the oxidative stress and thereby contribute to the development of NASH lesions [202]. Very low-density lipoproteins (VLDL) are lipoprotein particles that are produced in endoplasmic reticulum and further processed in Golgi apparatus. Their production and secretion is a complex and tightly controlled process. During the process, water-soluble triglycerides are translated to water-soluble form so that they can be exported from the liver to the circulation. High VLDL levels in circulation are often converted to atherogenic low-density lipoprotein (LDL) particles [156]. Insulin resistance enhances the synthesis and secretion of VLDL [203].

In hepatosteatosis, the ability of insulin to repress the production and secretion of VLDL is impaired, which contributes to hypertriglyceridemia and low high-density lipoprotein (HDL) level [204]. However, although the VLDL secretion rate is increased in NAFLD, it is not able to adequately compensate the fatty acid input. This is mainly for two reasons: first, due to the limited capacity of apoB100 secretion, which is an essential structural component of VLDL [205], VLDL secretion reaches a plateau phase in NAFLD whereas in BMI- and body fat-matched controls the secretion is linearly increased [156]. Moreover, due to the limited capacity to enhance the

apoB100 production, triglyceride content in each VLDL particle is increased; as a result, VLDL particles cannot penetrate the sinusoidal endothelial pores to get out of the liver [156]. Second, fatty acids from non-systemic sources (intrahepatic or visceral fat lipolysis or *de novo* lipogenesis) are responsible for the stimulation of VLDL secretion, whereas increased lipolytic rate from systemic sources (primarily from subcutaneous adipose tissue) is unable to stimulate secretion rates sufficiently [206]. In total, there are three sources of hepatic triglycerides. Donnelly *et al.* reported that in their study 59% of hepatic triglycerides accumulation is from serum FFAs, 26% from *de novo* lipogenesis and 15% from diet [207]. In summary, hepatosteatosis is formed when VLDL secretion and Acyl-CoA oxidation are overwhelmed by enhanced FFA influx and *de novo* lipogenesis. The lipid accumulation in the liver is mainly formed of triglycerides, which are not lipotoxic nor induce insulin resistance to the same extent as many other lipids do. Indeed, converting FFAs to triglycerides may be seen as the liver protecting itself from the toxic effects of the excess FFAs, cholesterols, diacylglycerols, phospholipids and its components (ceramides, sphingolipids, lysophosphatidyl choline) in the surrounding milieu leading to oxidative stress, insulin resistance and more severe NAFLD [208].

Heme oxygenase: an Overview

HO, a ubiquitously expressed inducible cellular stress protein, serves as a rate-limiting enzyme catalysing the oxidative degradation of heme to the bioactive molecules carbon monoxide (CO), biliverdin, and ferrous iron. Biliverdin is subsequently converted into bilirubin [209]. The ferrous iron is rapidly sequestered by ferritin and either pumped out of the cell by an ATPase pump or recycled for heme synthesis [210]. Two main isoforms of HO have been identified, the inducible isoform HO-1 and the constitutively expressed isoform HO-2 [210]. HO-1 can be induced by several stimuli, such as its substrate heme, heavy metals, inflammation, stress, hypoxia and other oxidants [211]. The anti-apoptotic and anti-inflammatory properties of HO-1 are largely due to these degradation products [212]. CO is reported to have several beneficial functions including relaxation of blood vessels [213], suppression of apoptosis [214], stimulation of blood vessel formation [215], and inhibition of pro-inflammatory cytokines [216]. Bilirubin is a potential antioxidant, and also inhibits NADP(H) oxidase, subsequently inhibiting superoxide anion production [217, 218].

Some, but not all [219] evidence suggest that induction of HO-1 has beneficial effects in several disease models including metabolic disorders such as insulin resistance, type 2 diabetes and obesity [210].Thus, the subsequent studies tried to identify effective approaches to induce HO-1 in human [220-222]. CoPP and hemin [223-225] were used to induce HO-1 in cell culture and pre-clinical models in most studies. Unlike CoPP, hemin is the derivative of heme which is a HO-1 substrate analog that can serve as both an inducer and a substrate for HO-1 [226]. The underlying mechanism for the induction of HO-1 typically involves reciprocal regulation of Bach1 and Nrf2 protein stability [227]. Both are basic leucine zipper transcription factors and the former represses HO-1 expression whilst the latter promotes its expression [227]. Interestingly, bivalent metals other than iron, including Cu, Zn, Sn and Co, are able to bind to the porphyrin ring within heme and the HO-specific binding region does not discriminate between these different metalloporphyrins [228]. As such, these alternate metalloporphyrins bind to and inhibit HO-1 enzyme activity because they are not degraded [229]. Hence, metalloporphyrins can induce HO-1 expression and compromise enzyme activity. CoPP is widely used to induce HO-1 activity as it is a strong inducer of HO-1 expression and weak inhibitor of HO-1 activity. In contrast, SnMP is used as a HO-1 inhibitor because it is a weak inducer of HO-1 expression but a strong inhibitor of HO-1 catalytic activity [228].

HO-1 and Obesity

Accumulating evidence has led to the suggestion that induction of HO-1 via CoPP or Hemin [224, 225] has a range of beneficial effects on obesity. For example, chronic HO-1 induction in obese mice prevents body weight gain, increases the number and decreases the size of adipocytes, increases circulating adiponectin levels and decreases circulating pro-inflammatory cytokine levels [230]. Studies show that CoPP administration to rats either subcutaneously or via intra-cerebroventricular injection led to an initial decrease in food intake of between 60-80%, compared with vehicle-treated animals [229], and subsequently results showed a 20 to 25% reduction of body weight compared with vehicle [231], but this reduction in body weight was eliminated when co-treated with HO inhibitor [230]. CoPP administration and HO-1 induction via other compounds have also been demonstrated to induce weight loss. Treatment with hemin has been demonstrated to lower body weight in Zucker diabetic fatty rats [232]. Consistent with

reduction in food intake and lower body weight, insulin sensitivity and glucose tolerance in obese mice were also improved [230]. Systemic induction of HO-1 has been reported to lower hyperglycemia and hyperinsulinemia in several models of obesity in both rats and mice [232]. A recent study also showed that MC4R-deficient obese mice treated with long-term (4-23 weeks of age) CoPP administration, exhibited increased oxygen consumption, and CO_2 and heat production [233]. Increased adiponectin has also been reported and has been proposed to underpin these beneficial effects [234, 235]. Studies have also shown that the above benefits can be reversed by SnMP treatment [236]. It has also been reported that the beneficial effects of HO-1 induction are correlated with the increase of phosphorylated AMP-activated protein-kinase (AMPK), Akt, insulin receptors and the increase of the glucose transporter-4 in both adipocytes and skeletal muscle [233]. Collectively, these results indicate that improvements in basal metabolism combined with decreased food intake may underlie the sustained weight loss observed in response to HO-1 induction *in vivo*, and adiponectin seems to be a key element in these beneficial effects of HO-1 induction. However, the underlying mechanism of how the HO-1 inducer affects food intake and basal metabolism requires further investigation.

HO-1 and adipocytes

Adipose tissue plays an important role in obesity. As individuals become obese, their adipocytes enlarge, and adipose tissue undergoes molecular and cellular changes which affect systemic metabolism. Pro-inflammatory factors are also increased with the increase of adipose tissue in obesity [237]. Recently, emerging studies have reported that chronic induction of HO-1 can significantly alter the phenotype of adipocytes [238]. Induction of HO-1 by CoPP or hemin administration in ob/ob mice or Zucker rats reduced adiposity and improved insulin sensitivity in diabetic animals [230, 239]. It was first reported by L'Abbate et al that HO-1 induction results in increased serum adiponectin in diabetic rats [240]. Subsequent studies showed that chronic HO-1 induction appears to remodel adipocytes, resulting in hyperplasia instead of hypertrophy [236]. Abraham and colleagues demonstrated that HO-1 induction in adipocytes not only leads to an increase in adiponectin, but also a decrease in inflammatory cytokines such as TNFα, IL-6, and IL1-β [236, 241]. HO-1 may relieve inflammation by reducing NF-κB, which plays an important role in the development of

insulin resistance. Moreover, the increased phosphorylation of AMPK and PPARγ [210], and decreased levels of monocyte chemoattractant protein-1 (MCP-1) [242], may also contribute to the reduced inflammation following HO-1 induction. As decreased serum adiponectin has been observed in subjects with obesity and its associated diseases [243]. Adiponectin-deficient mice exhibit diet-induced obesity and insulin resistance [244] which can be reversed by adiponectin administration . Therefore, it is thought that the increased levels of adiponectin are the mechanism by which induction of HO-1 results in improvement of the health status in obese subjects [234, 240, 245].

Genetic manipulation of HO-1

Genetic studies on the increase of HO-1 activity in adipocytes have been performed by two independent research groups by using the aP2 promoter to manipulate HO-1 expression in adipocytes via lentiviral or transgenic approaches. In the paper published by Cao et al, researchers used lentiviral (aP2-HO-1) construct via intracardiac injection, which successfully increased HO-1 expression in adipose tissue and attenuated high fat diet (HFD) induced body weight increase, decreased adiposity and also improved insulin sensitivity and adiponectin production [224]. Another paper published by Huang et al, used a classic transgenic approach to increase HO-1 in adipose tissue [244, 246]. In contrast to the previous report, this failed to attenuate HFD-induced obesity, insulin resistance or the increase in adiponectin. The explanation for these contrasting findings needs further investigation, but it is worth know that the aP2 promoter is switched on in the early stage of differentiation process, which means the increased HO-1 expression would also be seen in immature adipocytes, and it would also drive gene expression in cells other than adipocytes such as cardiomyocytes and macrophages [247]. In the papers published by Huang et al, transgenic overexpression of HO-1 via the P2 promoter resulted in an increase in HO-1 in peritoneal macrophages which is sufficient to increase M2 macrophage polarisation but not enough to protect against obesity decreased adiponectin and other metabolic dysfunctions [246].Collectively, these studies indicated there may be an association between reduced body weight and improved adiponectin levels. Moreover, recent literature published by Jais et al. provided the most challenging findings in this area [219]. Jais et al. found that HO-1 acts as a driver rather than a brake in obesity associated inflammation. In this study,

they found no evidence of a major role for HO-1 in adipocyte, muscle or pancreatic β-cells [219]. However, their results indicate that inhibition of HO-1, rather than induction, may represent effective therapeutic effects in both myeloid and hepatic cells [219]. Due to the discrepancy of these studies, further studies are necessary to determine the underlying mechanisms between HO-1, insulin sensitivity, adiposity, adiponectin and inflammation.

The side effects of metalloporphyrins

As discussed previously, metalloporphyrins can bind to porphyrin rings to compete for heme and inhibit enzyme activity. An exception is CoPP, which is a strong HO-1 inducer, and its HO-1 synthesis effect outweighs its inhibitory effect [248]. Therefore, the major concern of the use of metalloporphyrins is the potential cytotoxicity of heme after blockade of its metabolism. It has been reported that metalloporphyrins interact with many heme-containing enzyme systems, including soluble guanylyl cylase (sGC), nitric oxide synthase (NOS), and cytochrome P450 (CYP450) [249]. They have also been found to affect steroidogenesis, matopoiesis, and the iron status of the body [249]. However, the most prominent potential side effect is the photosensitivity of majority metalloporphyrins. The photosensitinzing property of metalloporphyrins causes the formation of triplet excited states which subsequently causes the formation of singlet oxygen [250]. The excess singlet oxygen reacts with many biological substrates, including amino acids, cholesterol and fatty acids) [250, 251]. It has been reported that the photophysical parameters and singlet oxygen-sensitizing ability of SnMP is strong, and therefore it is expected to have phototoxic effects [252]. It has also been reported that mortality was observed in rats when treated with SnMP and exposure to cool white fluorescent light simultaneously [253]. The underlying mechanisms are not known. Another major concern in the use of metalloporphyrins is that they reduce the CO and free iron status of cells; therefore, they may affect hemoproteins and other enzymes. It has been reported that SnMP decreases CYP450 activity and therefore affects CYP450-related enzymes of adrenal synthesis and drug metabolism in animal models [249]. However, the underlying mechanisms are not clear.

Chapter 2

N-acetylcysteine (NAC) ameliorates lipid-related metabolic dysfunction in bone marrow stromal cells-derived adipocytes

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Abstract

Recent experimental data suggest that fatty acids and lipotoxicity could play a role in the initiation and evolution of metabolic bone diseases such as osteoporosis. A functional bone marrow adipose tissue (BMAT) may provide support to surrounding cells and tissues or may serve as a lipid reservoir that protects skeletal osteoblasts from lipotoxicity.

The present study examined the effect of N-acetylcysteine (NAC), a powerful antioxidant and precursor of glutathione, commonly used to treat chronic obstructive pulmonary disease, on triglycerides accumulation in bone marrow stromal cells-derived adipocytes. Quantification of Oil Red O-stained cells showed that lipid droplets decreased following NAC treatment. Additionally, exposure of bone marrow stromal cells (HS-5) to NAC increased adiponectin, PPARγ, HO-1, SIRT-1 and increased beta-oxidation markers such as PPARα and PPARδmRNA levels. As there is now substantial interest in alternative medicine, the observed therapeutic value of NAC should be taken into consideration in diabetic patients.

Introduction

Obesity, diabetes and metabolic syndrome are linked to abnormal bone homeostasis. The balance between bone formation and resorption is compromised in diabetes, which may contribute to the high risk of fractures in diabetic patients [1-4].

Osteoporosis is a metabolic bone disease associated with a dysregulated bone remodeling resulting from an alteration of the balance between bone formation and bone resorption but although the association between osteoporosis and type I diabetes (T1D) is well established[5], the reported association between osteoporosis and type II diabetes (T2D) is less clear. Chronic hyperglycemia, observed in T2D, accelerates the non-enzymatic process of protein glycosylation, resulting in the formation and accumulation of advanced glycation end-products which contribute to skeletal fragility [6, 7].

Accumulative evidence has indicated that T2D is associated with a reduced bone turnover and consequently a poor bone quality caused by lower levels of bone formation and resorption [8, 9].

Interestingly, an increased bone marrow adiposity (BMA) has been reported in T1D and T2D; however, in T1D, it was observed a decrease in bone mass, whereas T2D is characterized by no change or higher bone mass and paradoxically increased risk of bone fractures [10, 11]. In the last decade, there has been increasing interest in the formation, function and potential endocrine roles of bone marrow adipose tissue (BMAT) [12-14].

BMAT develops postnatally and accounts for 50–70% of bone marrow volume in healthy adult humans. Historically BMAT was considered as an inert type of fat which accumulates in the bone marrow to fill empty space. Marrow adipocytes are no more regarded as simple filling cells, and their involvement in bone physiopathology is now considered. Indeed, BMAT can be considered a functional organ that can undergo pathologic changes and respond to diseases [15].

Furthermore, several studies support the notion that BMAT is significantly associated with skeletal health. In *vitro* studies have shown that adipocytes may directly influence osteoblast and osteoclast differentiation and function, through secretion of adipokines and free fatty acids, suggesting a direct effect of BMAT on bone turnover. BMAT adipocytes are situated in a unique microenvironment, surrounded by hematopoietic and skeletal lineage cells. Hyperglycemia has emerged as a major issue that threatens health and causes vascular and organ dysfunction. Recent reports indicate that hyperglycemia impairs bone marrow hematopoietic function and alters hematopoietic niche [16].

These observations suggest that BMAT may function to provide support to surrounding cells and tissues or may serve as a lipid reservoir, a storage site that protects skeletal osteoblasts from lipotoxicity or may exert systemic effects [17-19].

These findings have raised much interest in the potential role of BMAT supporting the concept that BMAT has an endocrine/paracrine function modulating marrow environment supporting bone remodeling and that this function is under similar regulatory axes as in peripheral adipose tissue.

Changes in bone fat metabolism may contribute to an increase in oxidative stress and in oxidized lipids associated with increased local inflammatory responses, resistance to anabolic effects of Wnt signaling and osteoporotic bone formation. Bone mineral density and biochemical markers of bone turnover are adversely affected in individuals with diabetes. Indeed, glucose intolerance and saturated fatty acids are associated with the attenuation of bone remodeling and turnover in animal models of diet-induced obesity and diabetes [20, 21].

Increasing evidence confirmed that oxidative stress plays a role in the triggering of bone disease associated with diabetes [22].

Moreover, levels of reduced glutathione (GSH), which represents the major intracellular reducing molecule, are decreased in the bone marrow after induction of diabetes.

N-acetylcysteine (NAC) is a thiol compound that stimulates glutathione-S-transferase activity; it acts as a scavenger of free radicals and as an antioxidant by restoring the pool of intracellular GSH. NAC has been commonly used to treat chronic obstructive pulmonary disease [23, 24] but in addition to its antioxidant and anti-inflammatory properties, there is a growing interest in the beneficial effects of NAC for treating metabolic disorders. It has been shown that NAC supplementation suppresses fructose and high sucrose diet-induced hyperglycemia, hyperinsulinemia, and improves peripheral insulin sensitivity [25-27].

Additionally, Yamada et al. demonstrated that NAC markedly promoted the differentiation of osteoblastic cells and accelerated bone regeneration [28]. Despite some data demonstrating that NAC inhibits murine pre-adipocyte differentiation [29], its anti-inflammatory effects on human mature and hypertrophic bone marrow adipocytes are not known.

The goal of the present study was to determine whether NAC treatment affect lipid metabolism and triglycerides accumulation in bone marrow stromal cells-derived adipocytes.

Material and Methods

Differentiation of human BMSC into adipocytes

HS-5, human bone marrow stromal cells, were purchased from American Type Culture Collection (Rockville, MD, USA). After thawing, HS-5 cells were resuspended in DMEM, supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic solution (Invitrogen) and plated in a 75 cm^2 flask at a density of 1 to 2 × 10^4 cells. The medium was replaced with adipogenic medium, and the cells were cultured for additional 19 days. The adipogenic media (Lonza, Basel, SW) consisted of complete culture medium supplemented with DMEM-high glucose (4.5 g/L), 10% (v/v) FBS, 10 µg/ml insulin, 0.5 mM dexamethasone (Sigma–Aldrich, St. Louis, MO), 0.5 mM isobutylmethylxanthine (Sigma–Aldrich, St. Louis, MO) and 0.1 mM indomethacin (Sigma– Aldrich, St. Louis, MO). Medium was changed every 3 days. In our experiments human BMSCs were cultured in the presence of NAC (10 mM) which was added once for the last 3 days of differentiation.

Cell viability assay

HS-5 cells were seeded at a concentration of 2×10^5 cells per well of a 96-well, flat-bottomed 200 µl microplate. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere and cultured for 24 h in the presence and absence of different concentrations (1,10,100 mM) of NAC. Four hours before the end of the treatment time, 20 µl of 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline (PBS) was added to each microwell. After incubation with the reagent, the supernatant was removed and replaced with 100 µl DMSO. The amount of formazan produced is proportional to the number of viable cells present. The optical density was measured using a microplate spectrophotometer reader (Thermo Labsystems Multiskan Italy) at $\lambda = 570$ nm.

Free radical scavenging activity

The free radical scavenging activity of NAC was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test. An aliquot of MeOH solution containing NAC 10 mM was added to a daily prepared methanol DPPH solution (final concentration 0.1 mM). The optical density change at 517 nm was measured, 20 min after the initial mixing, using a microplate spectrophotometer reader (Thermo Labsystems Multiskan Italy). Rutin (50µM) was used as reference. The scavenging activity was measured as the decrease in absorbance of the samples versus DPPH standard solution. The results were obtained from the average of three independent experiments, and are reported as mean radical scavenging activity percentage (%) ± SD.

Measurement of HO enzymatic activity in HS-5 cell line

Total HO activity in the cell lysate was determined by measuring the bilirubin formation using the difference in absorbance at 464–530 nm as previously described [30]. Reaction mixtures (500 µL) consisted of 20 mM Tris-HCl, pH 7.4, (1 mg/mL) cell lysate, 2 mg/mL biliverdin reductase, 1 mM NADPH, 2 mM glucose 6-phosphate (G6P), 1 U G6P dehydrogenase, 25 µM hemin. Incubations were carried out for 1 h at 37 °C in a circulating water bath in the dark. Reactions were stopped by adding 1 volume of chloroform. After recovering the chloroform phase, the amount of bilirubin formed was measured with a double-beam spectrophotometer as OD464-530 nm (extinction coefficient, 40 mM/cm-1 for bilirubin). One unit of the enzyme was defined as the amount of enzyme catalyzing the formation of 1 nmol of bilirubin/ mg protein/h.

Oil Red O staining

Staining was performed using 0.21% Oil Red O in 100% isopropanol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, adipocytes were fixed in 10% formaldehyde, stained with Oil Red O for 10 minutes, rinsed with 60% isopropanol (Sigma-Aldrich), and the Oil Red O eluted by adding 100% isopropanol for 10 minutes and the optical density (OD) measured at

490 nm, for 0.5 sec reading. Lipid droplets accumulation was examined by using inverted multichannel LED fluorescence microscope (Evos, Life Technologies, NY).

RNA extraction and qRT-PCR

RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was then synthesized with Applied Biosystem (Foster City, CA, USA) reverse transcription reagent. Quantitative real-time PCR was performed in 7900HT Fast Real-Time PCR System Applied Biosystems using the SYBR Green PCR MasterMix (Life Technologies). The primer sequences used are shown in Table I. The specific PCR products were detected by the fluorescence of SYBR Green, the double stranded DNA binding dye. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR

product and normalized with that of GAPDH by using comparative $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

Statistical significance ($p<0.05$) of differences between experimental groups was determined by the Fisher method for analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by either single-factor analysis of variance (ANOVA) for multiple groups or the unpaired -test for two groups, and the data are presented as mean ± standard deviation (SD).

Results

Figure 1

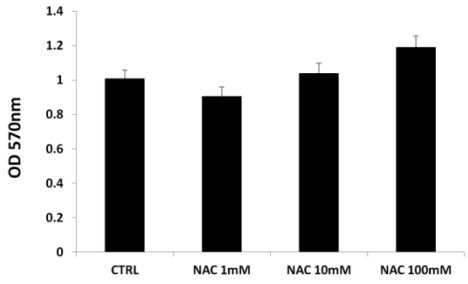

Figure 1. The cytotoxicity of NAC (1, 10, 100mM) on HS-5 cells. Cells were incubated with NAC for 24 h and cell viability was assessed using an MTT assay. Values represent the mean ± SD of 4 experiments.

Viability Assessment

The absence of cytotoxicity of NAC was assessed by the MTT assay, a widely recognized in vitro preliminary screening able to individuate time and concentration-dependent toxic effect on mammalian cell vitality and growth. After a 24 h incubation period to various concentrations of NAC (1, 10, 100 mM), MTT assay (Figure 1) did not reveal any cytotoxic effect of NAC against HS-5 bone marrow stromal cells.

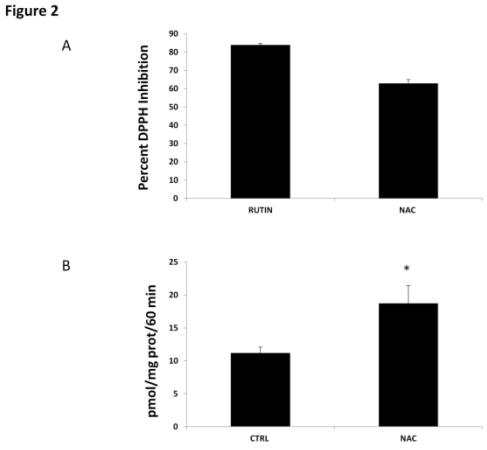

Figure 2. A) DPPH radical scavenging activity of NAC 10mM compared with the standard antioxidant Rutin 50μM. Results are expressed as percent DPPH inhibition. The data shown are the mean from three independent experiments. B) HO-1 activity in HS-5 cell lysate in absence and in presence of NAC 10mM. Values, expressed as pmol bilirubin/mg protein/60min, represent the means ± SD of four experiments performed in triplicate. Significant vs untreated control cells: *$p<0.05$.

Antioxidant activity of NAC

Because of its odd electron, DPPH gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. As shown in Figure 2A, NAC exhibited DPPH free radical scavenging activity in a cell-free system. As DPPH is a synthetic radical, we also investigated the antioxidant capacity of NAC by measuring heme oxygenase derived bilirubin production in a cellular system. Under our experimental conditions, exposed HS-5 cells to 10 mM NAC for 24 hr, showed a significant increase in heme oxygenase activity compared to untreated control cells (Figure 2 B).

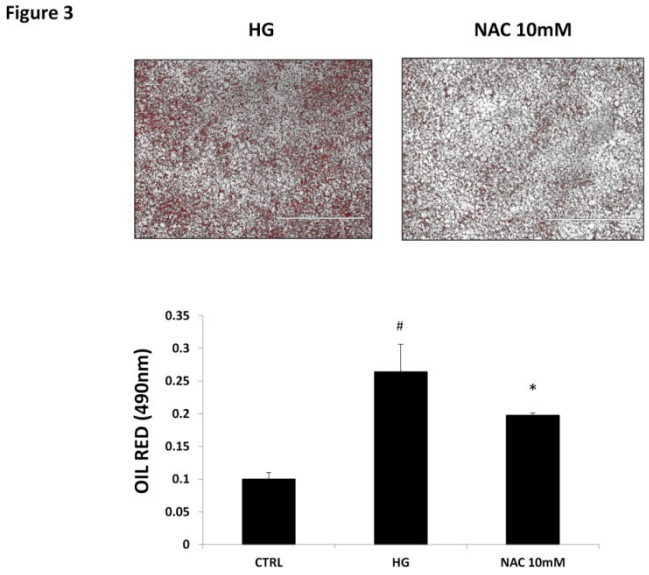

Figure 3. Representative Oil red O staining of HS-5 cells in presence and absence of NAC (10mM). Lipid content was measured as the relative absorbance of Oil Red O at day 19 after inducing adipogenesis as described in materials and methods (mean ± SD, $^{*}p < 0.05$ versus high glucose). Values represent the means ± SD of 4 experiments performed in triplicate.

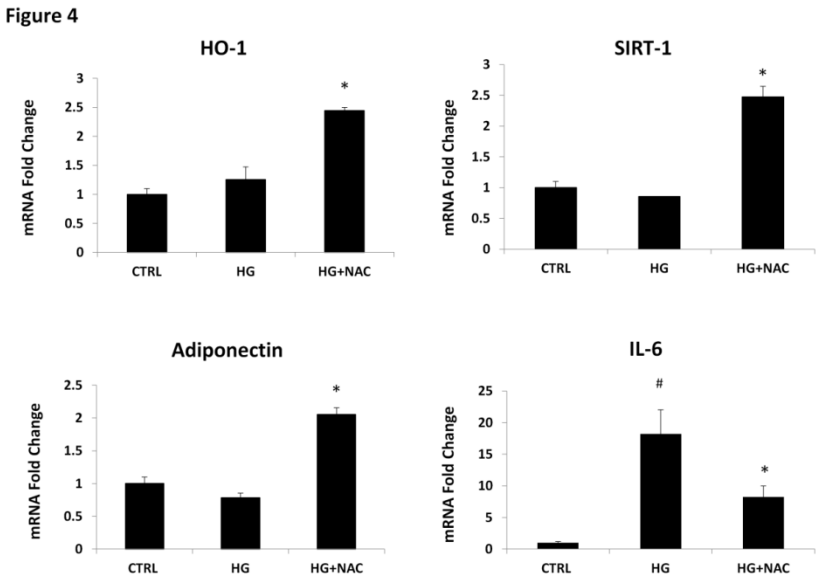

Figure 4. Analysis of gene expression by Real time PCR of HO-1, SIRT-1, Adiponectin and IL-6. All values are expressed as mean ± SD of four experiments ($n = 4$) in duplicate (*$p < 0.05$ versus high glucose; $^{\#}p < 0.05$ versus control).

Figure 5

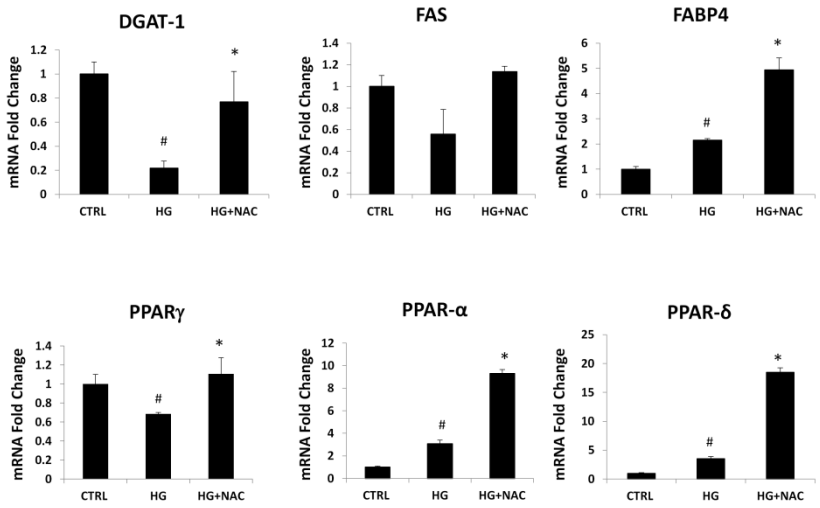

Figure 5. Analysis of gene expression by Real time PCR of lipogenic pathway. All values are expressed as mean ± SD of four experiments ($n = 4$) in duplicate (*$p < 0.05$ versus high glucose; $^{\#}p < 0.05$ versus control).

Effect of NAC on adipocyte lipid metabolism

We examined the effect of NAC on lipid accumulation after 19 days, using standard culture conditions by measuring Oil Red O-stained lipid droplet area (Figure 3). Quantification of Oil Red O-stained cells showed that lipid droplets decreased following NAC treatment. In addition to that, the expression of SIRT-1 and the antioxidant enzyme HO-1 in the presence of NAC significantly increased after 19 days (Figure 4), which was consistent with our previous results showing HO-1 induction decreased lipid droplets [31-33].

To further examine the mechanism by which NAC regulates lipid metabolism, we measured DGAT1, FABP4, FAS, PPARg, PPARα, PPARd, and adiponectin mRNA levels in adipocytes (Figures 4 and 5). Interestingly, as seen in Figure 5, DGAT1 mRNA expression was found to be significantly decreased in mature adipocytes compared to control cells and this effect was reversed by concurrent treatment with NAC. Our results, further demonstrated that the expression of PPAR was significantly downregulated after 19 days of differentiation and this effect was negated by the treatment with NAC. Also our data showed that FAS levels were not significantly altered in all groups but FABP4 levels were

increased by NAC treatment. Further, our results on beta-oxidation markers, such as PPARα and PPARδ, showed that NAC treatment increased both mRNA levels.

Adipose cell enlargement is associated with increased secretion of cytokines, which impairs the differentiation of pre-adipocytes and reduces adiponectin secretion. We examined the levels of IL-6 in NAC-treated HS-5, and found that IL-6 levels were significantly decreased. In contrast, adiponectin levels were increased following NAC treatment when compared to controls at day 19 (Figure 4).

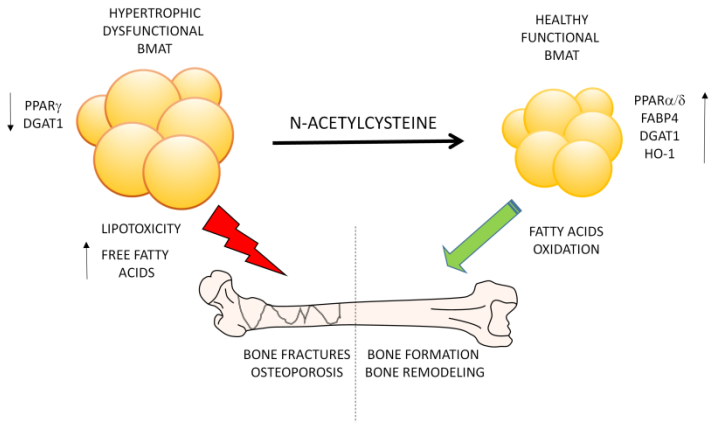

Figure 6. Proposed mechanisms demonstrating the role of NAC in the regulation of lipid metabolism. NAC restores the function of BMAT which may protect skeletal osteoblasts from lipotoxicity.

Discussion

Although there is extensive literature on the role of visceral, subcutaneous, and brown adipose depots in the development of insulin resistance and type II diabetes, few studies have investigated the function of BMAT as an adipose storage organ [34].

Studies are underway to examine the role of impaired glucose metabolism and its associated hyperglycemia and hyperinsulinemia on the biological functions of BMSC [35].

Evidence collected from animal and human studies indicate that impairment in adipose tissue metabolism correlates with decreased bone quality.

A recent clinical study demonstrated very clearly that adipocyte size is associated with vertebral fractures supporting the hypothesis that large adipocytes are inflamed and produce several pro-inflammatory cytokines which affect bone quality as well as progenitor cells[36-38]. Currently, there are several ongoing studies examining the potential positive effects of BMAT on bone mass. A recent and innovative hypothesis suggest that BMAT can be targeted to treat bone disease since BMAT could support bone formation and remodeling. Marrow fat may participate in lipid metabolism by clearing and storing circulating triglycerides, thereby providing a localized energy reservoir for osteogenesis during bone fracture healing. Recent experimental data suggest that FA and lipotoxicity could play a role in the initiation and/or evolution of metabolic bone diseases such as osteoporosis[39]. Although in small quantities relatively to white adipose tissue, marrow adipocytes produce leptin and adiponectin. Thus, it is reasonable to believe that BMAT has a local endocrine/paracrine function modulating marrow environment supporting bone remodeling and that this function is under similar regulatory axes as in peripheral fat.

Although NAC is a well-known thiol compound used in chronic obstructive pulmonary disease because it possesses a free sulfhydryl group through which it reduces disulfide bonds conferring antioxidant effects, NAC possess other beneficial effects on glucose and lipid metabolism [40]. Yang *et al.* reported that NAC administration partially reduces plasma triglycerides, total cholesterol, and LDL levels while increasing the HDL level in rats fed a high fat diet [41]. Short-term NAC administration in drinking water prevented high-sucrose induced weight gain and dyslipidemia. Collectively, these studies show that NAC is capable of restoring dysregulated glucose and lipid metabolism, suggesting its potential application in metabolic disturbance. Our results demonstrate that NAC exhibits an interesting antioxidant activity in cell-free and in cell systems. It was able to quench the synthetic DPPH radical and exhibited an increase in heme oxygenase activity as showed by rise in bilirubin formation. In adipose tissues, induction of HO-1 has been shown to reduce body weight, decrease pro-inflammatory cytokines, and increase PGC-1α mediated thermogenesis, thereby increasing energy uptake and the stimulation of mitochondrial FA oxidation [42-45]. To investigate the effects of NAC on human bone marrow adipocytes, HS-5 cells were exposed to NAC added to the differentiation medium for three days. HS-5 accumulated lipid droplets following exposure to the differentiation medium; however, consistent with previous reports on murine cells, lipid accumulation in the presence of NAC was reduced compared to untreated cells. To assess whether the reduced lipid droplets

formation, induced by NAC, could be explained by altered activation of typical key markers of mature adipocytes, PPAR, DGAT1, FABP4, FAS and adiponectin levels were investigated. Figures 4 and 5 show that NAC increases DGAT1, FABP4 and adiponectin levels. The latter represents a hormone with insulin-sensitizing, anti-inflammatory, and anti-apoptotic functions released by functional healthy adipocytes.

PPAR represents the master regulator of adipogenesis and increased PPARγ signaling in mature adipocytes is linked to improved insulin sensitivity [46].Recently, it has been shown that specific modulators of PPARγ activity, specifically those which protect Ser112 phosphorylation, induce beige-like profile in BMAT and in peripheral white adipose tissue and have either none or positive effect on bone, in contrast to thiazolidinediones which dephosphorylate Ser112 and have negative effects on bone structure [47, 48]. Our unexpected results on DGAT1 might support the concept that the function of DGAT1-mediated FA re-esterification is not to preserve TG mass but instead protects the endoplasmic reticulum from lipotoxic stress and associated adipose tissue inflammation. Indeed, DGAT levels are decreased in mature adipocytes (Figure 3), suggesting that hypertrophic adipocytes lose the esterification function essential to re-esterificate free fatty acids which represent the main cause of insulin resistance. In support of that, Dgat1-deficient mice have abnormal bone architecture and increased levels of osteoclast differentiation[49]. In addition to that, low FABP4 expression in adipose tissue could lead to less free fatty acids transport to β-oxidation, resulting in a fatty acids accumulation, which may exceed the adipose tissue storage capacity, resulting in excess fat “overspilled” to non-adipose tissues [50]. Further beneficial effects mediated by NAC are associated with an increase in mRNA levels of genes responsible for fatty acid oxidation and HO-1 induction. Indeed, NAC cause a strong induction of PPARα (8 fold of increase) and PPARδ (15 fold of increase) compared to untreated cells. These data support the beneficial interaction between bilirubin and PPAR alpha [51]. Several studies demonstrated that bilirubin treatment decreased body weight, body fat and increased lean body mass in wild-type but not in PPARα knockout mice [52, 53]. In conclusion, we show here in a cell-based model of adipogenesis that NAC entails reduced lipid accumulation comprised of smaller healthier adipocytes, reduced inflammation and improved adipokine secretion (Figure 6). These results provide direct evidence to support the use of NAC supplementation as an effective drug metabolic disorders.

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Chapter 3

Protective Effects of Caffeic Acid Phenethyl Ester (CAPE) and Novel Cape Analogue as Inducers of Heme Oxygenase-1 in Streptozotocin-Induced Type 1 diabeticrats.

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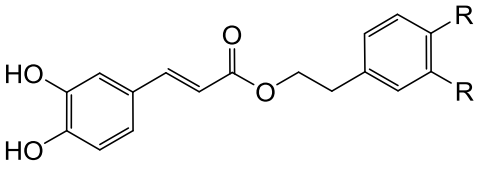
Abstract: Type 1 diabetes mellitus (T1D) is the chronic autoimmune disease resulting in the destruction of insulin producing β-cell of the pancreas with consequent insulin deficiency and excessive glucose production. Hyperglycemia results in increased levels of oxygen free radicals (ROS) and nitrogen species (RNS) with consequent oxidative/nitrosative stress and tissue damage. Oxidative damage of the pancreatic tissue may contribute to endothelial dysfunction associated with diabetes**.** Aim of the present study was to investigate if the potentially protective effect of phenethyl ester of caffeic acid (CAPE), one of the main components of propolis with a flavonoid-like structure, and of a novel CAPE analogue, as heme oxygenase-1 (HO-1) inducers, could reduce pancreatic oxidative damage induced by excessive amount of glucose, affecting the nitric oxide synthase/dimethylarginine dimethylaminohydrolase (NOS/DDAH) pathway in streptozotocin-induced type 1 diabetic rats. Our data demonstrated that inducible nitric oxide synthase/Gamma-Glutamyl-Cysteine Ligase (iNOS/GGCL) and DDAH dysregulation may play a key role in high glucose mediated oxidative stress whereas HO-1 inducers such as CAPE or its more potent derivatives may be useful in diabetes and other stress-induced pathological conditions.

1. Introduction

Diabetes mellitus (DM) is a chronic syndrome of impaired carbohydrate, protein, and fat metabolism caused by insufficient secretion of insulin and/or defects in insulin action in tissues due to insulin resistance. Type 1 diabetes mellitus (T1D) is the chronic autoimmune disease resulting in the destruction of insulin producing β-cell of the pancreas with consequent insulin deficiency and excessive glucose production [1, 2]. Although insulin resistance is traditionally linked to type 2 diabetes mellitus (T2D), intense inflammatory activities characterized by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits, and fibrosis may result also in T2D to loss of β-cells and reduced insulin production [3]. Moreover, irrespective of the type, DM is a complex metabolic disease, often associated with long-term complications, including vascular complications, affecting many tissues [4-9]. In the diabetic status, exposure to high levels of glucose cause a marked reduction in endothelial cell (EC)-released NO [10] with consequent vascular dysfunction [11]. Previous studies showed that endogenous arginine analogs may play a regulatory role in the arginine/NO pathway [12]. Asymmetric NG, NG-dimethyl-L-arginine (ADMA) is an endogenous inhibitor of all isoforms of Nitric Oxide Synthase (NOS). Elevated ADMA levels have been identified as a biomarker of endothelial dysfunction [13] suggesting that plasma ADMA is significantly associated with cardiovascular risk. ADMA metabolism is related to its generation from protein breakdown and to its cleavage by dimethylarginine dimethylaminohydrolase (DDAH) into citrulline and dimethylamine [14]. Two distinct isoforms of DDAH have been described so far, DDAH-1 and DDAH-2 with distinct tissue distribution [15, 16]. It has been reported that overproduction of reactive oxygen species (ROS) leads to downregulation of DDAH-1 and -2 as well as ADMA accumulation by inhibiting DDAH enzyme, which can be prevented by antioxidants [17, 18]. In most tissues, hyperglycemia results in increased levels of ROS and nitrogen species (RNS). Without adequate compensatory response by endogenous antioxidant systems, a redox imbalance occurs leading to the activation of specific pathways that can amplify the damage. It has been reported that in diabetic patients the increase in oxidative stress is associated with a decline in cellular antioxidant defenses [7]. The transcription factor called Nrf2 (nuclear factor erythroid-derived 2) is referred to as the "master regulator" of the antioxidant response; it modulates the expression of hundreds of genes, including those with a promoter region containing an antioxidant response element (ARE) [19], such as heme oxygenase-1 (HO-1), DDAH-1, DDAH-2, Gamma-Glutamyl-

Cysteine Ligase (GGCL) [20-23] and other antioxidant/detoxifying enzymes. The pharmacological manipulation of Nrf2 may represent a target in treating metabolic disorders such as diabetes. This research aims to elucidate some biochemical and metabolic aspects of the diabetes, identifying any changes in the capacity of antioxidant defense, in an experimental model *in vivo* of diabetes. In addition, although some experimental data showed unwanted effects of HO-1 induction in diabetic models [24-26], as it is also evident that all molecules capable of inducing the biosynthesis of HO-1 may represent potential protective agents, natural compounds and synthetic derivatives of natural molecules could be a valid approach for use as adjuvants in the antidiabetic therapy [27]. The phenethyl ester of caffeic acid (CAPE), a natural phenolic compound occurring in a variety of plants and derived from honeybee hive propolis has many beneficial properties (anti-carcinogenic, anti-viral, anti-inflammatory, anti-oxidant) [28, 29], however the mechanisms of pleiotropism of CAPE are not fully understood and are partially attributed to the ability to induce HO-1 expression [30]. Our previous, *in vitro*, study showed that CAPE and small focused series of CAPE analogues were HO-1 inducers. Some of tested compounds were more potent HO-1 inducers than CAPE.

Particularly, 3-(3,4-dihydroxyphenyl)-(2E)-2-propenoic acid 2-(3,4-dimethoxyphenyl) ethyl ester (VP961) was the most potent (Figure 1). Moreover VP961 is the first known compound able to activate directly HO-1 enzyme and to induce at the same time its protein expression [31].



CAPE: R = H
VP961: R = OCH_3

Figure 1. Chemical structure of CAPE and VP961-CAPE derivative.

Aim of the present study was to investigate if the potentially protective effect of CAPE as HO-1 inducer could reduce pancreatic oxidative damage induced by excessive amount of glucose, affecting the NOS/DDAH pathway in streptozotocin-induced type 1diabetic (STZ) rats. Moreover, because to date only limited structural CAPE analogues have been

examined *in vivo* [32, 33] the protective effect of CAPE derivative VP961, more potent *in vitro* than the parent compound CAPE as HO-1 inducer, was investigated in the same above mentioned animal model.

2. Results

2.1. Body weight, blood glucose content, food intake, water intake and volume of urine excreted

2.1.1: The Effects of CAPE and VP961 on animal body weight

Table 1 shows the time course of the changes in the body weight during the experimental period. The diabetic control rats (STZ) displayed a marked decrease in body weight respect to normal control rats. Body weight was significantly increased in CAPE- or VP961-treated STZ rats respect to diabetic control.

Groups	T0	8 days	15 days	21 days
	Body weight (g)	Body weight (g)	Body weight (g)	Body weight (g)
Control	231±3	265±5	300± 7	335±11
STZ	228±5	238±7*	262± 5*	280±3*
STZ/CAPE	220±3	256±9	291±3	329±4
STZ/VP961	226±5	253±8	291±4	318±6

Table 1. Effects of CAPE and VP961 on body weight during the experimental period. Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *$p < 0.05$ vs normal control rats**.**

2.1.2: The Effects of CAPE and VP961 on blood glucose content

Figure 2 shows the time course of the changes in blood glucose content during the experimental period. After two days a significant increase was observed both in diabetic control rats (STZ) and in CAPE- or VP961-treated STZ rats respect to normal control rats. A significant reduction in blood glucose content was observed in CAPE- or VP961-treated STZ rats respect to diabetic control rats after 8, 15 and 21 days of treatment.

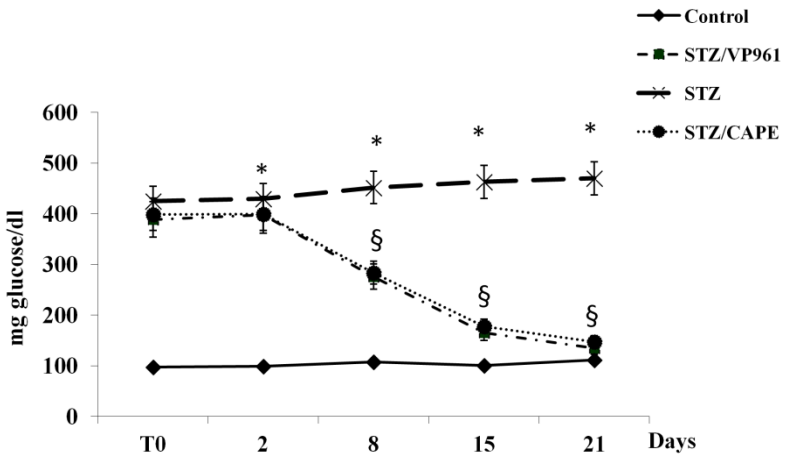


Figure 2. Effects of CAPE and VP961 on blood glucose content during the experimental period. Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *p < 0.05 vs normal control rats; § p < 0.05 vs diabetic control rats (STZ).

2.1.3: The Effects of CAPE and VP961 on water intake, volume of urine excreted and food intake

The time course of the changes in water intake and volume of urine excreted during the experimental period shows that after two days a significant increase was observed both in diabetic control rats and in CAPE- or VP961-treated STZ rats respect to normal control rats. A significant reduction in water intake and volume of urine excreted was observed in CAPE- or VP961-treated STZ rats respect to diabetic control rats (STZ) after 8, 15 and 21 days of treatment (Figure 3-4).

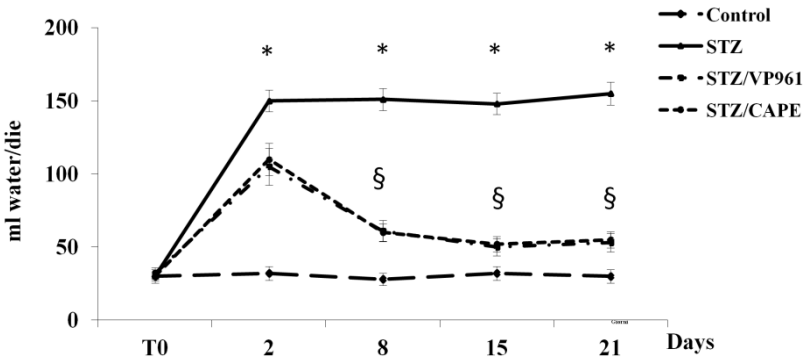


Figure 3. Effects of CAPE and VP961 on water intake during the experimental period. Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *p < 0.05 vs normal control rats; § p < 0.05 vs diabetic control rats (STZ).

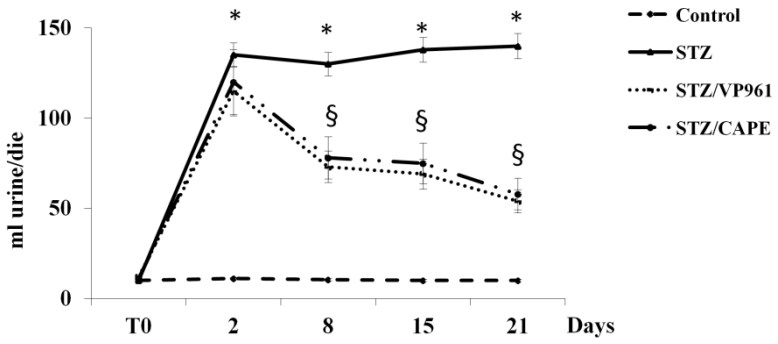


Figure 4. Effects of CAPE and VP961 on volume of urine excreted during the experimental period. Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *p < 0.05 vs normal control rats; § p < 0.05 vs diabetic control rats (STZ).

The food intake of normal rats was higher respect to STZ rats (Normal Control=25±2 g/day; Diabetic Control rats (STZ)= 35± 3 g/day). The food intake of STZ rats treated with CAPE or VP961 was similar to that of normal rats.

2.2. Plasma insulin, RSH, LOOH, ADMA and Nitrite/Nitrate levels

As shown in Table 2, the plasmatic insulin and RSH levels were significantly lower in the diabetic control rats (STZ) than that in non-STZ rats. Treatment with CAPE or VP961 increased significantly these levels. The levels of LOOH, oxidative stress biomarker, in the plasma of diabetic control rats (STZ) were significantly elevated compared with non-STZ rats; however, these levels on receiving CAPE or VP961 were significantly decreased. STZ rats had increased plasmatic ADMA and NO_2^-/NO_3^- levels compared to the normal control group. CAPE or VP961 treatment in STZ rats significantly reduced ADMA and NO_2^-/NO_3^- levels respect to control STZ rats.

PLASMA	Insulin (ng/ml)	RSH (nmoles/ml)	LOOH (nmoles/ml)	ADMA (nmoles/ml)	NO_2^-/NO_3^- (nmoles/ml)
Control	1.0±0.03	140±10	15± 2	0.1±0.02	0.75±0.03
STZ	0.45±0.05 *	80±7*	30± 5*	0.9±0.03*	1.5±0.05*
STZ/CAPE	0.82±0.03 **	130±9**	18±3**	0.6±0.02**	0.8±0.03**
STZ/VP961	0.78±0.07**	120±8**	17±4**	0.3±0.02**	0.7±0.04**

Table 2. Plasmatic insulin, RSH, LOOH, ADMA and NO_2^-/NO_3^- levels. Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *p < 0.05 vs normal control rats; ** p < 0.05 vs diabetic control rats (STZ).

2.3. Pancreatic RSH, LOOH, ADMA and Nitrite/Nitrate levels

Concerning the pancreatic RSH content, the diabetic control rats (STZ)showed a marked decrease compared with non-diabetic control rats. This content was significantly increased by CAPE or VP961 treatment as shown in Table 3. The levels of pancreatic LOOH, oxidative stress biomarker, in diabetic control rats (STZ) were significantly elevated compared with non-STZ rats; however, these levels on receiving CAPE or VP961 were significantly decreased. STZ rats had increased pancreatic ADMA and NO_2^-/NO_3^- levels compared to the normal control group. CAPE or VP961 treatment in STZ rats significantly reduced ADMA and NO_2^-/NO_3^- levels respect to control STZ rats (Table 3).

PANCREAS	RSH (nmoles/mg prot.)	LOOH (nmoles/mg prot.)	ADMA (nmoles/mg prot.)	NO_2^-/NO_3^- (nmoles/mg prot.)
Control	28±2	0.2±0.03	20±0.8	4±0.9
STZ	12±1*	1±0.04*	200±5*	12±2*
STZ/CAPE	27±2**	0.4±0.02**	50±4**	6±0.8**
STZ/VP961	26±3**	0.3±0.03**	53±3**	5±0.9**

Table 3. Pancreatic RSH, LOOH, ADMA and NO_2^-/NO_3^- levels. Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *p < 0.05 vs normal control rats; ** p < 0.05 vs diabetic control rats (STZ).

2.4. Pancreatic HO-1, DDAH-1, GGCL, iNOS protein expressions

The expression levels of antioxidant enzyme-related proteins, such as HO-1 and GGCL, in diabetic control rats (STZ) were very low (Figure 5, Panels B-C). More in detail, HO-1 protein was weakly expressed both in STZ- rats and in non-STZ rats, however CAPE or VP961 administration in STZ rats resulted in a significant upregulation (Figure 5-Panel B).

The expression levels of GGCL in STZ-rats were significantly lower than those of non-STZ rats, as shown in Figure 5 (Panel C). The decreased protein expression of GGCL in STZ rats was increased by the CAPE or VP961 administration.

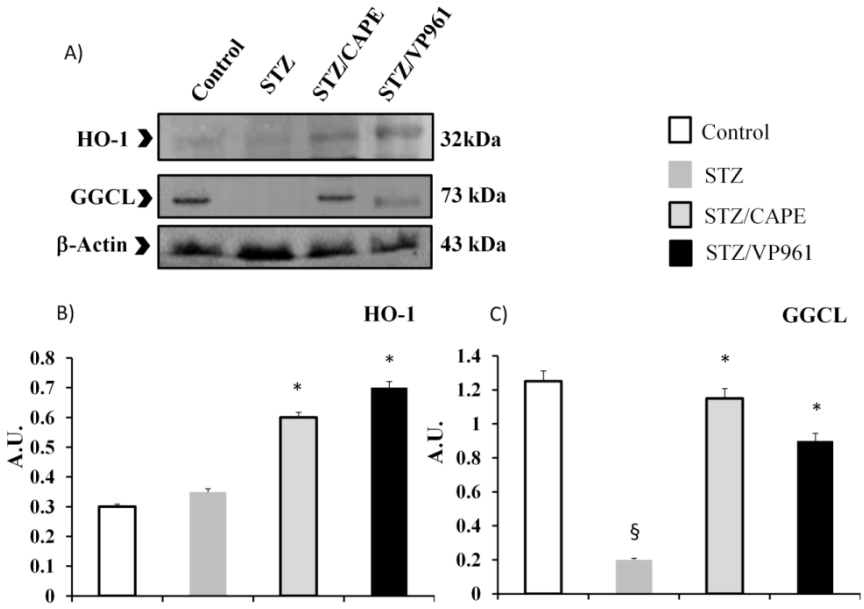


Figure 5. Representative western blotting of HO-1 and GGCL protein expressions (Panel A). Densitometric quantification of HO-1 and GGCL protein expressions in the pancreas of non-STZ rats (Control), STZ rats and CAPE or VP961 treated STZ rats (CAPE/STZ; VP961/STZ) (Panel B-C). Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *$p < 0.05$ vs diabetic control rats (STZ); § $p < 0.05$ vs normal control rats.

The expression levels of iNOS protein in diabetic control rats was significantly higher than those of non-STZ rats, as shown in Figure 6 (Panel B). The increased protein expression of iNOS in STZ rats was decreased by the CAPE or VP961 administration.

The expression levels of DDAH-1 protein in diabetic control rats were significantly lower than those of non-STZ rats, as shown in Figure 6 (Panel C). The decreased protein expression of DDAH-1 in STZ rats was increased by the CAPE or VP961 administration.

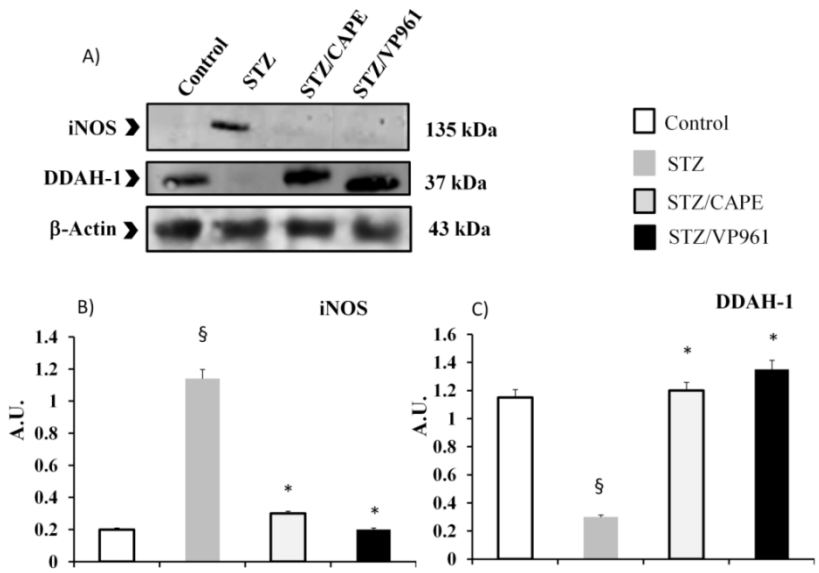


Figure 6. Representative western blotting of iNOS and DDAH-1 protein expressions (Panel A). Densitometric quantification of iNOS and DDAH-1 protein expressions expressions in the pancreas of non-STZ rats (Control), STZ rats and CAPE or VP961 treated STZ rats (CAPE/STZ; VP961/STZ) (Panel B-C). Values are mean±standard deviation (S.D.) of three independent experiments performed in triplicate. *$p < 0.05$ vs diabetic control rats (STZ); § $p < 0.05$ vs normal control rats.

3. Discussion

ROS and RNS are well recognized for playing a dual role in human pathology as both deleterious and beneficial species [34]. In addition, it is often difficult to distinguish whether oxidative reactions occurring during a disease process are the cause, by participating in the initial pathogenetic mechanisms of tissue damage, or if they appear only as one of the final effects of the process [35]. Attempts were made to reduce oxidative damage related to diabetes complications, but results of administration of antioxidants were disappointing **[36, 37]**; for these reasons, currently researches are aiming at the identification of also referred to as "indirect antioxidants", and able to stimulate and strengthen endogenous antioxidant defenses **[38, 39]**. In recent years much attention has been focused on phyto-constituents present in fruits, vegetables, and medicinal herb and, in particular, on some plant secondary metabolites such as phenolic and terpene compounds [40]. There are now many published studies on the antioxidant activities or to the ability of enhancing endogenous antioxidant defenses by modulating the cellular redox state of plant-derived substances **[41-48]**, but their potential beneficial effects on human health are not

confined to their antioxidant action; in fact, numerous and interesting biological activities could reveal new roles of these compounds in the prevention and treatment of certain diseases, such as metabolic syndrome and/or diabetes complications [37, 49, 50]. However, it is important to note that most of the studies were conducted using cell models, while few results were obtained using *in vivo* models **[36, 37]**. Type 1 diabetes, leads to high blood glucose levels (hyperglycemia) that can cause serious health complications **[51]**. Although hyperglycemic damage is a multifactorial process, data in literature suggest that oxidative/nitrosative stress and stress-activated signaling pathways might represent a unifying hypothesis [7]. In diabetic patients, the overproduction of ROS and RNS is associated to iNOS overexpression which might contribute to stress-induced pancreatic cell death **[52, 53]**.

In our experimental conditions, the significant increase of plasmatic and pancreatic LOOH and nitrite/nitrate levels, markers of oxidative/nitrosative stress, induced by low insulin content and consequent hyperglicemia, may be related to upregulation of pancreatic iNOS protein.

Moreover, oxidative stress is also related to depletion of antioxidant defenses, which also contributes to many of complications of diabetes, including vascular complications. It has been reported that, the overproduction of free radicals could cause damage and apoptosis of pancreatic islet β-cells and reduction of insulin secretion **[54]**. Bruce C.L. et al reported that HO-1 mRNA expression is significantly reduced in T2D patients **[55]** whereas, upregulation of the HO-system increases pancreatic β-cell insulin release and reduces hyperglycemia in different diabetic models **[56]**. *In vitro* and *in vivo* studies demonstrated that CAPE has many beneficial properties including anti-hyperglycemic and antioxidant properties **[57-61]**. In our experimental conditions body weight of CAPE or VP961-treated STZ rats was significantly increased compared to the diabetic control rats. Moreover, treatment of STZ rats with CAPE or VP961 significantly reduced blood glucose levels, increased plasmatic insulin levels, decreased plasmatic and pancreatic LOOH and nitrite/nitrate levels respect to control STZ rats. The reduction of plasmatic and pancreatic nitrite/nitrate levels may be related to iNOS downregulation in CAPE or VP961 treated STZ rats. These results suggest that the effects of CAPE or VP961 may be due to protecting from damage of the pancreatic tissue. Moreover, the significant increase of plasmatic and pancreatic LOOH induced by low insulin content and consequent hyperglicemia, may be related to downregulation of pancreatic antioxidant defences, both enzymatic than

nonenzymatic, such as HO-1, GGCL and RSH. Under physiological conditions Nrf2 locates in the cytoplasm and binds to its inhibitor kelch-like ECH associated protein 1 (KEAP1). Upon exposure of cells to natural phenolic compounds, Nrf2 is free from KEAP1 and translocates into the nucleus to bind to antioxidant-responsive elements (ARE) in the genes encoding antioxidant enzymes such as heme oxygenase-1 (HO-1) DDAH-1, DDAH-2, Gamma-Glutamyl-Cysteine Ligase (GGCL) [20-22] and other antioxidant/detoxifying enzymes. Our experimental data showed that HO-1 protein at basal levels was weakly expressed in pancreatic tissue of control rats. These data are in agreement with data of Li et al. (PMID:2984948) [62]. Since HO-1 protein was weakly expressed also in pancreatic tissue of STZ rats any downregulation could not be detectable. However the levels of HO-1 protein were significantly increased by CAPE or VP961-administration. According to Ye et al [63] HO-1 induction may be protective of pancreatic β-cells because of the scavenging of free heme, the antioxidant effects of the end-product bilirubin, or the generation of carbon monoxide, which might have insulin secretion-promoting effects and inhibitory effects on nitric oxide synthase. Moreover, VP961 *in vivo* was, slightly but significantly, more potent than CAPE as HO-1 inducer. In our experimental conditions in pancreas GGCL protein was downregulated in STZ rats, but the levels of this protein was significantly increased by CAPE or VP961-administration. The increased expressions of GGCL induced in STZ rats treated with CAPE or VP961 are related to increased levels of plasmatic and pancreatic RSH and to decreased levels of plasmatic and pancreatic LOOH. Our results demonstrate that *in vivo* CAPE resulted more potent than VP961 as GGCL inducer.

It has been reported that overproduction of ROS leads to downregulation of DDAH-1 and -2 as well as ADMA accumulation by inhibiting DDAH enzyme, which can be prevented by antioxidants [17, 18]. Numerous experimental data have showed that DDAH activities, are crucial in the regulation of ADMA metabolism **[64-66]** and in prevention of endothelial dysfunctions. Newsholme P. et al reported that oxidative stress and ADMA accumulation, could lead to pancreatic β-cell dysfunction and decreased insulin secretion, thus compounding the problematic metabolic status of diabetes **[67]**. In our experimental conditions, also DDAH-1 protein, the main isoform of DDAH expressed in pancreas, was downregulated in STZ rats, but the levels of this protein was significantly increased by CAPE or VP961-administration. The increased expressions of DDAH-1 induced in STZ rats treated with CAPE or VP961 may be due to Nrf2 translocation into the nucleus and to its binding to antioxidant-responsive elements (ARE) in the genes encoding DDAH-1.

DDAH1 upregulation is related to decreased levels of plasmatic and pancreatic ADMA. Our results demonstrate that VP961 resulted *in vivo* more potent than CAPE as DDAH-1 inducer. Then, overall our data demonstrated that in animal model of T1D, CAPE or VP961 treatment, may reverse the diabetic-induced oxidative stress in rat pancreas.

4. Materials and Methods

4.1. Animal model

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of "Catania University" and experiments were approved by the Animal Ethics Committee of "MINISTRY OF HEALTH (Directorate General for Animal Health and Veterinary Medicines) (Italy)". 30 days-old Wistar rats were purchased from Charles River Labs (Lecco, Italy). The rats were maintained under a 12-h light/dark cycle, and housed in a controlled temperature (24±2 °C) and humidity (50±5 %) environment. After several days of adaptation, the rats were divided into normal and diabetic groups. The experimental diabetes was induced by intraperitoneal (i.p.) injection of streptozotocin (50 mg/kg body weight in a 10 mM citrate buffer -pH 4.5-). One week after the injection, we verified the occurrence of hyperglycemia; animals with blood glucose > 140 mg/dl were placed in individual metabolic cages; body weight, amount of water and food taken, and volume of urine excreted were daily recorded. Non-fasting blood samples were collected twice per week by tail bleeding into heparinized tubes. In the plasma samples the glucose concentrations were determined. Rats were distributed in four groups: group I included a lot of six animals untreated that were considered as normal control group; group II included six diabetic animals considered as diabetic control group; group III included six diabetic animals orally treated with a non-toxic dose (30mg/Kg) of the alcoholic extract of CAPE and group IV included six diabetic animals orally treated with a non-toxic dose (30mg/Kg) of the alcoholic extract of CAPE derivative VP961. Control groups (diabetic and non-diabetic rats) received the same volume of ethanol as vehicle.

After 21 days, animals were sacrificed by an overdose of anesthetic and blood and pancreas tissues were immediately removed and frozen for biochemical assays.

4.2. Measurement of glucose and insulin in the plasma

Plasmatic glucose and insulin levels were measured using respectively commercial glucose ELISA kit (CrystalChem, Netherlands) and commercial insulin ELISA kit (ALPCO, USA) in accordance with the manufacturer's instructions. Results are reported respectively as mg glucose/dl of plasma and ng insulin/ml of plasma.

4.3. Plasmatic and pancreatic Nitrite/Nitrate determination

Quantification of Nitrite, the stable metabolite of nitric oxide, was measured colorimetrically via Griess reaction. Aliquots of plasma or pancreas homogenates were preincubated for 30 min at room temperature with 50 μM nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich,St.Louis,MO) and 24 mU nitrate reductase (Roche Diagnostics Gmbh, Mannheim, Germany), and then the samples were treated with 0.2 U lactate dehydrogenase (Roche) and 0.5 mol sodium pyruvate for 10 min. The coloration was developed adding Griess reagent (Merck KGaA, Darmstadt, Germany; 1:1, vol/vol). Finally, after 10 min at room temperature, absorbance was recorded by 96-well plate microtiter at λ540 nm. Nitrite levels were determined using a standard curve and expressed as nanomoles of NO_2^-/NO_3^- per ml of plasma or NO_2^-/NO_3^- per milligram of protein. Protein concentration was measured using TAKE 3 nanodrop.

4.4. Plasmatic and pancreatic ADMA determination

Plasma and tissue ADMA concentration was determined in plasma or pancreas homogenates by using a commercially available enzyme-linked immunosorbent assay kit (DLD Diagnostika GmbH, Hamburg, Germany) according to the manufacturer's instructions. Results are reported as nmoles ADMA/ml of plasma or nmoles ADMA/mg prot.

4.5. Determination of plasmatic and pancreatic Lipid Hydroperoxide Levels

Plasma and pancreatic levels of lipid hydroperoxide were evaluated following the oxidation of Fe^{+2} to Fe^{+3} in the presence of xylenol orange at λ560 nm as previously described [68]. Results are reported as nmoles LOOH/ml of plasma or nmoles LOOH/mg prot.

4.6. Thiol Group Determination

Plasma and pancreatic levels of thiol groups were measured, in 200 µl of plasma or pancreatic homogenate, by using a spectrophotometric assay as previously described [68]. Results are reported as nmoles RSH/ml of plasma or nmoles RSH/mg prot.

4.7. Western blotting

Western blotting analysis was performed as previously described [69, 70]. Briefly, tissues were homogenized in lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% v/v Triton X-100, 1% phenylmethylsulfonyl fluoride (PMSF), 0.05 mM pepstatin A and 0.2 mM leupeptin) and tissue homogenates (30 µg proteins) were loaded onto 12% SDS-polyacrylamide (SDS-PAGE) gels and subjected to electrophoresis (120 V, 90 min). The separated proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After transfer, the blots were incubated with Li-COR blocking buffer for 1 h, followed by overnight incubation with primary antibodies directed against HO-1 (1:1000) [Enzo Life Sciences, Plymouth Meeting, PA)], GGCL (1:1000) [Abcam, Cambridge, United Kingdom], DDAH-1 (1:5000) [Calbiochem EMD Biosciences (Darmstadt, Germany)], iNOS (1:1000) (SantaCruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA). After washing with TBS, the blots were incubated for 1 h with the secondary antibody (1:1000). Protein detection was carried out using a secondary infrared fluorescent dye-conjugated antibody absorbing at 800 and 700 nm. The blots were visualized using an Odyssey Infrared imaging scanner (LI-COR Biosciences) and quantified by densitometric analysis performed after normalization with β-actin. Results are expressed as arbitrary units (A.U.).

4.8. Statistical analysis

Data are reported as mean±standard deviation (S.D.) values of at least three independent experiments. The results were analyzed for statistical significance using ANOVA followed by Bonferroni's post hoc test. A p-value < 0.05 was considered as significant.

5. Conclusions

Overproduction and/or insufficient removal of free radicals result in different pathological conditions including diabetes [71]. Diabetes mellitus increases oxidative stress in pancreatic tissue. Oxidative damage of the pancreatic tissue may contribute to endothelial dysfunction associated with diabetes. It can be concluded that CAPE and VP961 inhibit lipid peroxidation and regulate antioxidant enzyme-related proteins in STZ rats. Moreover, iNOS/GGCL and DDAH dysregulation, may play a key role in high glucose mediated oxidative stress whereas HO-1 inducers such as CAPE or its derivatives may be useful in diabetes and other stress-induced pathological conditions.

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Chapter 4

The effects of olive leaf extract from a Sicilian cultivar in an experimental model of hepatic steatosis

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Abstract

Olive oil is a well known product for its health benefit, but the leaf has also been used as a traditional medicine in the Mediterranean for centuries. Olive leaves contain a great variety of chemical substances belonging to phenolic acids, phenolic alcohols, flavonoids and secoiridoids, and many other pharmacological active compounds with an important antioxidant effects such as oleuropein (OE), hydroxytyrosol (HT), tyrosol, cumaric acid, ferulic acid, caffeic acid, vanillic acid, rutin, verbascoside, luteolin, quercetin, dimethyloleuropein and ligstroside. Characterization of these compounds demonstrated that they can play an important role in human health, because of their ability to improve glucose homeostasis, ameliorate dyslipidemia and reduce inflammatory cytokine. The aim of this study was to investigate the effect of Olive leaf extract (OLE) from Sicilian cultivar in an *in vitro* model of hepatic steatosis to evaluate the protective effects again free fatty acids accumulation in hepatocytes. We report here that OLE treatment ameliorated the lipid metabolism, and this effect was coupled with a parallel decrease in number of lipid droplets and a concomitant increase in FABP-4, SIRT-1 and HO-1 expression. Furthermore, OLE treatment induced a significantly reduction of the inflammatory cytokines IL-1β and TNF-α.

Introduction

Olive oil is a well-known product for its health benefit, but the leaf has also been used as a traditional medicine in the Mediterranean for centuries. Olive leaves contain a great variety of chemical substances belonging to phenolic acids, phenolic alcohols, flavonoids and secoiridoids, and many other pharmacological active compounds with antioxidant effects such as oleuropein (OE), hydroxytyrosol (HT), tyrosol, ferulic acid, caffeic acid, vanillic acid, cumaric acid, rutin, quercetin, dimethyloleuropein and ligstroside (Talhaoui et al. 2015) . In human diet and health they can play an important role due to their ability to to improve glucose homeostasis, ameliorate dyslipidemia and reduce inflammatory cytokine (Lockyer et al. 2015; Wainstein et al. 2012; Yoon et al. 2015). Benavente-Garcia et al, demonstrated that olive phenolics show a synergic behaviour in their radical scavenging capacity (Benavente-Garcia et al. 2002). Recently, it has been demonstrated that Olive leaf extract (OLE) attenuates obesity in high-fat diet-fed mice by modulating the expression of molecules involved in adipogenesis and thermogenesis (Shen et al. 2014). In particular, this study demonstrates that OLE exerts beneficial effects against the excessive accumulation of fat in the liver, typical of hepatic steatosis. The liver is a central metabolic organ and regulates several key aspects of lipid metabolism (by oxidation of fatty acids, lipogenesis and lipoprotein uptake and secretion) in response to nutritional and hormonal signals (Rui 2014). Steatosis, liver inflammation and fibrosis have been associated with an excessive triglyceride accumulation in the liver, insulin resistance and increases in visceral adipose tissue, mediated by increased free radical formation and free oxygen radical species, and modulated by genetic susceptibility (Day and James 1998; Hijona et al. 2010) . Non-alcoholic fatty liver diseases (NAFLD) is currently the most chronic liver disease associated with obesity, insulin resistance, hyperlipidemia and the metabolic syndrome and enhanced risk of cardiovascular disease and mortality (Nassir and Ibdah 2016).

Insulin resistance causes increased lipolysis thus leading to high levels of plasma fatty acids (FA), as well as increased FA uptake by hepatocytes, which results in the formation of intracellular lipid droplets (Marchesini et al. 1999). Hepatic lipid accumulation can progress from simple steatosis to non-alcoholic steatohepatitis (NASH), which includes hepatocellular injury, inflammation and fibrosis (Pais et al. 2011). In addition, further severe complications may occur, such as liver cirrhosis and hepatocellular carcinoma (Onnerhag et al. 2014). Ongoing clinically therapeutic schedules for NAFLD include

insulin sensitization agents, hypolipidemic drugs, antihypertensive agents, cell-protective agents, anti-inflammatory cytokine, antioxidants, and other types of medications (Rusu et al. 2015). The present study explored the therapeutic effects of Olive leaf extract (OLE) on NAFLD in a lipid-accumulation HepG2 cell model induced by FA, which *in-vitro* simulated the pathological process of clinical NAFLD.

Materials and methods

Sample preparation

Samples of olive leaves (*Olea europea L.*) from sicilian variety named "Nocellara dell'Etna" was employed. The leaves were collected at the same time, during pruning period, from sicilian organic farming. The extract was obtained in accordance to Lee-Huang et al. with some modifications (Lee-Huang et al. 2003). Briefly, sicilian olive leaves (OLEs), previously dehydrated at 40 °C, were extracted by an acqueous solvent, 100 mL of hot water was employed for the extraction of 5 g of leaves. Successfully the samples were stored in the dark.

Total polyphenols content

Total polyphenol content of OLE was evaluated by the Folin-Ciocalteu method (Vázquez, Janer, & Janer, 1973), with some modifications. Briefly, 1.25 mL portion of Folin-Ciocalteu (Fluka Analytical) reagent was mixed with 0.25 mL of the sample; after 3 min, 2.5 mL of a sodium carbonate solution (20%) was added to the mixture and the reaction was kept in the dark for 1 h. The absorbance was spectrophotometrically measured at 725 nm, using Perkin elmer lambba 25 (UV-VIS) spectrometer. Caffeic acid (Fluka) was used as reference standard for calibration curve (0.02 – 0.9 mg/mL; y = 1.1429x + 0.0185, where x and y represent the caffeic acid concentration (mg/mL) and absorbance at 725 nm, respectively; r^2 = 0.9995). Contents of total phenolic compounds in OLE were expressed as caffeic acid equivalents in milligram per gram of dried leaf (Vázquez A 1973).

Antioxidant capacity

Radical scavenging activity was determined on OLE by the DPPH as previously described (Brandwilliams et al. 1995). Briefly, a methanol 1,1-Diphenyl-2-picryl-hydrazyl DPPH• (Sigma-Aldrich, USA) solution (100 µM) was used and 3 mL of solution was mixed with 70 µL of leaf extract. The samples were incubated for 60 min at room temperature, then the decrease in absorbance at 515 nm (*A*E) was measured spectrophotometrically. DPPH radicals have a maximum absorption at 515 nm, the peak disappears with reduction by an antioxidant compound. A blank sample, containing 70 µL of methanol in the DPPH• solution, was used as reference. The experiment was carried out in triplicate and Trolox equivalent antioxidant capacity (TEAC) value was derived for each OLE. In addition Radical scavenging activity (RSA%) was calculated using the following equation:

RSA% = [(*A*B-*A*E)/*A*B] × 100 (*A*B= absorbance of the blank sample, and *A*E= absorbance of the leaf extract). RSA% values were expressed as *µ*mol Trolox equivalents/g of leaf extract.

Cell culture

HepG2 cells are a well-characterized human hepatoma cell line expressing a variety of liver function. Consequently, this cell line treated in culture with monousatured or satured fatty acid represents the main *in vitro* NAFLD human model

(Garcia-Ruiz et al. 2015; Gomez-Lechon et al. 2007). HepG2 cell cultures have been shown to retain many characteristics of normal differentiated quiescent hepatocytes, including cholesterol and bile acid metabolism (Javitt 1990) but, although they retain estradiol responsiveness, they have reduced estrogen receptor α (ERα) expression (Marino et al. 2002). HepG2 cells were maintained in DMEM supplement with 10 % FBS, 1 % Penicillin and Streptomycin (GIBCO) solution, and then incubated at 37 °C in a 5 % CO_2 humidified atmosphere (Jeong et al. 2016). For the experiments, the cells were seeded in a 24-well cell culture plate at a density of 5×10^5 cells for per well and incubated for 24 h and then washed with PBS and incubated with high glucose DMEM for 12 h for serum starvation. Then, the medium was changed to DMEM with 3 mM FA (palmitic acid and oleic acid 2:1) 5 % BSA and OLE ("Nocellara" Sicilian variety), containing 0.27 mg caffeic acid/mL and 0.37 mg oleuropein/mL. The cells were treated for 24 h in incubator to induce hepatic steatosis and to observe the OLE effects.

Oil Red O staining

Staining was performed using 0.21% Oil Red O in 100% isopropanol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, hepatocytes were fixed in 10% formaldehyde, stained with Oil Red O for 10 minutes, rinsed with 60% isopropanol (Sigma-Aldrich), and the Oil Red O eluted by adding 100% isopropanol for 10 minutes and the optical density (OD) measured at 490 nm, for 0.5 sec reading. Lipid droplets accumulation was examined by using inverted multichannel LED fluorescence microscope (Evos, Life Technologies, NY).

RNA extraction and qRT-PCR

RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA) (Tibullo et al. 2013). First strand cDNA was then synthesized with Applied Biosystem (Foster City, CA, USA) reverse transcription reagent. Quantitative real-time PCR was performed in Step One Fast Real-Time PCR System Applied Biosystems using the SYBR Green PCR MasterMix (Life Technologies) (Malaguarnera et al. 2011). The primer sequences used are shown in Table 1. The specific PCR products were detected by the fluorescence of SYBR Green, the double stranded DNA binding dye. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH by using comparative $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

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

Results

Partial characterization of OLE extracts

Olive leaves dried samples were extracted by acqueous solvent. The results show that OLE from "*Nocellara dell'Etna"* contains polyphenols with radical scavenging activity, expressed as RSA% and TEAC value (Table 2). The extract obtained by an acqueous method contains phenolic compounds that were identified and quantified by HPLC analysis and oleuropein (46.25 mg/g of dried leaves) is the most abundant antioxidant compound, followed by hydroxytyrosolglucoside and ligstroside (15 mg/g and 9.7 mg/g of dried leaves respectively). The extract contained also luteolin-7-O-glucoside, verbascoside, rutin, and hydroxytyrosol, and caffeic acid, chlorogenic acid (data not shown).

Figure 1

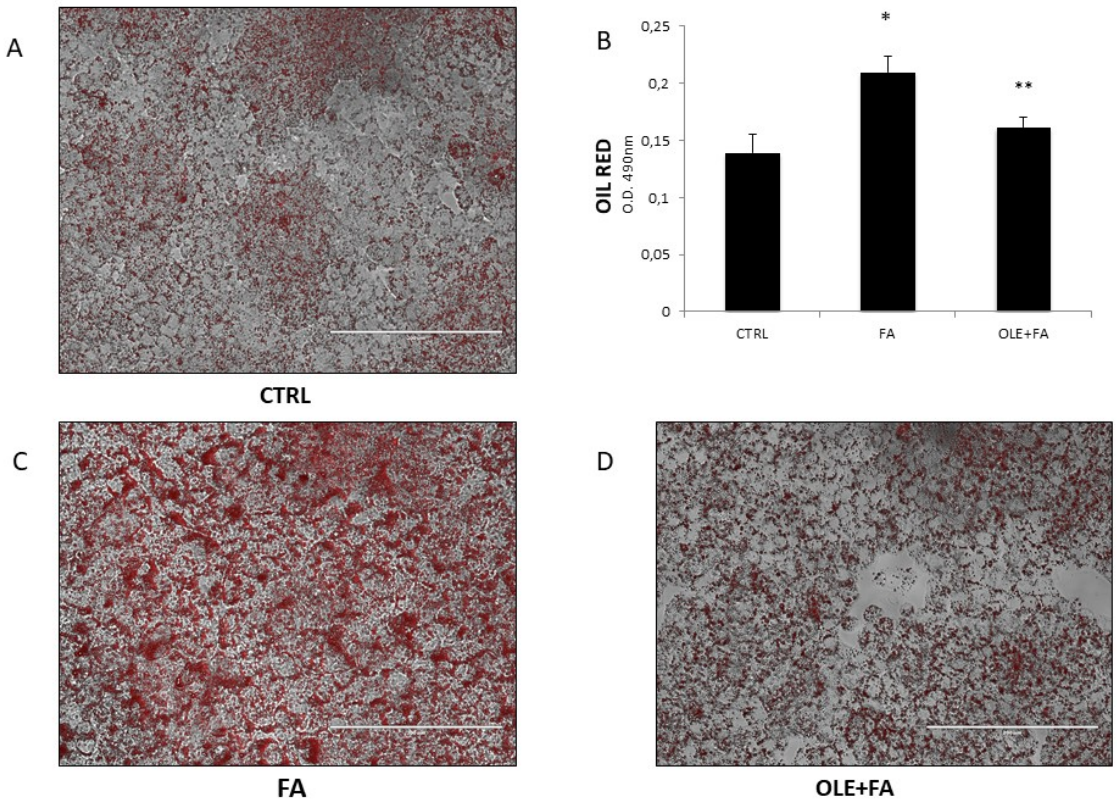

Figure 1. Lipid droplets accumulation measured by Oil red staining in hepatocytes in presence or absence of OLE. Lipid droplets accumulation was measured as the relative absorbance (OD 490nm) of Oil Red at 24h after addition of FA as described in materials and methods. All values are expressed as mean ± SEM of four experiments (n=4) in duplicate. (* $p<0.05$ versus untreated; (**$p<0.05$ versus FA).

Effects of OLE on lipid droplets formation

We treated HepG2 cells with FA or with a combination of FA and OLE. Then, we examined the effect of OLE on lipid accumulation after 24h using standard culture conditions by measuring Oil Red O-stained lipid droplets. Figure 1 shows the positive Oil Red O staining in hepatocytes after 24h of treatment with FA. The absorbance of HepG2 cells in each group is shown in Figure 1 and it was measured after the process of Oil Red O staining and extraction with isopropanol.

Quantification of Oil Red O staining cells showed that lipid droplets accumulation decreased following OLE co-treatment.

Figure 2

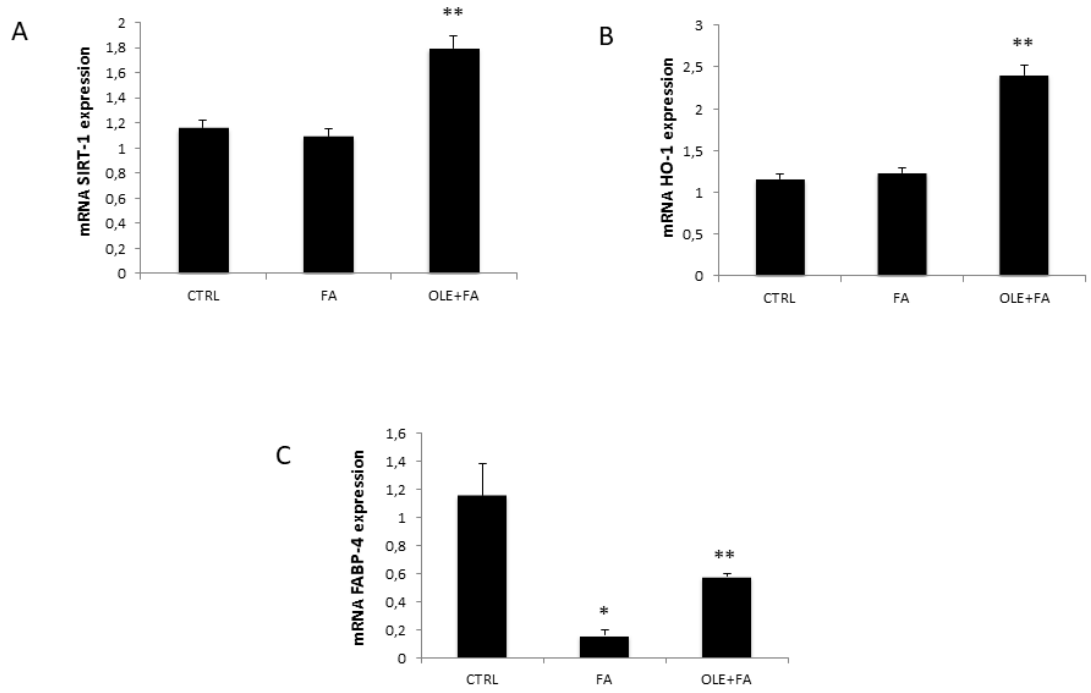

Figure 2. Analysis of gene expression by Real time PCR of SIRT1 (A), HO-1 (B) and FABP4 (C). All values are expressed as mean ± SEM of four experiments (n=4) in duplicate. (** $p<0.05$ versus FA).

Effect of OLE on FABP4, HO-1 and SIRT1 gene expression

To investigate the molecular signals that might regulate lipid metabolism in our model, we analyzed the mRNA levels of fatty acid binding protein 4 (FABP4), NAD-dependent

deacetylase sirtuin-1 (SIRT-1) and heme oxygenase 1 (HO-1). As seen in Figure 2A and 2B, levels of SIRT-1 and HO-1 were not changed after exposure to FA. In contrast, FABP4 levels were reduced in FA-treated cells (Figure 2C). OLE treatment significantly increased the levels of SIRT1, HO-1 and FABP4 compared with cells treated with FA alone. These observations suggest that OLE can ameliorate lipid metabolism and in addition to that, the expression of the antioxidant enzyme HO-1, in presence of OLE, significantly increased after 24 h (Figure 2B), which was consistent with our previous published results showing decreased lipogenesis after HO-1 induction (Vanella et al. 2013).

Figure 3

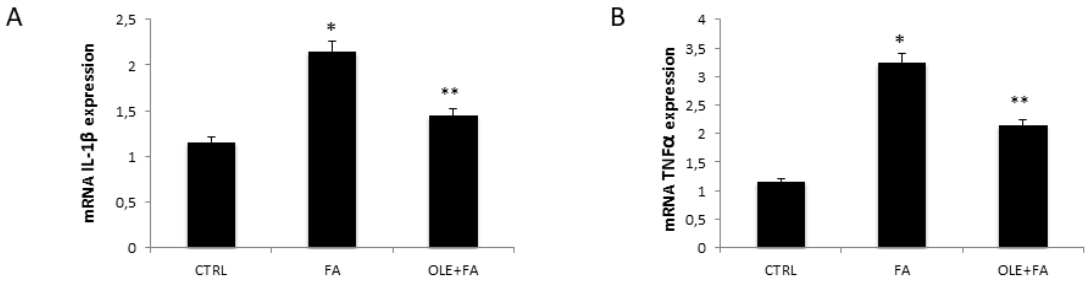

Figure 3. Analysis of gene expression by Real time PCR of the cytokines IL-1β (A) and TNFα (B). All values are expressed as mean ± SEM of four experiments (n=4) in duplicate. (* $p<0.05$ versus untreated; **$p<0.05$ versus FA).

Effects of OLE on inflammatory cytokines

In order to study the effects of OLE on inflammation, we investigated IL-1β and TNF-α levels. Cells treated with FA, in the absence of OLE, displayed a significant increase in IL-1β and TNF-α (Figure 3). In contrast, cells treated with FA for 24 h in the presence of OLE, showed a significant decrease in IL-1β and TNF-α levels compared to FA treatment alone (Figure 3).

Discussion

Recently, more attention has been paid to the antioxidant properties of polyphenols contained in natural substances and their application in biomedical field (Cirillo et al. 2016). Numerous studies focused the possibility to use natural extracts for pharmacological treatment or adjuvant in diseases characterized by oxidative stress (Li Volti et al. 2011; Pittala et al. 2017). An example is represented by oleuropein, a phenolic compound that can decrease the production of monocytic inflammatory mediators, decreasing the production of IL-1β in human whole blood cultures stimulated with monocytes-triggered by LPS (Miles et al. 2005). Oleuropein is endowed also with antithrombotic and anti-atherogenic properties, which, at least in part, depend on its anti-inflammatory and anti-oxidative activities (Manna et al. 2004).

As the leading cause of hepatic dysfunction worldwide, the typical feature of NAFLD includes hepatocyte intracellular lipid accumulation, which resulted from an imbalance between lipid synthesis and oxidation (Byrne and Targher 2015). HepG2 cells have been successfully used to establish a fatty liver cell model, which can be used to screen for preventive and therapeutic drugs and simultaneously explore fatty liver pathogenesis (Mantzaris et al. 2011). In this study we evaluated if a specific extract of olive leaves (OLE) from Sicilian cultivation could have any protective effects in an *in vitro* model of hepatic steatosis. Our results show that this extract, characterized by high polyphenols content, presents a strong antioxidant activity. We demonstrate that OLE was able to reduce triglycerides accumulation stored inside the hepatocytes, previously treated with 3mM FA. We hypothesize that this effect was mediated by induction of FABP4, which regulates energy balance and lipid signal and it is responsible of the transport, esterification and β-oxidation of fatty acids. FABP4 regulate the transfer of fatty acids from cytosol to the lipid droplets for storage or to the mitochondria and peroxisomes for the oxidation (Hotamisligil and Bernlohr 2015). Additionally, exposure of HepG2 to high FA levels increased pro-inflammatory cytokines mRNA levels. To evaluate OLE anti-inflammatory capacity, we measured the expression of IL-1β and TNF-α, two major pro-inflammatory cytokines induced by fat accumulation (Purushotham et al. 2009). TNF-α plays a key role in the pathogenesis and disease progression of NAFLD (Bahcecioglu et al. 2005; Tokushige et al. 2005). Overexpression of TNF-α mRNA in liver tissue has been reported

in severely obese patients with NASH. It was also reported that treatment of HepG2 cells with FA resulted in increased production of TNF-α mRNA (Crespo et al. 2001).

In addition, Ma et al., have reported that IL-1β and TNFα increased cholesterol accumulation in both HepG2 and primary hepatocytes, showing that inflammatory stress reduced intracellular cholesterol efflux by inhibiting PPARa, LXRa and ABCA1 gene and protein expression *in vitro* and *in vivo*, suggesting that inflammatory stress also accelerates the progression of fatty liver in an NAFLD model by reducing intracellular cholesterol efflux (Ma et al. 2008).

Our results showed an over expression of IL-1β and TNFα following the addition of fatty acids in the culture medium, while the co-treatment with OLE reduced the expression of both cytokines.

In our study we have also evaluated SIRT-1 and HO- 1 levels. SIRT1, a mammalian ortholog of Sir2 (silent information regulator 2), is an NAD-dependent deacetylase that acts as a master metabolic sensor of NAD+ and modulates cellular metabolism (Blander and Guarente 2004; Gerhart-Hines et al. 2007; Leibiger and Berggren 2006). Additionally, it has been implicated in the control of energy metabolism through deacetylation of PGC-1α (proliferator-activated receptor α coactivator 1) (Blander and Guarente 2004; Rodgers et al. 2005; Rodgers and Puigserver 2007).

SIRT1 plays a key role in the development of NAFLD through its involvement in the regulation of both lipid and carbohydrate metabolism (Hirschey and Zhao 2015; Tobita et al. 2016). Studies *in vivo* and *in vitro* characterized this protein as a metabolic sensor that has the potential to improve NAFLD (Mariani et al. 2015; Sodhi et al. 2015). SIRT1 was significantly lower in an obese group with severe liver steatosis compared to a group with mild steatosis, and both groups had lower concentration of SIRT1 in the plasma compared to control lean patients (Mariani et al. 2015). The Heme-oxygenase system (HO) is one of the key cellular-antioxidant defenses that lowers ROS by the breakdown of heme (pro-oxidant) to carbon monoxide (CO) and biliverdin (BV) (Abraham et al. 2016; Di Noia et al. 2006). BV is rapidly reduced to the antioxidant, bilirubin (Cao et al. 2011). Induction of HO-1 increases the phosphorylation of AMP-activated protein kinase (AMPK), and decrease fatty acid synthase (FAS) resulting in an augment of insulin sensitivity and in a decrease of fatty acid levels (Chau et al. 2010; Stienstra et al. 2007). Furthermore, the induction of HO-1 attenuated the development of fatty liver and decreased lipid droplet size in obese mice. It has been shown that the HO-1-SIRT1 pathway is capable to attenuate

hepatic steatosis, regulating genes involved in lipid metabolism and suppressing pro-inflammatory pathways (Sodhi et al. 2015). Our results showed an over expression of HO-1 and SIRT1 in the OLE group, and in accordance to our previous published results (Li et al. 2015), confirmed that HO-1, a critical anti-oxidant protein, together with SIRT1 may counteract lipid accumulation in the liver.

In conclusion, the present study demonstrated that OLE can reduce HepG2 intracellular FA through the improvement of lipid metabolism and a decreased numbers of lipid droplets in hepatocytes.

Given the role of OLE in preventing lipogenesis, finally the present data demonstrate a new function of OLE in the prevention of NAFLD, following an extensive exposure of hepatocytes to oleic and palmitic acid.

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Chapter 5

Inhibition of Heme Oxygenase antioxidant activity exacerbates hepatic steatosis and fibrosis *in vitro*

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Abstract: The progression of NAFLD and the development of hepatic fibrosis is caused by changes in redox balance, leading to an increase of reactive oxygen species (ROS) levels. NAFLD patients are at risk of progressing to nonalcoholic steatohepatitis (NASH), associated to cardiovascular diseases (CVD), coronary heart disease and stroke. Heme Oxygenase-1 (HO-1) is a potent endogenous antioxidant gene that plays a key role in decreasing oxidative stress. The present work was directed to determine whether use of an inhibitor of HO-1 activity affect lipid metabolism and fibrosis process in hepatic cells. Oil Red assay and mRNA analysis were used to evaluate the triglycerides content and the lipid metabolism pathway in HepG2 cells. ROS measurement, RT-PCR and Soluble collagen assay were used to assess the intracellular oxidant, the fibrosis pathway and the soluble collagen in LX2 cells. The activity of HO-1 was inhibited using Tin Mesoporphyrin IX (SnMP). Our study demonstrates that a non-functional HO system results in an increased lipid storage and collagen release in hepatocytes. Consequently, an increase of HO-1 levels may provide a therapeutic approach to address the metabolic alterations associated with NAFLD and its progression to NASH.

1. Introduction

Hepatic steatosis is a common liver disease characterized by the presence of triglycerides vesicles, accumulating within hepatocytes [1]. It is associated with dyslipidaemia, obesity and insulin resistance, despite a diet with low alcoholic drinks consumption: this conditions it is known as non-alcoholic fatty liver disease, or NAFLD [2-7]. NAFLD has become a critical problem for public health, because of the involving of other collateral cardiometabolic diseases, including diabetes and hypertension [8-12]. NAFLD patients are at risk of progressing to nonalcoholic steatohepatitis (NASH) and ultimately cirrhosis; they are also at higher risk of cardiovascular diseases (CVD), including coronary heart disease and stroke[13]. NAFLD confers increased cardiovascular disease risk independent of traditional cardiovascular risk factors and metabolic syndrome [14]. The abnormal accumulation of lipids in the liver causes a nonalcoholic steatohepatitis (NASH) with progressive liver damage characterized by inflammation and oxidative stress, that could lead to advanced fibrosis or cirrhosis [15-17]. In case of repeated damage, liver parenchyma could responde with an excessive extracellular matrix (ECM) accumulation, due to activation of Hepatic Stellate Cells (HSCs) in perisinusoidal space [18,19]. After differentiation in myofibroblast-like cells, HSCs play a key role in ECM remodeling through the overexpression of α-smooth muscle actin (α-SMA), that lead to hepatic fibrosis [20-23]. An imbalance of ECM synthesis and degradation is caused by the activity of many mediators, such as mitogen-activated protein kinase (MAPK), integrins and various growth factors [24,25]. Transforming growth factor β (TGF-β) is a key mediator in fibrotic matrix increase [26] as the main pro-fibrogenic cytokine, promotes the accumulation of ECM through both activation of SMAD-dependent and -independent pathways, and regulation of enzymes like metalloproteinases [27-30]. The progression of NAFLD and the development of hepatic fibrosis is caused by changes in redox balance, leading to an increase of reactive oxygen species (ROS) levels [31-33]. Heme oxygenase (HO) is a microsomal enzyme involved in oxidative stress control, that catabolizes heme into biliverdin, ferrous iron (Fe^{2+}) and carbon monoxide (CO) [34-36]. HO exerts anti-oxidant effect through its products, that possess many biological protective properties involved in regulation of inflammation and apoptosis [37]. Cytoprotective actions of HO and its by-products can be harmful, especially when translated into pathophysiological processes like tumorigenesis [38-42].

Humans own two isoenzymes of HO, namely HO-1 and HO-2, encoded by the HMOX1 and HMOX2 genes, respectively. Also known as heat shock protein 32, HO-1 is induced in a range of cells and in several organs, in response to inflammation and oxidative stress, while HO-2 is constitutively expressed [43-47]. It has been shown that HO-1 ensures protective effect on liver cells under injury conditions, and an induction of HO-1 is even involved in prevention of liver fibrosis development [48,49]. Conversely, low levels of HO-1 are related to severe oxidative stress and organ failure, showed by iron deposits in the damaged liver [50]. The aim of this study is to investigate the role of HO in two of the main processes involved in the NASH pathology, using the human hepatocellular carcinoma cell line (HepG2) treated with free fatty acids (FFA), and the human hepatic stellate cells (LX2) treated with TGF-β, as steatosis and fibrosis models, respectively.

2. Materials and Methods

2.1 Cell culture

HepG2 cells retain many characteristics of normal differentiated quiescent hepatocytes. They were widely used in several studies as NAFLD in vitro model, administrating fatty acids [51-53]. HepG2 cells were maintained in DMEM supplement with 10 % FBS, 1 % Penicillin and Streptomycin solution and incubated at 37 °C in a 5 % CO_2 humidified atmosphere. For the experiments, the cells were seeded in 24-well plates at a density of 5 $\times 10^5$ cells per well. Then the cells were treated for 24 hours with DMEM containing FFA 2mM (palmitic acid and oleic acid 2:1) in presence or absence of Tin- Mesoporphyrin IX (SnMP) 5μM, alone or in combination with Cobalt Protoporphyrin (CoPP) 5μM, for 2 hours as pre-treatment.

The LX2 cells were maintained in medium DMEM low glucose supplemented with 10% FBS and 1% Penicillin and Streptomycin solution. For this study, the cells were seeded in 6-well plates and then treated with 5ng/ml TGF-β to induce their activation and the collagen release, in presence or absence of SnMP 5μM and CoPP 5μM.

2.2 Oil Red O staining

Staining was performed using 0.21% Oil Red O in 100% isopropanol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, hepatocytes were fixed in 10% formaldehyde, stained with Oil Red O for 10 minutes, rinsed with 60% isopropanol (Sigma-Aldrich), and the Oil Red O eluted by adding 100% isopropanol for 10 minutes and the optical density (OD) measured at 490 nm, for 0.5 sec reading. Lipid droplets accumulation was examined by using inverted multichannel LED fluorescence microscope (Evos, Life Technologies, NY).

2.3 ROS measurement

Determination of ROS was performed by using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA); 100 μM DCFH-DA, dissolved in 100% methanol was added to the cellular medium and the cells were incubated at 37 °C for 30 min. Under these conditions, the acetate group is not hydrolyzed [54]. The fluorescence [corresponding to the oxidized radical species 2′,7′-dichlorofluorescein (DCF)] was monitored spectrofluorometrically (excitation, λ=488 nm; emission, λ=525 nm). The total protein content was evaluated for each sample, and the results are reported as percentage increase in fluorescence intensity (FI)/mg protein with respect to control untreated cells.

2.4 Sircol collagen assay

Total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay (Biocolor, Belfast, UK). For these experiments, confluent cells in 6-plate wells were incubated for 24h with 5ng/ml of TGF-β (Sigma). One mL of Sirius red stain, an anionic dye that reacts specifically with basic collagen side chain groups, was added to 400 µL of supernatant and incubated with gentle rotation for 30 min at room temperature. After centrifugation at 12,000 g for 10 min, the collagen-bound dye was dissolved again after addition of 1 mL of 0.5 M NaOH and absorbance at 540 nm was measured using a microplate spectrophotometer reader (Synergy HT, BioTek). The absorbance was directly proportional to the amount of newly formed collagen in the cell culture supernatant.

2.5 RNA extraction and qRT-PCR

RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA) [4]. First strand cDNA was then synthesized with Applied Biosystem (Foster City, CA, USA) reverse transcription reagent. Quantitative real-time PCR was performed in Step One Fast Real-Time PCR System Applied Biosystems using the SYBR Green PCR MasterMix (Life Technologies) [5]. The specific PCR products were detected by the fluorescence of SYBR Green, the double stranded DNA binding dye. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH by using comparative $2^{-\Delta\Delta Ct}$ method.

2.6 Statistical analyses

Statistical significance ($P < 0.05$) of differences between experimental groups was determined by the Fisher method for analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by either single-factor analysis of variance (ANOVA) for multiple groups, or the unpaired t-test for two groups, and the data are presented as mean ± SD.

3. Results

3.1 Effect of HO Inhibition on hepatic fatty storage

HepG2 cells were treated with FFA 2 mM in order to create an in vitro model of hepatic steatosis.

After 24h an Oil Red O staining was performed to evaluate the amount of lipid droplet. Figure 1 shows as the FFA treatment was able to increase the triglycerides storage in HepG2 cells compared to control untreated group. The group pre-treated with SnMP showed a significative increase of lipid droplets amount compared to the CTRL and FFA groups, while in the group pre-treated with the combination of SnMP and CoPP this effect was partially reversed, indicating the involvement of HO system in the lipid metabolism regulation.

Figure 1

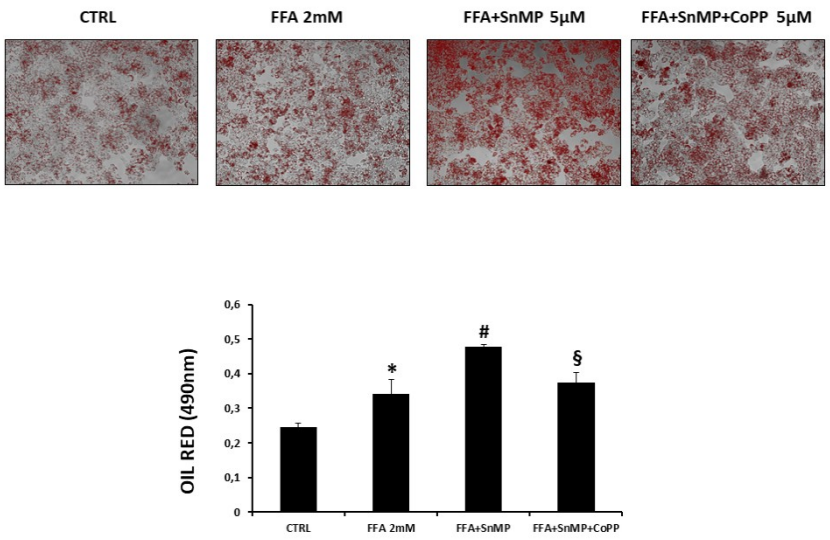

Figure 1. Effect of HO inhibition on oil droplets formation in Hepatocytes. We measured the effect of SnMP 5µM treatment on lipogenesis in presence of FFA. *p<0.05 vs. CTRL, # p<0.05 vs. FFA 2mM, § p<0.05 vs. FFA+SnMP

3.2 Effect of HO regulation on Lipid metabolism pathway

To support the Oil Red data, We analyzed the mRNA levels of lipid metabolism pathway genes. Figures 2A-C showed an increase of Diglyceride acyltransferase 1 (DGAT-1), Sterol regulatory element-binding transcription factor 1 (SREBP-1) and Fatty acid synthetase (FAS) gene expression in the group with SnMP treatment compared to control group, suggesting an increased synthesis of cholesterol, fatty acids and triglycerides. The co-treatment with CoPP, a strong inducer of HO-1 expression, reversed the SnMP effect on DGAT-1 and SREBP-1 genes, but did not affect FAS expression. We also analyzed HO-1 levels (Figure 2D), that were increased in the FFA group when compared with the control, probably because the FFA treatment causes a moderate oxidative stress. As expected, in the SnMP group, HO-1 was markedly increased compared to FFA group because, as previous studies demonstrated, SnMP decrease the HO activity, but increase its protein expression [55,56]. The group with both compound SnMP and CoPP showed a synergic effect with a strong increase of HO-1 expression compared with all other groups. Sirtuin 1 expression (SIRT1) didn't show difference in mRNA expression, in both groups FFA and

FFA-SnMP when compared with the control, but the co-administration of CoPP showed a significantly increase compared with other groups (Figure 2E). That result is in according with several published studies that demonstrates the positive relation between HO and SIRT1 genes expression [57].

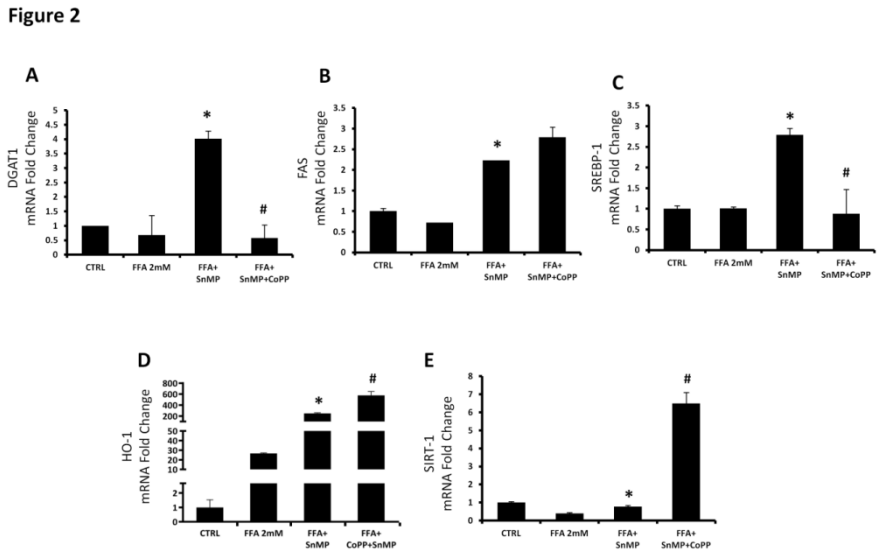

Figure 2. . mRNA expression of DGAT1 (A), FAS (B), SREBP-1 (C), HO-1 (D) and SIRT1(E) of HepG2 control cells, cells treated with FFA 2mM and cells treated with SnMP 5uM alone or in combination with CoPP 5uM. Results are mean ± SD, $^{*}p<0.05$ vs. FFA 2mM, $^{\#}p<0.05$ vs. FFA+SnMP

3.3 Hepatic fibrosis in vitro model

We investigated another main process that characterized the NAFLD physiopathology creating an in vitro model of hepatic fibrosis, administrating to LX2 cells the TGF-β protein, that is known to activate fibroblast resulting in Collagen release [58]. We treated LX2 cells with 5ng/ml of TGF-β and we measured the Reactive Oxygen Species (ROS) generation and the Soluble Collagen release. ROS production was increased of 72% after 1 hour in the group with TGF-β 5ng compared to control (Figure 3A). We measured the soluble collagen using a colorimetric kit (Sircol) and we showed a significant increase of collagen release in the group treated with TGF-β 5ng compared to control. Furthermore, we analyzed the mRNA expression of the main genes involved in the Collagen production as Collagen type 1 alpha 1 (COL1A1), Alpha smooth muscle actin (α-SMA), SMAD3,

SMAD4, SMAD7 and TIMP-1. As showed in the Figure 4, in all the genes the expression was markedly increased by TGF-β 5ng treatment compared to the control.

Figure 3

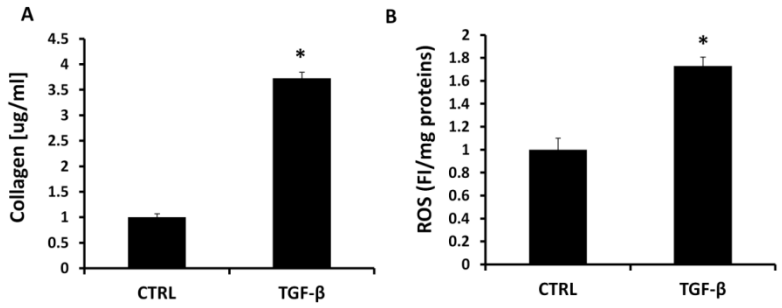

Figure 3. (A) Soluble collagen in LX2 cells activated with TGF-β 5ng for 24 h. Soluble collagen measurement are expressed as μg/ml. Values represent the means ± SD of three experiments performed in triplicate. *p<0.05, significant result vs. untreated LX2 cells. (B) Intracellular oxidants in LX2 cells activated with TGF-β 5ng for 1 h. Results are mean ± SD, *p<0.05 vs. CTRL.

Figure 4

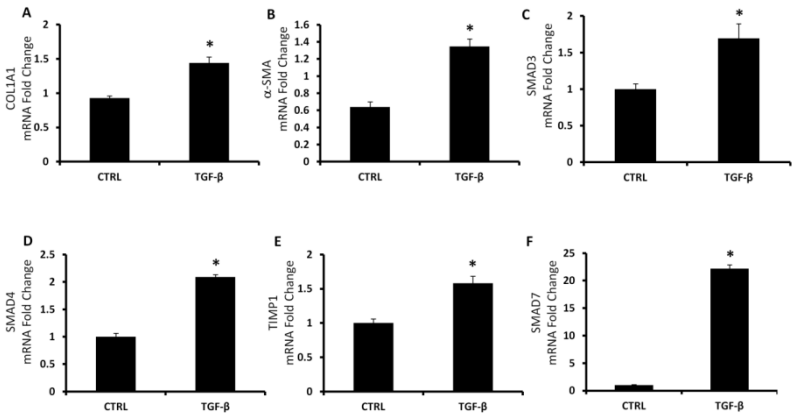

Figure 4. mRNA expression of fibrosis pathway. COL1A1 (A), α-SMA(B), SMAD3 (C), SMAD4 (D), TIMP1 and SMAD7(E) of LX2 control cells and cells treated with TGF-β 5ng. Results are mean ± SD, *p<0.05 vs. CTRL.

3.4 Effect of HO Inhibition on TGF-β induced Soluble Collagen release

We investigate the role of HO in this fibrosis using SnMP and CoPP, both at the concentration of 5µm. We treated the LX2 cells with these compounds for 2 hours before the administration of TGF-β 5ng and we obtained a significant increase in collagen release levels after 3 hours in the group with SnMP compared to TGF-β group (Figure 5). The effect was reversed by the HO-1 inducer CoPP.

Figure 5

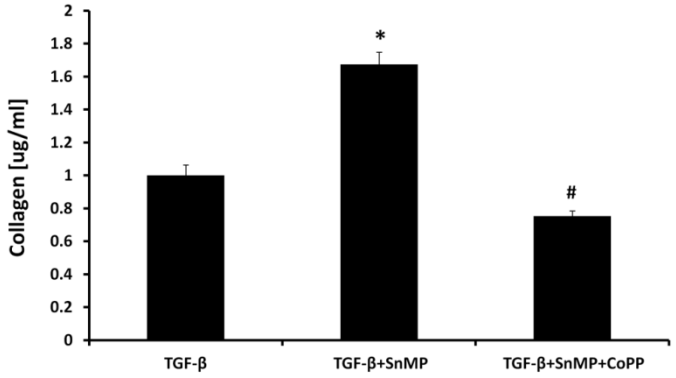

Figure 5. Soluble collagen in LX2 cells treated with TGF-β 5ng for 24 h in presence or absence of a 2 hours pre-treatmemt with SnMP 5µM alone or in combination with CoPP 5µM . Soluble collagen measurement are expressed as µg/ml. Values represent the means ± SD of three experiments performed in triplicate. *$p<0.05$ vs. TGF-β ; # $p<0.05$ vs. TGF-β+SnMP.

4. Discussion

Considering the complexity of NAFLD and its rising prevalence globally, it's of primary importance find new protein targets for the regulation of the pathways involved in this pathology. In NAFLD, an increase in hepatic FFAs uptake, lipid synthesis, impaired β-oxidation, and decrease in lipid export facilitates accumulation of fat in the liver [59,60]. In order to study the role of HO system in the main NASH pathological aspects, we propose two different *in vitro* model for steatosis and fibrosis. Primary hepatocytes, derived from human liver samples, are an ideal in vitro model for studying hepatosteatosis, but the difficulty to obtain normal clinical liver samples lead us to use HepG2 cells as an alternative cellular model [52]. Whereas oleic and palmitic acid represent the main fatty acids in the triglycerides (TG) content of steatotic patients, we treated HepG2 cells with a combination of these fatty acids to simulate NAFLD [61]. To assess how HO affect the hepatocytes lipid metabolism, we cultured the HepG2 cells with a well known HO activity inhibitor named SnMP, alone or in combination with the strong HO inducer CoPP. Figure 1 showed that FFA was able to increase the intracellular lipid droplets content compared to untreated cells, in particular in the cells that received the pre-treatment with SnMP, suggesting that HO inhibition impairs the lipid metabolism in hepatocytes. SREBP, characterized by the three isoforms SREBP-1a, SREBP-1c and SREBP-2, plays a key role on regulation of various genes expression involved in cholesterol and lipid metabolism. SREBP-1c represents the major isoform that controls FA synthesis in the liver and is regulated by a series of nutritional and hormonal stimulus through transcriptional and post-transcriptional mechanisms. Yahagi et al. showed that knockout SREBP-1c ob/ob mice presented a significant reduction in the hepatic expression of lipogenic genes preventing liver steatosis [62]. Conversely, overexpression of SREBP-1c results in raised levels of FAS, Acetyl CoA carboxylase (ACC) and Stearoyl-CoA desaturase (SCD) causing an increase in lipogenesis [63] that, in concert with an augmented hepatic FFAs uptake, is known to contribute TG accumulation in the hepatocytes [64]. In HepG2 cells treated with FFA, we observed a significant increase of SREBP-1c, DGAT-1 and FAS levels after SnMP treatment which it is associated with an increase of fatty acid storage. Conversely, co-administration of CoPP and SnMP reversed DGAT and SREBP-1c mRNA levels, confirming that HO can affect TG formation and storage in hepatocyte's cytoplasm. As the most extensively studied sirtuin, Sirt1 has a prominent role in metabolic tissues, such as the

liver, skeletal muscle and adipose tissues. Sirt1 overexpression in the liver can deacetylate a range of substrates, including SREBP-1c, PGC-1α and FoxO1 proteins, and can result in a pronounced effect on glucose and lipid metabolism [65,66]. Previous studies indicated that the overexpression of Sirt1 protects against HFD-induced hepatic inflammation by decreasing the NFκB-mediated induction of inflammatory cytokines [67]. Consistent with previous published results, our data showed a positive regulation of Sirt1 by HO-1 induction [68]. Despite SnMP did not affect Sirt1 gene levels, induction of HO-1 by CoPP treatment significantly increased Sirt1 mRNA. The crosstalk between HO-1 and Sirt1 may be considered as a pivotal axis against oxidative stress caused by hyperglycemia and hyperlipidemia, and it is essential to protect the liver from steatosis. The results reported here extend our previous findings that upregulation of HO-1 in hepatocytes results in the negative regulation of lipogenesis [68-71]. In order to evaluate the detrimental role of HO-1 inhibition on liver fibrosis, we first established an in vitro model of liver fibrosis using TGF-β as a fibrotic agent in human stellate hepatic cells. In chronic liver diseases, hepatic stellate cells have been considered as a primary target for active TGF-β, thereby cell treatment with TGF-β contributes to their activation and subsequent fibrogenesis. The increase of ROS levels mediated by TGF-β treatment (Figure 3) and lipid peroxidation products contribute to collagen release causing the onset and progression of fibrosis [72]. As shown in Figure 4, the induction of α-SMA, COL1A1 and SMADs represents reliable markers of HSCs activation to myofibroblast-like cells with direct contribution to hepatic fibrogenesis. In the present article, we showed that HO activity inhibition further increased collagen release from activated LX2 compared to cells treated exclusively with TGF-β. Furthermore, we found that increased HO-1 levels by CoPP reversed the effect mediated by SnMP and reduced the levels of soluble collagen released from activated LX2. This study provides additional evidences for a link between liver disorders and HO system. Consequently, increase of HO-1 levels may provide a therapeutic approach to address the metabolic alterations associated with NAFLD and its progression to NASH.

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Chapter 6

Development of NASH in Obese Mice is Confounded by Adipose Increase in Inflammatory NOV and Oxidative Stress

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Abstract

Aim: Non-alcoholic steatohepatitis (NASH) is the consequence of insulin resistance, fatty acid accumulation, oxidative stress and lipotoxicity. We hypothesize that an increase in the inflammatory adipokine NOV decreases antioxidant Heme Oxygenase 1 (HO-1) levels in adipose and hepatic tissue, thus becoming a major contributor to NASH in obese mice. ***Methods:*** Mice were fed a high fat diet (HFD) and obese animals were administered an HO-1 inducer with or without an inhibitor of HO activity to examine levels of adipose-derived NOV and possible links between increases in the synthesis of inflammatory adipokines and hepatic pathology. ***Results:*** NASH mice displayed decreased HO-1 activity, increased levels of hepatic heme, NOV, MMP2, hepcidin, and increased NAS scores and hepatic fibrosis. Increased HO-1 is associated with a decrease in NOV, improved hepatic NAS score, ameliorated fibrosis, and increases in mitochondrial integrity and insulin receptor phosphorylation**.** Adipose tissue function is disrupted in obesity as evidenced by an increase in pro-inflammatory molecules such as NOV and a decrease in adiponectin. Importantly, increased HO-1 levels are associated with a decrease of NOV, increased adiponectin levels, increased levels of thermogenic and mitochondrial signaling associated genes in adipose tissue. ***Conclusions:*** These results suggest that the metabolic abnormalities in NASH are driven by decreases in hepatic HO-1 that is associated with an increase in the adipose-derived pro-inflammatory

adipokine NOV in this obese mouse model of NASH. Concurrently, induction of HO-1 provides protection against insulin resistance as seen by the increases in insulin receptor phosphorylation. Pharmacological increases in HO-1 or decreases in NOV may have potential as a therapeutic approach in preventing fibrosis, mitochondrial dysfunction, and the development of NASH.

Introduction

Metabolic syndrome and its associated pathologies of obesity, insulin resistance (IR), and dyslipidemia are often accompanied by liver involvement, defined as nonalcoholic fatty liver disease (NAFLD) [1]. NAFLD's progression to nonalcoholic steatohepatitis (NASH), characterized by low grade inflammation, cell ballooning, and mitochondrial dysfunction is a primary risk factor for development of fibrosis and cirrhosis and therefore an important area of clinical research [2]. Reactive oxygen species (ROS) can induce lipid peroxidation leading to inflammation and liver damage [3,4]. Inflammation in metabolic syndrome is unlike the acute inflammation observed in infection and traumatic injury. This inflammation, also called chronic, low grade inflammation, is provoked when the capacity for adipocytes to store fat is overwhelmed resulting in the production of inflammatory cytokines leading to metabolic inflammation [5]. Fatty acids released from hypertrophic, dysfunctional, and insulin resistant adipocytes, together with increased hepatic de-novo lipogenesis and impaired (FA) fatty acid export, cause an accumulation of triglycerides in the liver leading to lipotoxicity [6,7]. Steatotic livers are more sensitive to increased ROS and oxidative stress, leading to mitochondrial dysfunction, decreased levels of hepatocyte antioxidants, and inflammation, and culminating in NASH and fibrosis [8,9]. Increased calorie intake and obesity leads to an increase in tissue fat mass through adipocyte hyperplasia and hypertrophy, subsequently resulting in a decrease in adiponectin and an increase of inflammatory TNF-α causing IR, inflammation, and oxidative stress in the liver [10].

Induction of HO-1, an antioxidant gene highly expressed in Kupffer cells and in all organs including fat tissues, confers advantageous effects in metabolic syndrome [11]. Cobalt protoporphyrin (CoPP) induction of HO-1 partially contributes to phosphorylation of the insulin receptor, improving insulin sensitivity [12,13]. Additionally, HO-1 acts through the degradation products of pro-oxidant heme to bilirubin and biliverdin, antioxidants that have been shown to increase mitochondrial fusion, while also serving to improve adipocyte

function and remodeling by increasing adiponectin expression levels [14,15]. Humans with low levels of HO-1 suffer severe oxidative stress and organ failure and demonstrate iron deposits in the liver [16,17]. CoPP has been used to prevent body weight gain, increase oxygen consumption, and decrease fasting blood glucose in rats, and mice [18]. Recently, CoPP treatment has been shown to decrease expression of pro-apoptotic protein, abridge percolation of inflammatory cells, and to reduce AST and ALT levels in IR induced liver damage [19,20], Moreover, HO-1 induction increases mitofusion over fission related proteins and improves mitochondrial quality control [21].

In adipose tissues, induction of HO-1 has been shown to reduce body weight, decrease NOV, and increase PGC-1α mediated thermogenesis, thereby increasing energy uptake and the stimulation of mitochondrial FA oxidation. Adipose PGC-1α serves as a key moderator of energy metabolism and promotes mesenchymal stem cell differentiation into brown fat adipocytes [22] and the browning of white fat to a distinct phenotype known as brite fat, which aids in the prevention of the development of metabolic syndrome and type 2 diabetes mellitus (T2DM) [23].

Another important component of metabolic syndrome is an associated increase in the levels of the pro-oxidant heme. Intracellular heme levels play a central role in the regulation of many cell functions [24,25,26]. Inflammatory increases in IL-6 upregulates hepcidin and iron trapping in Kupffer cell diseases [1]. The resulting increase in cellular heme decreases levels of PGC-1α, lipid metabolism, and adipogenesis [27].

The recently discovered inflammatory adipokine, NOV/CCN3 gene (nephroblastoma overexpressed) [28], is also shown to be repressed in conditions of increased HO-1 levels [29]. This protein plays key roles in inflammation, wound healing, fibrosis, and cancers [28]. NOV is involved in the adhesion, migration, proliferation, differentiation, and survival of different cell types [28], and modulates the expression of inflammatory molecules [30,31]. NOV−/− mice fed a HFD have less steatosis compared to WT mice, and hepatic triacylglycerol content is reduced by approximately threefold [32].

We hypothesize that the development of NASH is the result of combined decrease of HO-1, increased NOV, and inflammation of adipose tissues leading to the impairment of mitochondrial function. We further propose that induction of HO-1 in adipose tissues will have positive impact on hepatic tissue and reverse these negative effects on NOV levels, decreasing NASH scores and increasing mitochondrial integrity and function.

Methods

Eight week–old C57Bl6 male mice were fed a HF diet for 20 weeks, a time frame in which the manifestations of NASH are present. Mice were divided into four groups of 6 animals each: 1) control normal chow diet; 2) HF diet; 3) HF diet treated for the last 8 weeks with cobalt protoporphyrin (CoPP) (once/week); 4) HF diet treated for the last 8 weeks with CoPP and the last 3 weeks with tin mesoporphyrin (SnMP), an inhibitor of HO activity (twice/week). At the end of the 20-week period, blood glucose and alanine aminotransferase (ALT) were measured as described [29,33].

Histopathological examination of hepatic tissue, NAS score evaluation and Hepatic lipid droplet analysis.

Liver samples from each experimental group were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin wax and sectioned (6 μm thick). The main liver histopathological features commonly described in NAFLD including steatosis, inflammation, hepatocyte ballooning and fibrosis were scored according to the NAFLD histologic activity score (NAS) system, and lipid droplet analysis was performed as previously described [33-35]. Briefly, double-blinded analysis identified the degree of steatosis and NASH (grade 0 ≤ 5%; 1 = 5–33%; grade 2 = 34%–66%; grade 3 ≥ 66%), lobular inflammation (0: no foci, 1 < 2 foci per 200x field, 2: 2 to 4 foci per 200x field, and 3: foci per 200x field), hepatocyte ballooning (0: none; 1: rare or few; 2: many) and fibrosis (0: no fibrosis, 1: perisinusoidal or periportal fibrosis, 2: perisinusoidal and portal/perioral fibrosis, 3: bridging fibrosis, and 4: cirrhosis) [34].

Real-time qPCR, western blot analysis, HO-activity, heme measurement, and O2 consumption.

Total RNA and protein were extracted from liver and visceral adipose tissue and gene expression analysis was performed as described [29,33]. HO-activity and heme levels were determined as described [23,33,35]. Mouse oxygen consumption was assessed as described [29].

Statistical analyses.

Statistical significance between experimental groups was determined by Student's t-test for pairwise comparison between groups or by ANOVA with Tukey-Kramer post-hoc analysis for comparison between multiple groups. The data are presented as means ± SEM and the null hypothesis was rejected at $p<0.05$.

Results

Table 1- NAS score

NAS pathological score factors	Control	HF	HF + CoPP	HF + CoPP + SnMP
Steatosis	0 (0.67%)	2 (42.24%)	1 (10.28%)	2 (33.05%)
Inflammation	0 (no foci)	2 (2-4 foci/field)	1 (<2 foci/field)	2 (2-4 foci/field)
Ballooning	1 (rare)	2 (many)	1 (rare)	2 (many)
Fibrosis	0 (no fibrosis)	3 (bridging fibrosis)	0 (no fibrosis)	2 (perisinusoidal and portal fibrosis)
NAS value	1	9 NASH	3	8 NASH

Table 1

Histopathological NAS score evaluation.

Degree of steatosis and NASH (grade 0 ≤ 5%; 1 = 5–33%; grade 2 = 34%–66%; grade 3 ≥ 66%), lobular inflammation (0: no foci, 1 < 2 foci per 200x field, 2: 2 to 4 foci per 200x field, and 3: foci per 200x field), hepatocyte ballooning (0: none; 1: rare or few; 2: many), and fibrosis (0: no fibrosis, 1: perisinusoidal or periportal fibrosis, 2: perisinusoidal and portal/perioral fibrosis, 3: bridging fibrosis, and 4: cirrhosis).

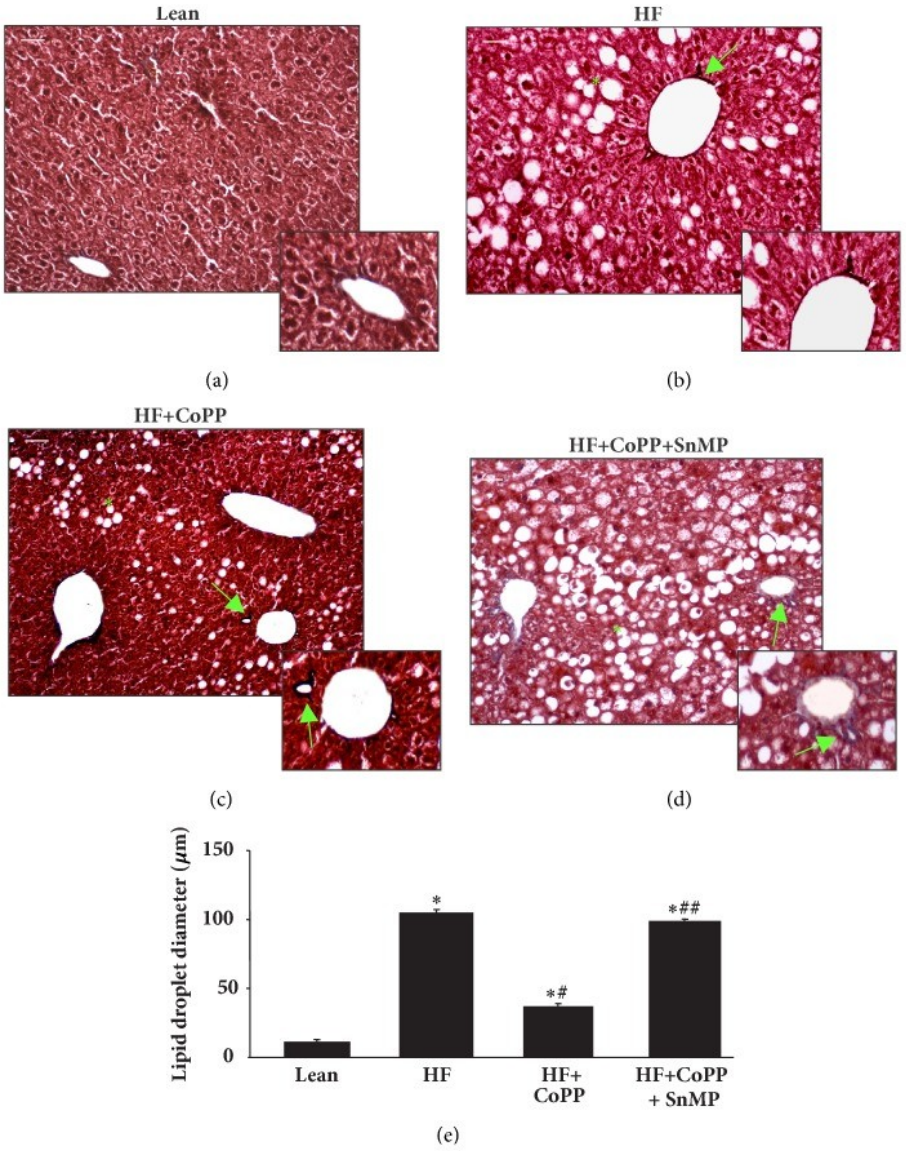

Figure 1. HO-1 induction prevents fibrosis and decreases NASH score.

Liver of lean (A), HF fed (B), HF fed treated with CoPP (C) and HF fed treated with CoPP and SnMP (D) in mice and (E) graph summarizes the morphometrical analysis of liver lipid droplet diameter. * $p< 0.05$ vs lean; # $p< 0.05$ vs HF fed and + $p< 0.05$ vs HF fed mice + CoPP. Masson's trichrome staining. Bar 20 μm. The arrow shows hepatic perivascular fibrosis and (*) indicates the steatosis of control lean mice, HF fed mice, HF fed mice + CoPP and HF fed mice + CoPP + SnMP.

HO-1 induction prevents fibrosis and decreases NASH score.

The HF mice revealed a higher NAS score (NAS: 9) with elevated steatosis, moderate lobular inflammatory loci, significant hepatocyte ballooning and fibrosis (Table 1; Figure 1 A-E). Increased HO-1 expression with CoPP improved this score (NAS: 3), diminished all the pathological parameters, and resulted in mild steatosis, rare inflammatory loci and

ballooning, and no fibrosis. Inhibition of HO activity in HF mice caused perisinusoidal steatosis and ballooning, and portal fibrosis (NAS: 8). Furthermore, the adverse effect of hepatosteatosis was confirmed by detailed morphometrical analysis of liver lipid droplet diameter (Figure 1 A-E). From these results we conclude that HO-1 can prevent lipid droplet formation in the liver, ultimately preventing the development of NAFLD and NASH in obese mice.

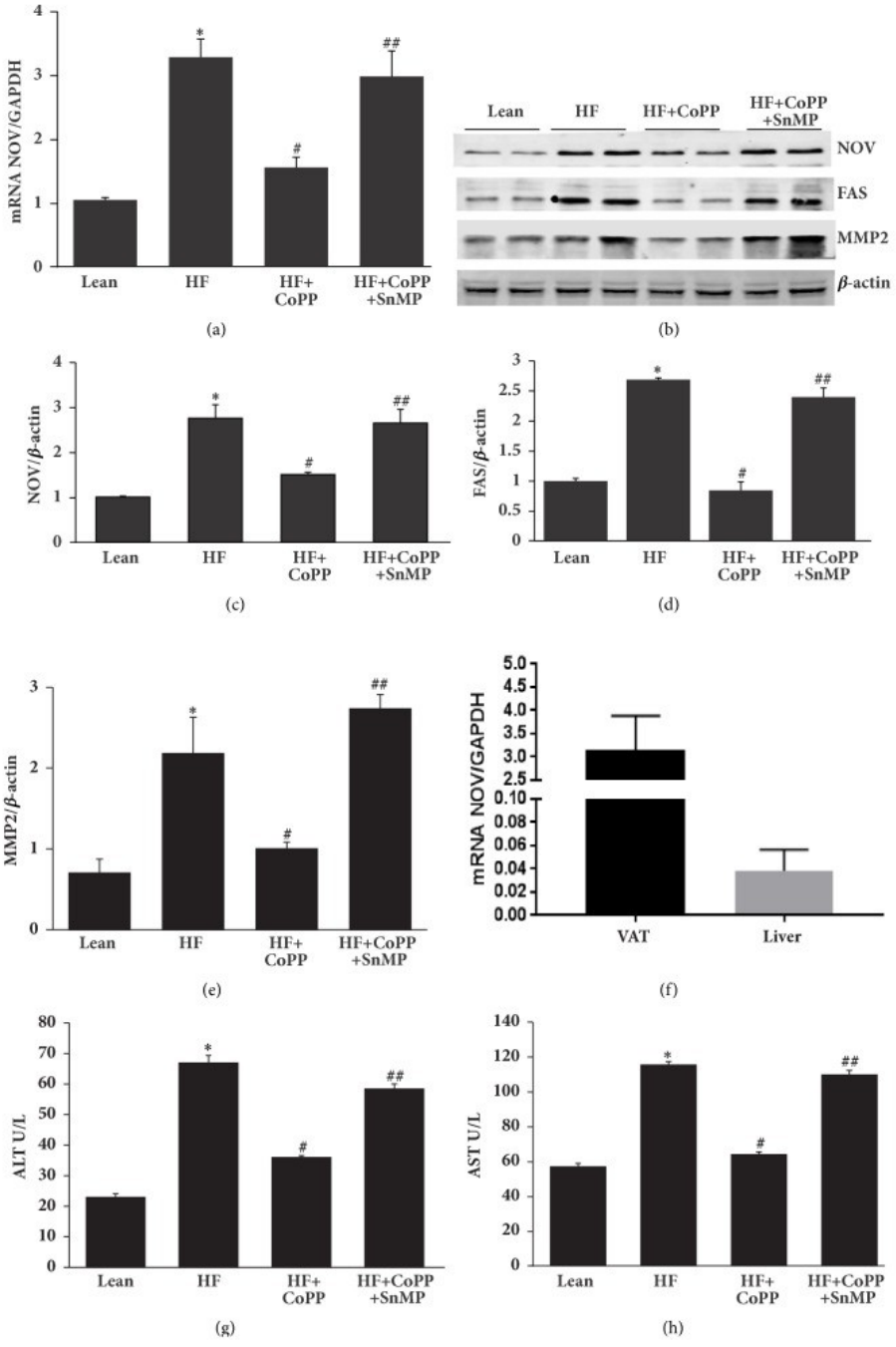

Figure 2. Induction of HO-1 decreases NOV and fibrotic markers and improves ALT and AST. (A) The mRNA expression of NOV, (B) representative western blots and densitometry analysis of (C) NOV, (D) FAS, and (E) MMP2 and levels of (F) AST(U/L), and (G) ALT(U/L) in hepatic tissues of control lean mice, HF fed mice, HF fed + with CoPP and HF fed mice + CoPP + SnMP. (H) NOV mRNA levels in visceral adipose tissue (VAT) and liver of lean mice. Results are mean ± SE, n=6, *p<0.05 vs lean mice, #p<0.05 vs HF fed mice, ##p<0.05 vs HF fed mice + CoPP.

Induction of HO-1 decreases NOV and fibrotic markers and improves ALT and AST. As seen in Figure 2 F, levels of NOV in the lean mouse are significantly higher in visceral adipose tissue (VAT) than in liver tissue. The HF diet increased the expression of hepatic NOV/ CCN3 mRNA and protein content (Figure 2 A, B and C) as compared to lean mice ($p<0.05$). Increase in HO-1 expression normalized NOV expression levels, an effect blocked by inhibitor of HO activity; SnMP-treatment ($p<0.05$) (Figure 2 A, B and C) ($p<0.05$). Similarly FAS protein expression was significantly ($p<0.05$) elevated in HF fed mice and normalized by CoPP (Figure 2 B and D). Fibrotic protein signaling in hepatic tissue of obese mice as measured by the expression of MMP2 was reduced by increased HO-1 ($p<0.05$), an effect that was prevented by inhibition of HO activity (Figure 2 B and E). Obese mice developed impaired liver function as indicated by increased levels of serum AST ($p < 0.05$) and ALT ($p < 0.05$), all of which was normalized by HO-1 induction ($p<0.05$) (Figure 2 G and H). HO-1 induced reduction in AST and ALT was eliminated by administration of SnMP (Figure 2 G and H). Taking all of these findings together it can be concluded that a decrease in levels of the pro-inflammatory adipokine NOV via HO-1 induction, mitigates the development of fibrotic markers that contribute to the NASH phenotype.

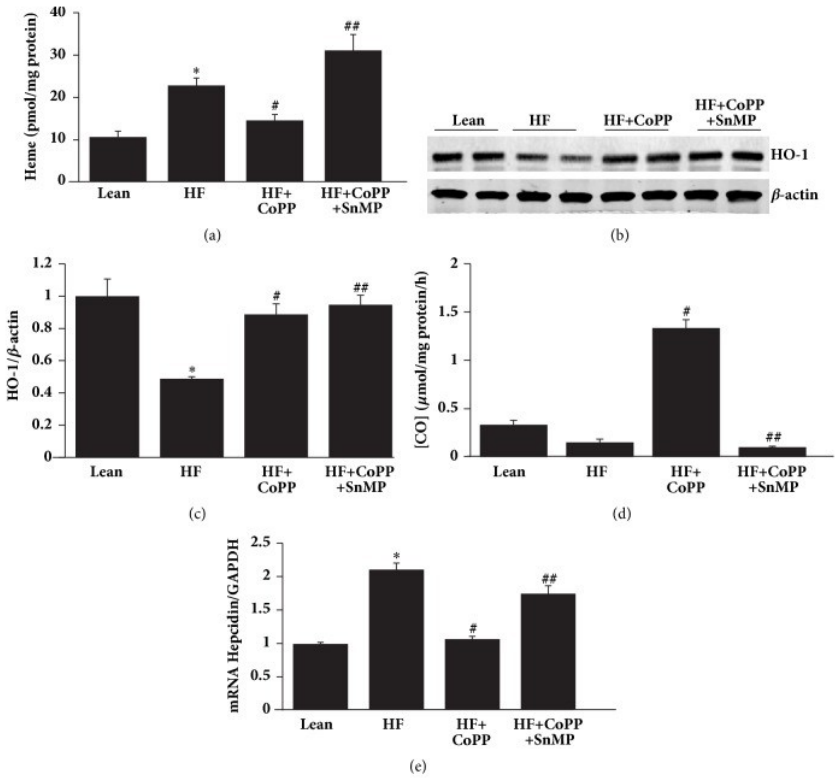

Figure 3. Increase of HO-1 decreases heme levels and hepcidin expression. (A) Heme (pmol/mg protein) content, (B) Representative western blots of HO-1 (C) densitometry analysis of HO-1 and (D) CO production (μmol/mg protein/h), and (E) mRNA expression of Hepcidin in hepatic tissues of control lean mice, HF fed mice, HF fed mice + CoPP and HF fed mice + CoPP + SnMP, Results are mean ± SE, n=6, *p<0.05 vs lean mice, #p<0.05 vs HF fed mice, ##p<0.05 vs HF fed mice treated with CoPP.

Induction of HO-1 decreases heme and hepcidin expression.

In accordance with our hypothesis our results indicate that NASH livers have significantly ($p<0.01$) increased heme levels as compared to control lean mice fed a normal chow diet. Induction of HO-1 decreased heme levels as compared to the HF fed diet group ($p<0.01$). The favorable effects of induction of HO-1 were reversed by SnMP (Figure 3 A). Western blot analysis demonstrated that hepatic tissues of mice fed a HF diet for 20 weeks had significantly ($P<0.05$) decreased levels of HO-1 protein as compared to hepatic tissues of mice fed a normal chow diet (Figure 3 B and C). CoPP treatment for 8 weeks increased liver levels of HO-1 as compared to mice fed a HF diet alone, $p<0.01$ (Figure 3 B and C). The positive effects of increased HO-1 levels were reversed by SnMP (Figure 3 B and C). Of note is the fact that SnMP does not prevent an increase in HO-1 protein expression but rather inhibits HO activity [36]. HO activity in hepatic tissue was increased by CoPP and decreased by SnMP in HF fed mice, $p<0.05$ (Figure 3 D). As seen in Figure 3 E, the

hepcidin mRNA level was increased in NASH livers of HF fed mice, as compared to lean mice. Increased HO-1 expression significantly, $p<0.05$, reduced the expression of hepcidin, an effect which was reversed by SnMP ($p<0.05$).

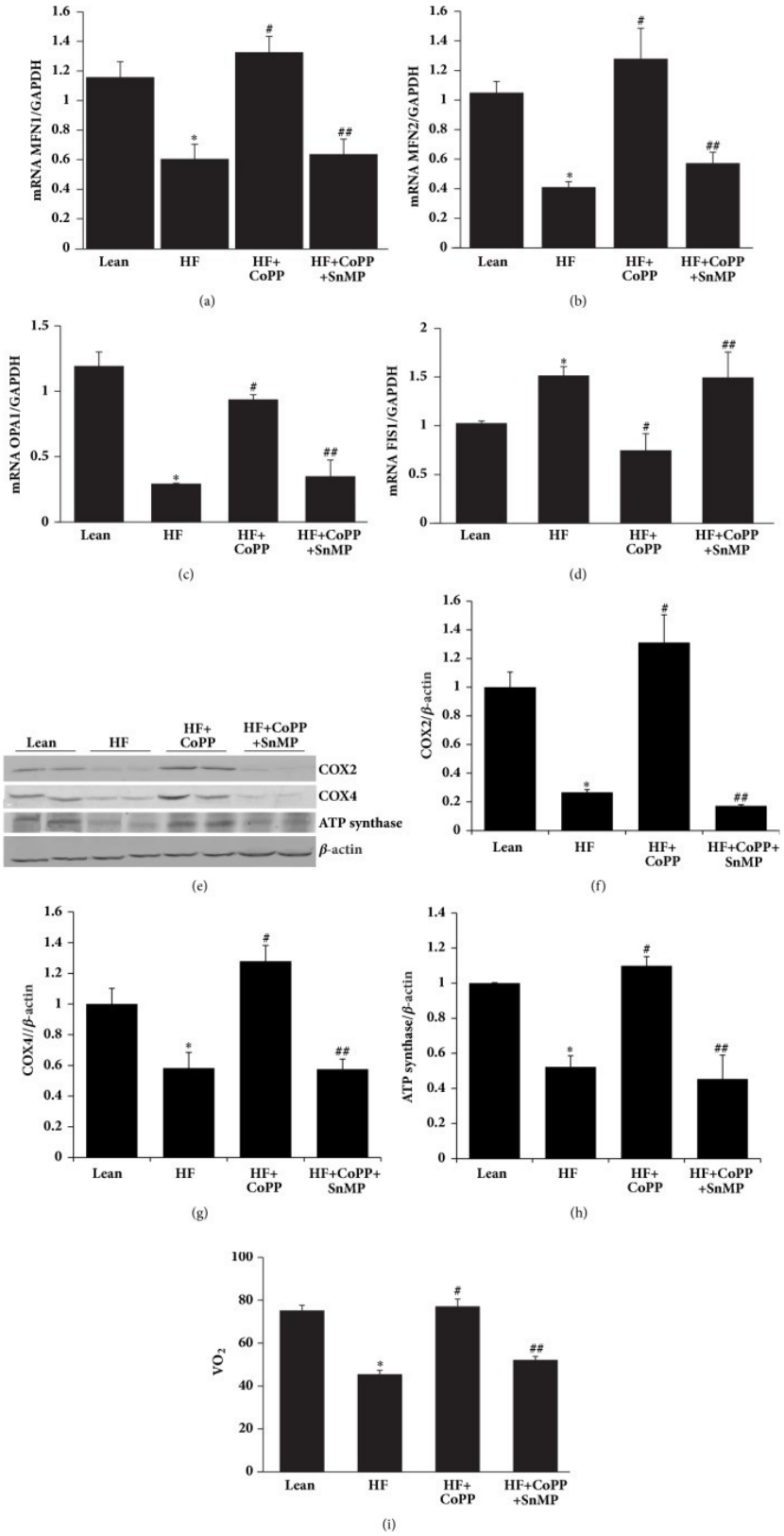

Figure 4. Increase of HO-1 expression augments mitochondrial integrity. mRNA expression of (A) MFN1, (B) MFN2, (C) OPA1, and (D) FIS1. (E) Representative western blots and densitometric analysis of (F) COX2, (G) COX4, and (H) ATP synthase. (I) Total body oxygen consumption (VO2) of mice, in hepatic tissues of control lean mice, HF fed mice, HF fed mice + CoPP and HF fed mice + CoPP + SnMP, Results are mean ± SE, n=6, *p<0.05 vs lean mice, #p<0.05 vs HF fed mice, ##p<0.05 vs HF fed mice + CoPP

Increase of HO-1 expression augments mitochondrial integrity.

MFN1, MFN2 and OPA1 expression levels were increased, while FIS1 mRNA was decreased by HO-1 activation (p <0.05) an effect that was reversed in SnMP treated mice (p<0.05) (Figure 4 A-D). These results indicate that HO-1 is a powerful inducer of mitochondrial fusion (the merge of dysfunctional to functional), and an inhibitor of mitochondrial fission. This increase in fusion (MFN1 & 2) and decrease in fission (FIS1) contributes to an increase in overall mitochondrial function, leading to a decrease in adiposity in HFD fed mice, a consequent reduction in obesity, and a concurrent reduction in the development of NASH. Mitochondrial expression of COX2 and COX4 as well as ATP synthase were reduced in obese mice as compared to lean mice (p<0.05), effects that were reversed by increased HO-1 (p<0.05) (Figure 4 E-H). Oxygen consumption in obese mice was decreased as compared to lean mice (p<0.05) (Figure 4 I). However, CoPP-treatment of obese mice normalized O2 consumption (p<0.05), an effect blocked by SnMP (Figure 4 I).

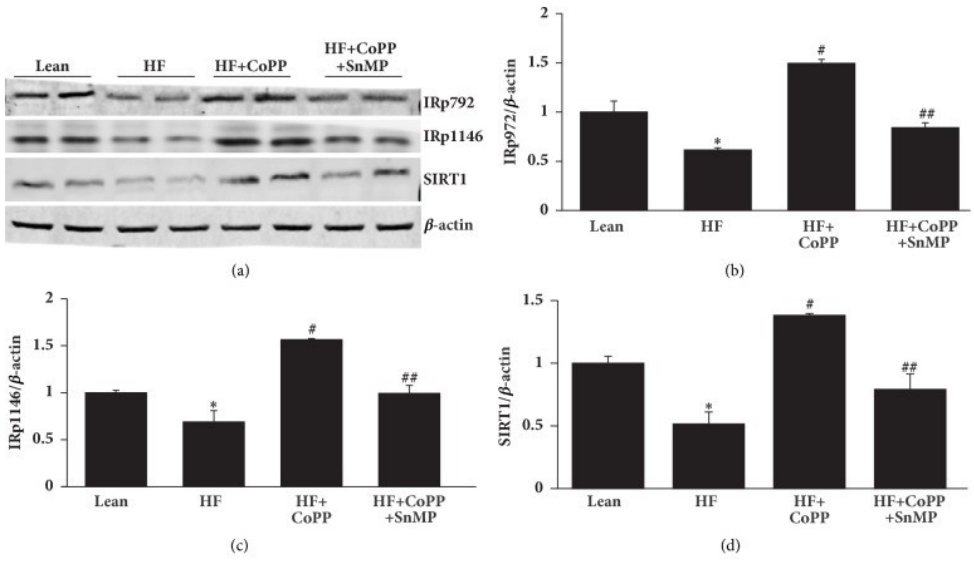

Figure 5. HO-1 up-regulation increases hepatic pAKT, pAMPK and insulin receptor phosphorylation levels. Representative Western Blots (A), and densitometry analysis of (B) IRp972, (C) IRp1146, and (D) SIRT1 in hepatic tissues of control lean mice, HF fed mice, HF fed mice + CoPP and HF fed mice + CoPP + SnMP,

Results are mean ± SE, n=4, *p<0.05 vs lean mice, #p<0.05 vs HF fed mice, ##p<0.05 vs HF fed mice + CoPP.

HO-1 up-regulation increases phosphorylation of the insulin receptor in the liver of obese mice. Obese mice had decreased expression levels of IRp-Tyr 972 and IRp-Tyr 1146 ($p<0.05$), as well as levels of SIRT1, as compared to control lean mice. HO-1 induction ameliorated the effect of HFD on insulin receptor phosphorylation and significantly increased IRp-Tyr 972, and IRp-Tyr 1146, as well as SIRT1 levels ($p<0.05$) (Figure 5 A-D), MFN1, and MFN2. These effects were reversed by inhibition of HO-1 by SnMP (Figure 5 A-F). The reversal of these beneficial effects corroborates the role of HO-1 expression and HO activity in mediating the beneficial effects of CoPP. As stated earlier some of the characteristics of NAFLD and NASH include low grade inflammation, mitochondrial dysfunction, and insulin resistance. In the figure we show that HO-1 successfully reverses insulin resistance seen in HFD fed mice by its increase in IRp-Tyr 972 and IRp-Tyr 1146.

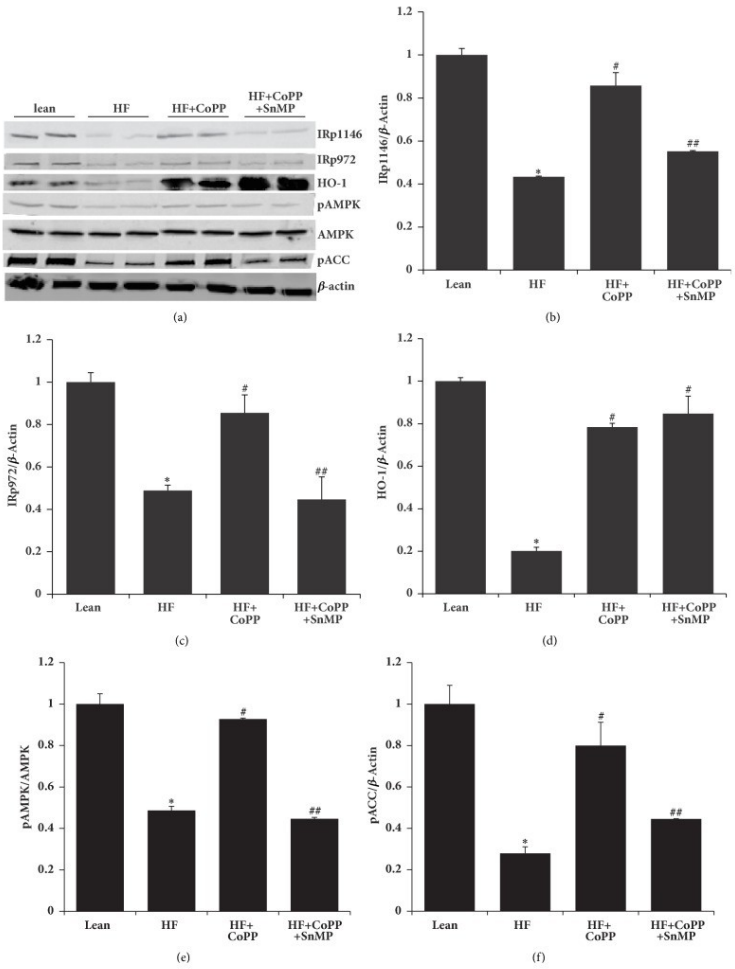

Figure 6. Adipose tissue HO-1 up-regulation increases phosphorylation of both insulin receptor and ACC. Representative western blot (A) and densitometry analysis of (B) IRp1146, (C) IRp972, (D) HO-1, (E)

pAMPK, (F) pACC. Results are mean ± SE, n=4, *p<0.05 vs lean mice, #p<0.05 vs HF fed mice, ##p<0.05 vs HF fed mice + CoPP

Adipose tissue HO-1 up-regulation increases phosphorylation of both the insulin receptor and acetyl-CoA-carboxylase (ACC). To test the possibility that NOV-mediated increases in adipose inflammation in turn decreases insulin receptor phosphorylation in an obese mouse model, we examined the effects of HO-1 on insulin receptor phosphorylation in the adipose tissue. The results show that adipose tissue of obese mice had decreased phosphorylation levels of IRp-Tyr 1146, IRp-Tyr 972, AMPK, and ACC and reduced expression of HO-1 as compared to control lean mice ($p<0.05$) (Figure 6 A-F). The negative effects associated with obesity were normalized in obese mice following induction of HO-1 ($p<0.05$) (Figure 6 A-F) and reversed by inhibition of HO activity with treatment with SnMP ($p<0.05$) (Figure 6 A-F).

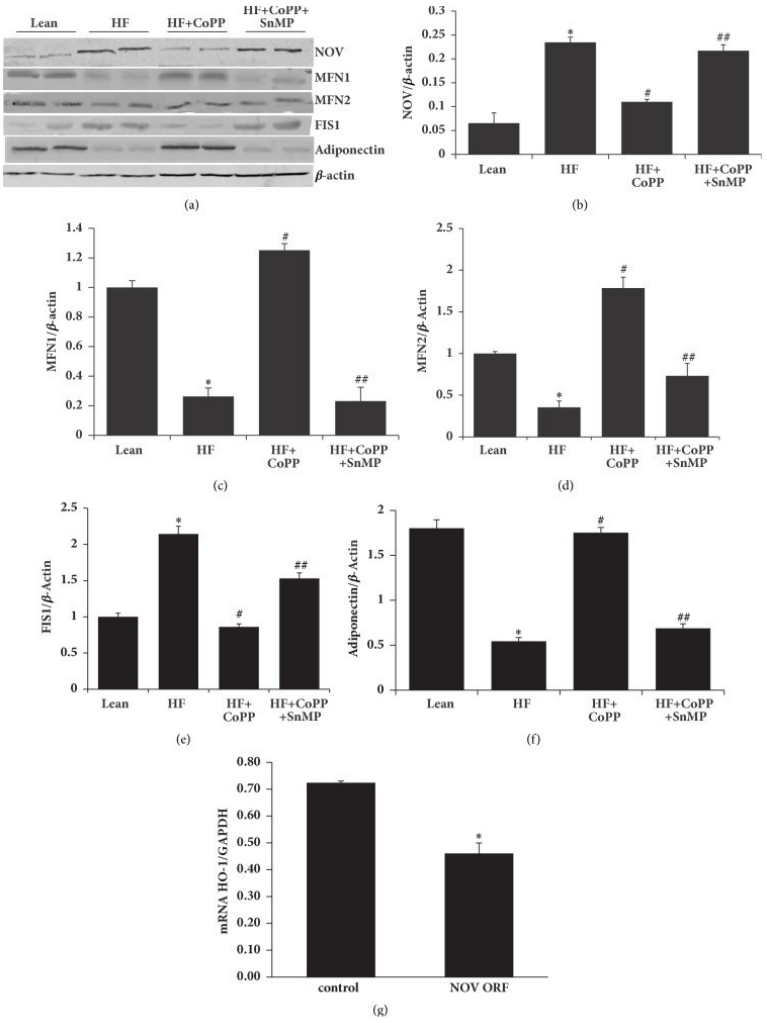

Figure 7. Adipose tissue HO-1 upregulation increases anti-inflammatory adiponectin and mitochondrial fusion-associated proteins, while decreasing pro-inflammatory NOV and the mitochondrial fission-associated

protein, FIS1. (A) Representative Western blots, and densitometry analysis of (B) NOV, (C) MFN1, (D) MFN2, (E) FIS1 and (F) Adiponectin.

Adipose tissue HO-1 upregulation increases anti-inflammatory adiponectin and mitochondrial fusion-associated proteins, while decreasing pro-inflammatory NOV and the mitochondrial fission-associated protein, FIS1. To further test the possibility of potential cross talk between adipose and hepatic tissue we examined the effects of HO-1 on adipose mitochondrial function as it related to the pro-inflammatory adipokine NOV . Inflamed adipose tissue from untreated obese mice expressed elevated levels of NOV. As seen in Figure 7 A and B, the NOV level in visceral adipose tissues of HF diet fed mice was elevated as compared to the levels in lean mice ($p<0.05$). HO-1 induction decreased visceral adipose tissue NOV levels ($p<0.05$), suggesting that induction of HO-1 reprograms white adipose tissue to beige, resulting in less inflammation (Figure 7 A and B).

More importantly, as mitochondrial integrity in liver tissue of HF diet fed mice was increased by HO-1 induction and as mitochondrial function is very important also for the health of adipose tissue, we assessed the levels of mitochondrial fusion and fission proteins in the visceral adipose tissue. As seen in Figure 7 A, C-E, the levels of MFN1 and MFN2 were decreased, while FIS1 was increased in obese mice as compared to lean mice, $p <0.05$. HO-1 induction normalized these levels, an effect reversed by concomitant SnMP-treatment ($p<0.05$) (Figure 7 A , C-E). Healthy adipocytes express the anti-inflammatory adipokine, adiponectin. As seen in Figure 7 A and F, the adiponectin level in visceral adipose tissues of HF diet fed mice was decreased as compared to the levels in lean mice ($p<0.05$). CoPP-mediated HO-1 induction normalized visceral adipose tissue adiponectin levels, an effect that was prevented by SnMP-treatment ($p<0.05$) (Figure 7 A and F).

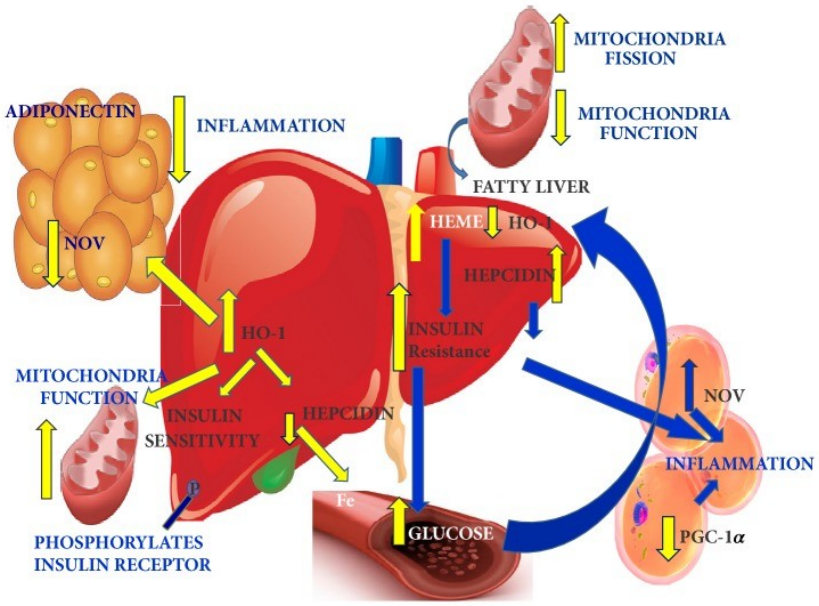

Figure 8

Schematic description of the HO-1 mediated induction of adipose tissue of HF fed mice. As seen in right side panel, decreased HO-1 resulted in an increase of hepatic heme, hepcidin, which is associated with the development and progression of obesity-induced NASH, fibrosis via mitochondrial dysfunction (fission and decreases in COX 2 and 4). Fat expansion was associated with remodeling marked by an increase in adipocyte hyperplasia and hypertrophy leading to proinflammatory molecule NOV, which is associated with a decrease of PGC-1α and insulin receptor phosphorylation and eventually development of fibrosis and NAS. In the left panel, induction of HO-1 reduced the severity of steatosis, inflammation, and fibrosis through decrease in NOV and an increase of adiponectin that resulted in the improvement of hepatic mitochondrial integrity, pAMPK-pAKT, and insulin receptor phosphorylation that all in concert leads to an improvement in hepatic function and steatosis.

Discussion

The primary findings of this study are the following: 1) The increase of the pro-inflammatory adipokine NOV and decrease of HO-1 in hepatic and adipose tissue of obese mice is associated with mitochondrial dysfunction and the development and progression of obesity-induced NASH. 2) Fat expansion is associated with remodeling marked by an increase in pro-inflammatory molecules and oxidative stress, and a decrease in PGC-1α and insulin receptor phosphorylation with the eventual development of metabolic abnormalities. 3) TNF and NOV are substantially increased while adiponectin is decreased in obese mice. 4) Reduction of heme through the pharmacological induction of HO-1 reduces the severity

of steatosis, inflammation, and fibrosis through the improvement of hepatic mitochondrial function.

The potential beneficial role of decreasing NOV in obesity and metabolic syndrome has been recently described [29]. Activation of NOV appears to be a key component of the inflammatory and fibrotic response in the liver of obese mice, and adiposity mediated increase of NOV appears to be involved in hepatic IR and in the pathophysiology of the inflammation and the resulting fibrosis. In NOV -/- obese mice there is a reduction in body weight, a decreased expression of pro-inflammatory cytokines and chemokines, and increased levels of PGC-1α and UCP1. In our obese mouse model, increased levels of HO-1 reduced NOV mRNA, as well as inflammation and markers of fibrosis to the levels of lean animals. This effect is reversed by inhibiting HO activity, indicating the pivotal role of HO-1 and HO activity in the regulation of obesity and metabolic syndrome. We speculate that a close crosstalk exists between adipose dysfunction and the development of fibrosis and NASH

HO-1 induction plays an important role in amelioration of oxidative stress [37]. In both humans and mice, low levels of HO-1 lead to organ damage [16,38], Moreover overexpression of HO-1 lowers levels of the inflammatory mediators TNFα and IL-6 in mouse liver [39]. A decrease in HO activity causes mitochondrial lipid peroxidation and mitochondrial dysfunction, while induction of HO-1 upregulates mitochondrial transcription factor A [21], all of which supports the contention that a reduction in HO activity results in mitochondrial dysfunction and increased insulin resistance [29]. The decrease in mitochondrial function leads to a decrease in beta oxidation in the liver which allows fat to accumulate resulting in a “fatty liver” [40,41].

Mitochondrial dysfunction is also a key player in the generation of ROS [42], which results in abnormal respiration [40]. Oxidative stress in NAFLD/NASH is associated with the reduced expression of PGC-1α in adipose tissue, negatively affecting mitochondrial biogenesis, thereby resulting in the mitochondrial dysfunction that is seen in the development of IR [43]. PGC-1α targets SIRT3, a mitochondrial deacetylase, which promotes mitochondrial biogenesis, suppression of ROS [44], and mitochondrial FA oxidation [45]. PGC-1α adipocyte knockout mice develop IR and glucose intolerance, and consequently elevated levels of circulating lipids and cholesterol [46] . This demonstrates the existence of PGC-1α crosstalk between adipocytes and the liver thereby correlating adipocyte mediated release of inflammatory cytokines with hepatic insulin resistance and

steatosis. With HO-1 induction, levels of PGC-1α and markers of mitochondrial fusion increase in adipose tissue, oxidative stress decreases, and lipogenesis and liver function in obese mice improve.

A HFD increases the expression of the FIS1 gene, which regulates mitochondrial fission, while concomitantly reducing the expression of those genes responsible for mitochondrial quality control and fusion processes, fueling ROS generation, and causing tissue inflammation [47]. An inverse relationship exists between MFN2 mRNA levels in skeletal muscles and BMI. Consistent with these observations, skeletal muscle from obese subjects present an altered, fragmented mitochondrial network, associated with nutrient oxidation, respiratory chain defects, and IR [48]. Additionally, liver MFN2 levels are decreased in obesity, but increased by increasing HO-1 levels, thereby reducing the severity of NASH. In particular, increased levels of HO-1 decreases steatosis, abolishes fibrosis, and the NAS score is reduced. In agreement with our data, NASH and fatty liver are both associated with IR, but NASH alone is associated with mitochondrial structural defects [49]. Finally, since hepcidin is released from adipose tissue and is upregulated by NOV, it follows that there would be increased hepcidin in individuals with higher BMI and metabolic abnormalities who would be more likely to develop NASH [1]. Thus, considering only a small percentage of patients with NAFLD progress to NASH, an increase in hepcidin might explain the conversion and associated mitochondrial dysfunction and inflammation. Additionally, differences may exist in patients with fatty liver based on different genetic backgrounds of these individuals (increased heme-NOV and a decrease in HO-1 expression).

In conclusion, these data identify that a decrease in hepatic HO-1 and an increase in adipose-derived NOV activation can be key mediators in the development and progression of obesity induced fibrosis and NASH. Decreased heme levels result in improved mitochondrial function and decreased FAS with an overall reduction in NOV/inflammation, fibrosis and NASH scores (Summarized in Figure 9). As the pursuit of a reliable surrogate marker of inflammation and fibrosis continues, liver histopathology is reflected by the NAFLD Activity Score, which remains the gold standard end-point of therapeutic efficacy. However, future pharmacologic targeting of the HO-1/NOV axis may prove fruitful in reducing the severity of a disease process that is increasing significantly in prevalence.

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Chapter 7

Epoxyeicosatrienoic Intervention Improves NAFLD in Leptin Receptor Deficient Mice by an Increase in HO-1-PGC1α -mitochondrial Signaling

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is associated with obesity and is considered to be an inflammatory disorder characterized by fatty acid accumulation, oxidative stress, and lipotoxicity. We have previously reported that epoxyeicosatrienoic acid-agonist (EET-A) has multiple beneficial effects on cardiac, renal and adipose tissue function while exhibiting both anti-inflammatory and anti-oxidant activities. We hypothesized that EET-A intervention would play a central role in attenuation of obesity-induced steatosis and hepatic fibrosis that leads to NAFLD. **Methods**: We studied the effect of EET-A on fatty liver using db/db mice as a model of obesity. Mice were fed a high fat diet (HFD) for 16 weeks and administered EET-A twice weekly for the final 8 weeks. **Results**: db/db mice fed HFD significantly increased hepatic lipid accumulation as manifested by increases in NAS scores, hepatic fibrosis, insulin resistance, and inflammation, and decreases in mitochondrial mitofusin proteins (Mfn 1/2) and anti-obesity genes Fibroblast growth factor 21 (FGF21) and Cellular Repressor of E1A-Stimulated Genes 1 (CREG1). EET-A administration reversed the decrease in these genes and reduced liver fibrosis. Knockout of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in EET-A treated mice resulted in a reversal of the beneficial effects of EET-A administration. **Conclusions:** EET-A intervention diminishes fatty acid accumulation, fibrosis, and NFALD associated with an increase in HO-1-PGC1α and increased insulin receptor phosphorylation. A pharmacological strategy involving EETs may offer a potential therapeutic approach in preventing fibrosis, mitochondrial dysfunction, and the development of NAFLD.

INTRODUCTION

In concert with the increase in the prevalence of obesity, nonalcoholic fatty liver disease (NAFLD) has become the main cause of chronic liver disease worldwide in both adults and children (Benedict and Zhang, 2017). NAFLD is considered an important component of the metabolic syndrome and is characterized by a broad spectrum of fatty liver disorders that result in severe liver disease and cirrhosis (Lonardo et al, 2018; Vigano et al, 2018). Inflammation and oxidative stress are major risk factors for the development of NAFLD (Anania et al, 2018). EET analogues such as EET-A and EET-B have demonstrated cardioprotective effects in an animal model (Neckar et al, 2018). In addition , they have anti-arrhythmic and anti-hypertensive properties (Cervenka et al, 2018; Khan et al, 2014) and prevent renal fibrosis (Yeboah et al, 2018). The CYP-450-derived EET pathway represents a novel therapeutic target for modulating renal dysfunction in liver cirrhosis (Skibba et al, 2017), highlighting the potential of EET analogues as therapy for the treatment of cardiovascular disease (CVD) (Imig, 2012a) and obesity. EET analogues inhibit sEH, the enzyme that degrades EETs. sEH inhibition alleviates HFD-induced hepatic steatosis, coupled with an anti-inflammatory effect in adipose tissue (Liu et al, 2012; Lopez-Vicario et al, 2015; Wells et al, 2016). Our group has demonstrated that EET-A administration results in an increase in scaffolding proteins, potentiating a signaling cascade that increases HO-1 and mitochondrial l signaling and decreases inflammatory adipokines such as NOV (Burgess et al, 2012; Cao et al, 2017; Schragenheim et al, 2018), Specifically, we have demonstrated that EET-A is upstream of PGC1α and is associated with a decrease in adipose in mitochondrial-derived superoxide and oxidative stress through (Waldman et al, 2016) and circulating inflammatory adipokines NOV (Cao et al, 2017; Singh et al, 2016). Furthermore, overexpression of HO-1 with both pharmacological compounds and gene transfer prevents ischemia/reperfusion (I/R) injury , hyperglycemia and metabolic dysfunction (Reviewed in (Abraham et al, 2016; Abraham and Kappas, 2008) (Prawan et al, 2005)). Additionally, in mice, the constitutive overexpression of PGC-1α in cardiac tissue activates mitochondrial biogenesis and proliferation (Arany et al, 2005). Mice that are lacking PGC-1α in adipose tissue and are fed HFD develop insulin resistance and exhibit increased levels of circulating lipids (Kleiner et al, 2012). Moderate expression of PGC1α is sufficient to increase insulin sensitivity (Benton et al, 2008) and improve cardiac remodeling (Whitehead et al, 2018), increase fatty acid oxidation, and

enhanced. The current strategy is to examine the effect of an EET analog on the function of scaffolding proteins, affecting signaling cascade including PGC1α-HO-1, leading to attenuation of development steatosis fibrosis and mitochondrial dysfunction. To this end, we used lentiviral vector to deliver PCG1silencing (Ln PGC1α sh) vector and EET-A to test the hypothesis that EET-A intervention is associated with a reduced hepatic lipid accumulation, inflammatory adipokines, fibrosis and progression of NAFLD

MATERIALS AND METHODS

Animal Protocols

All experimental protocols were performed following an institutionally (NYMC) approved protocol in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*.

We used four-week-old db/db mice from Jackson Laboratories (Bar Harbor, ME). Db/db were fed a HFD (western diets, containing 58% fat, 25.6%carbohydrate and 16.4 % protein with total calories of 23.4kJ/g (Harlan, Teklad Lab Animal Diets, Indianapolis, IN) for an additional 16-weeks. HFD used to expedite development substantial hepatic lipid uptake and fibrosis. Mice were divided into 3 treatment groups as follows: Group 1) Control mice were injected intraperitoneally with water and lentivirus vector, Group 2) injected intraperitoneally with EET-A [EET-A is sodium(S,Z)-2-(13-(3-pentylureido)tridec-8-enamido)succinate], twice/per week for 8 weeks with a dose of 1.5 mg/100 g of body weight as previously described (Cao et al, 2017), and Group 3) received EET-A as above plus 2 bolus injections of 40-70 × 10^6 TU/mouse in 80-100 µl PGC-1α (sh) lentivirus (Dharmacon, Lafayette, CO) into the retro orbital vein at 3-days and 5-weeks after the start of EET-agonist injections. After 8 weeks of treatment mice were euthanized with ketamine (100 mg/kg)/xylazine (10 mg/kg) injection followed by cervical dislocation. Body weight was measured at sacrifice (Table 1).

Fasting Blood Glucose and Blood Pressure

Blood glucose was measured following a 6-h fast 2-days after the last injection of EET-A. Blood pressure was determined using the CODA tail-cuff system (Kent Scientific, Torrington, CT). All measurements were taken at the same time of day, and the systolic blood pressure was recorded (mmHg)

Histopathological Analysis of Liver Tissue, NAS S3121core and Hepatic Lipid Droplet Evaluation

Samples of liver tissue were collected from each group and fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin wax and sectioned for histopathological analysis. The samples were cut by microtome (6 µm thick) and then the liver sections were stained in hematoxylin-eosin staining for overall assessment of parenchymal architecture, hepatocyte abnormalities (including ballooning) and inflammatory infiltration and for the evaluation of lipid droplets. Furthermore, Masson trichrome staining was assessed for the evaluation of the liver fibrosis (identifying in blue the collagen fibers and in red the hepatocyte cytoplasm). The fibrosis was calculated using a computerized image analyzer (Image Pro Premier 9.1, MediaCybernetics Inc., Rockville, USA) evaluating 20 randomly chosen liver fields per experimental animal as previously descried. NAFLD is characterized by liver histopathological features including steatosis, hepatocyte ballooning, inflammation and fibrosis**.** These features were scored according to the NAFLD histologic activity score (NAS) system, and the analysis of lipid droplets was performed as previously described (Liu et al, 2018; Singh et al, 2017). Briefly, double-blinded analysis identified the degree of steatosis and NASH (grade 0 ≤ 5%; 1 = 5–33%; grade 2 =

34%–66%; grade 3 ≥ 66%), lobular inflammation (0: no foci, 1 < 2 foci per 200x field, 2: 2 to 4 foci per 200x field, and 3: >4 foci per 200x field), hepatocyte ballooning (0: none; 1: rare or few; 2: many) and fibrosis (0: no fibrosis, 1: perisinusoidal or periportal fibrosis, 2: perisinusoidal and portal/perioral fibrosis, 3: bridging fibrosis, and 4: cirrhosis).

Real-time qPCR

Total RNA extraction and purification from liver tissue was performed with a RNeasy Mini Kit (Qiagen), as indicated by the manufacturer (Liu et al, 2018). RNA quantity and purity were determined using a Biotek™ plate reader and the Take3™ plate (Biotek, Winooski, VT), measuring the absorbance by the A260/A280 ratio. First-strand cDNA was then synthesized with a High Capacity cDNA Reverse Transcription Kit (Foster City, CA, USA) reverse transcription reagent. Gene expression was assessed using TaqMan® Fast Universal Master Mix (2x) and TaqMan probes, on a 7500 HT Fast Real-Time PCR System (Applied Bio systems). For each gene, the relative mRNA expression was normalized using GAPDH as an invariant control. Successively, the relative quantification was obtained comparing the db/db control vs. treatment.

Western Blot Analysis

Frozen liver tissues were ground under liquid nitrogen and resuspended in RIPA lysis buffer that included protease and phosphatase inhibitors (Complete™ Mini and PhosSTOP™). Homogenates were centrifuged at 12000xg for 12 minutes at 4ºC, supernatants were collected and protein concentrations measured. Protein detection was performed using a secondary infrared fluorescent dye conjugated antibody. The primary antibodies used in this study were obtained from THREE companies, Danvers, MA Cell signaling for SirT1 (D1D7) Rabbit mAb #9475, Phospho-AMPKα (Thr172) (40H9) Rabbit mAb #2535, AMPKα (23A3) Rabbit mAb #2603, β-Actin (13E5) Rabbit mAb #4970 and CCN3 Rabbit mAb #8767 were procured from Abcam, Cambridge, UK, for Heme Oxygenase 1 Rabbit mAb (ab13243) CREG1 Rabbit mAb (ab191909) while phospho-IR (Tyr972) Antibody (07-838) was obtained from Sigma, St. Louis, MO. The blots were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor Science Tec) and quantified by densitometry analysis performed after normalization with β-actin. Results were expressed as arbitrary units (AU).

Statistical Analysis

Statistical significance between experimental groups was determined by ANOVA with Tukey-Kramer post-hoc analysis for comparison between multiple groups (GraphPad Prism). The data are presented as means ± SEM and $p < 0.05$ was considered statistically significant.

RESULTS

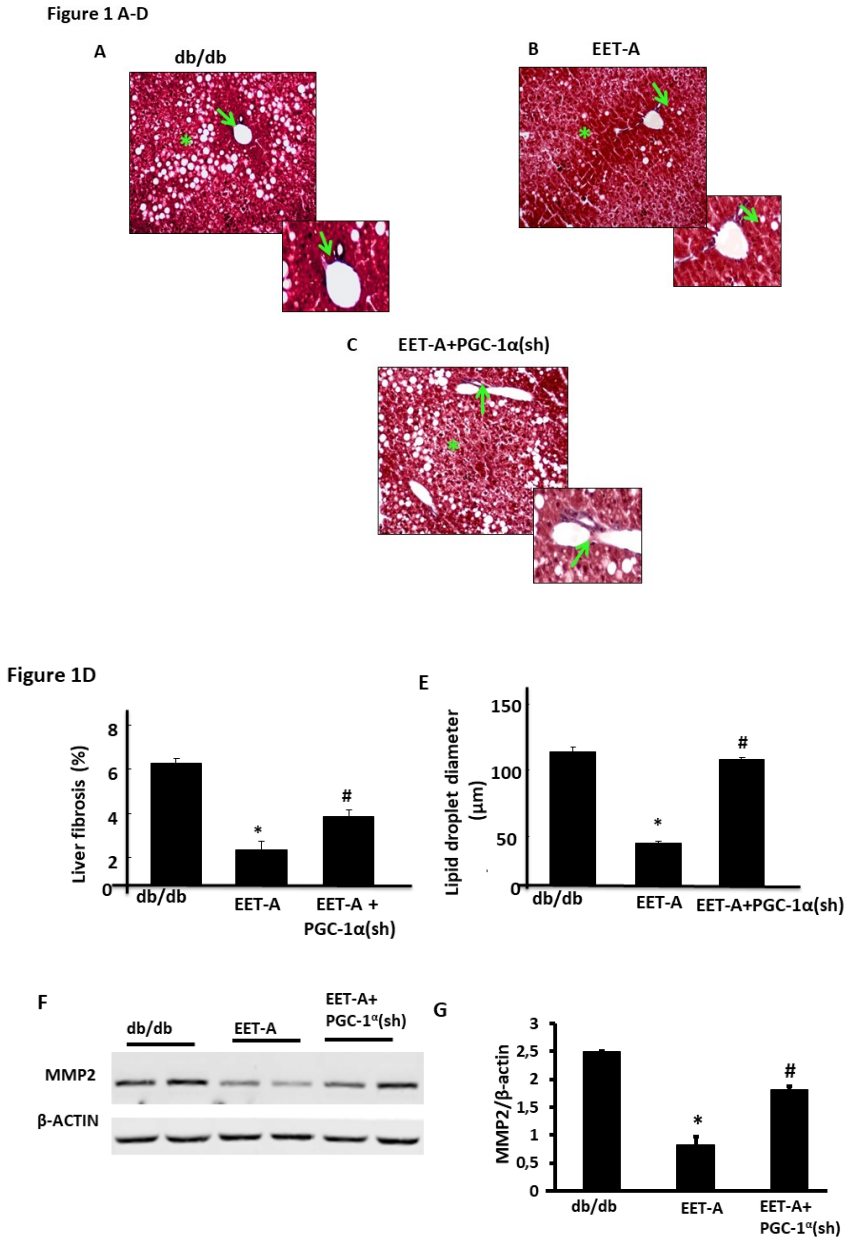

Figure 1. EET-A prevents fibrosis and decreases NASH score. Liver samples were paraffin-wax fixed and sectioned (6 µm thick). db/db (A), EET-A (B), EET-A+PGC-1α(sh) (C). Masson's trichrome staining. Bar 20 μm. The arrow shows hepatic perivascular fibrosis of control db/db mice, EET-A group and EET-A+PGC-1α(sh). The graph summarizes the morphometrically analysis of fibrosis and liver lipid droplet diameter (D and E, respectively). Representative western blotting for MMP-2 protein levels (F); densitometry analysis of MMP-2 (G). Results are mean ± SE, n=6, *$p < 0.05$ vs. db/db control, #$p < 0.05$ vs. db/db mice treated with EET-agonist.

EET-agonist Improves Physiological Parameters in Obese Mice

Blood glucose was measured following a 6-h fast; glucose levels in the Control group of diabetic obese mice were 340 ± 19 mg/dl; in the group with EET-A glucose levels were 112 ± 5 mg/dl, and in the EET-A group co-treated with plus Lentivirus PGC-1α, 245 ± 7 mg/dl (Table1). Blood pressure values were: 160 ± 5 mmHg in the db/db Control group, 115 ± 9 mmHg with EET-A treatment, and 135 ± 11 mmHg with EET-A plus injection of Lentivirus PGC-1α (Table 1).

EET-agonist Reverse Fibrosis and Decreases the NASH Score and MMP-2 Protein Levels

Livers of lean mice show no evidence of steatosis (0.54% of cells were positive for intracellular lipid accumulation), ballooning is sporadic and there are no signs of inflammatory foci or fibrosis (Figure 1A). In contrast, livers from obese mice exhibited a high NAS score (NAS: 9) with elevated steatosis (39.4% cells positive for lipid accumulation), moderate lobular inflammation, significant hepatocyte ballooning, and fibrosis (Figure 1B). EET-A improved the NAS score (NAS: 3) ameliorating all NASH parameters as evidenced by mild steatosis (6.83% cells positive for lipid accumulation), the rare occurrence of inflammatory loci and ballooning and reverse fibrosis (Figure 1C). Ln-PGC-α (sh) treatment reversed the beneficial effects of EET-A and caused perisinusoidal steatosis (37.4% cell positive for lipid accumulation) with increased ballooning and portal fibrosis (NAS: 8) (Table2) (Figure 1D). Moreover, hepato-steatosis was confirmed by analyzing liver lipid droplet diameter and morphology (Table 2, Figure 1E). We conclude that EET-A reduced lipid droplet formation in the liver, progression of NAFLD to NASH in obese mice. Moreover, MMP-2 protein levels were decreased by EET-A treatment mice group as compared to db/db mice (Figure 1F and G, respectively), however the effect was reversed by Ln-PGC-1α (sh).

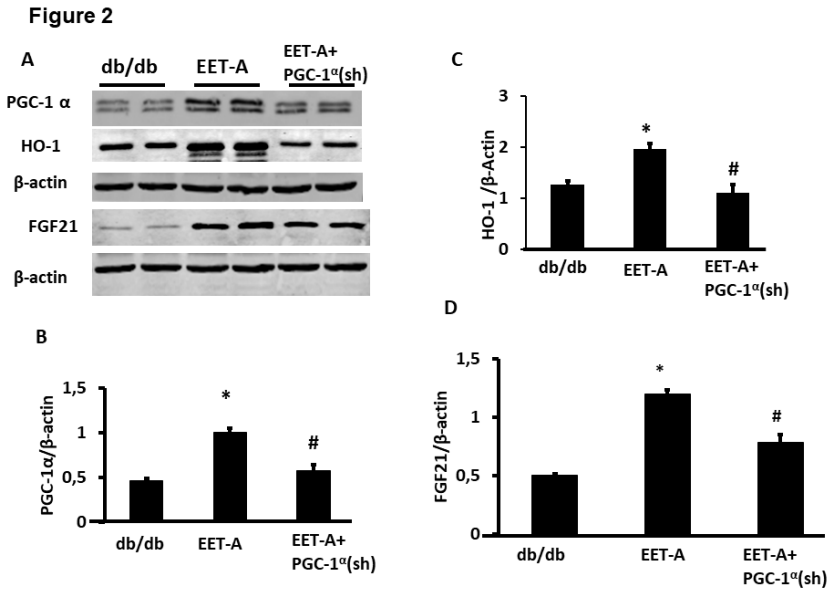

Figure 2. Effect of EET-agonist treatment on energy metabolism regulators in liver tissue of db/db mice. Representative western blots for PGC-1α, HO-1, FGF21 and β-Actin as control (A). Densitometry analysis of PGC-1α (B), HO-1 (C), and FGF21 (D) of db/db control mice, db/db mice treated with EET-agonist, and PGC-1α-deficient db/db mice treated with EET-agonist. Results are mean ± SE, n=6, *$p < 0.05$ vs. db/db control, #$p < 0.05$ vs. db/db mice treated with EET-agonist.

Effect of EET Agonist on PGC-1α, HO-1 and FGF21 Protein Levels

Figure 2A-B displayed that EET-A increased both PGC1-α as compared to db/db mice and this effect was reversed by Ln-PGC-1α (sh). As we expected the knockout PGC1-α protein was associated with a decrease in PGC1α proteins levels (Figure 2A-B). EET-A resulted in a upregulation of HO-1 protein levels (Figure 2A-C) as compared with control db/db mice. The EET-A beneficial effect was reversed by decreasing PGC1a levels using Ln-PGC-1α (sh). The EET-A treatment was efficient in upregulates the FGF21 protein levels (Figure 2A-D) as compared to db/db mice. The Ln-PGC-1α (sh) decreased the FGF21 protein levels compared to EET-A mice (Figure 2A-D).

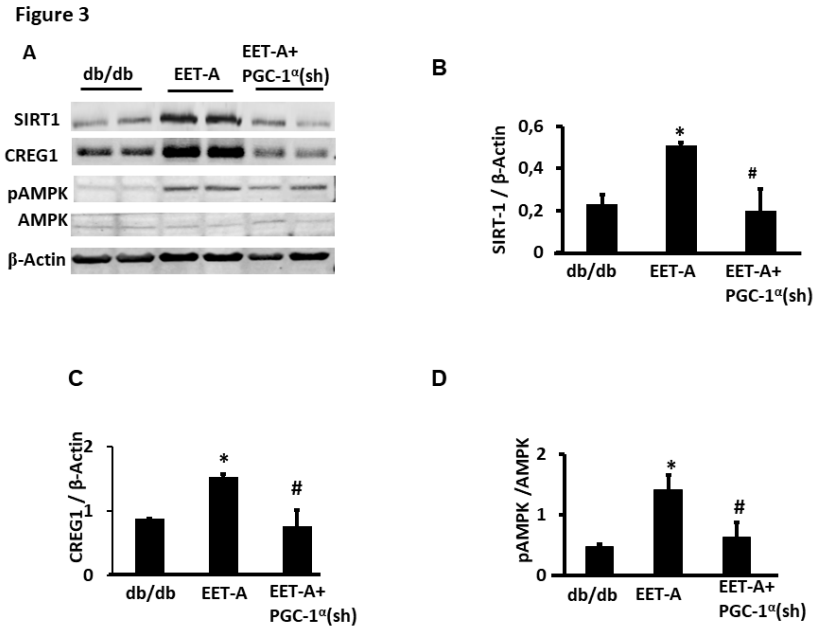

Figure 3. Effect of EET-agonist treatment on levels of SIRT1, CREG1 and pAMPK/AMPK in liver tissue of db/db mice. Representative western blots (A) and densitometry analysis of (B) SIRT1, (C) CREG1, (D) pAMPK/AMPK of db/db mice treated with EET-agonist, and PGC-1α-deficient db/db mice treated with EET-agonist. Results are mean ± SE, n=6, *$p < 0.05$ vs. db/db control, #$p < 0.05$ vs. db/db mice treated with EET-agonist.

Effect of EET Agonist on SIRT1, CREG1 and pAMPK Protein Levels

These findings were corroborated at the protein expression for SIRT1 (Figure 3A-B). To examine a potential link between EET-A and CREG we observed that EET-A markedly increased CREG1 protein expression as compared with control db/db mice ($p < 0.05$), (Figure 3A-C) which was prevented by Ln-PGC-1α (sh). We examined levels of pAMPK under the above experimental conditions. As shown in Figure 3A-D, EET-A-increased the phosphorylation status of liver AMPK by ∼40%, as compared to db/db control mice. In PGC-1α-deficient mice the EET-A effect on AMPK phosphorylation was markedly abrogated (Figure 3A-D).

Figure 4

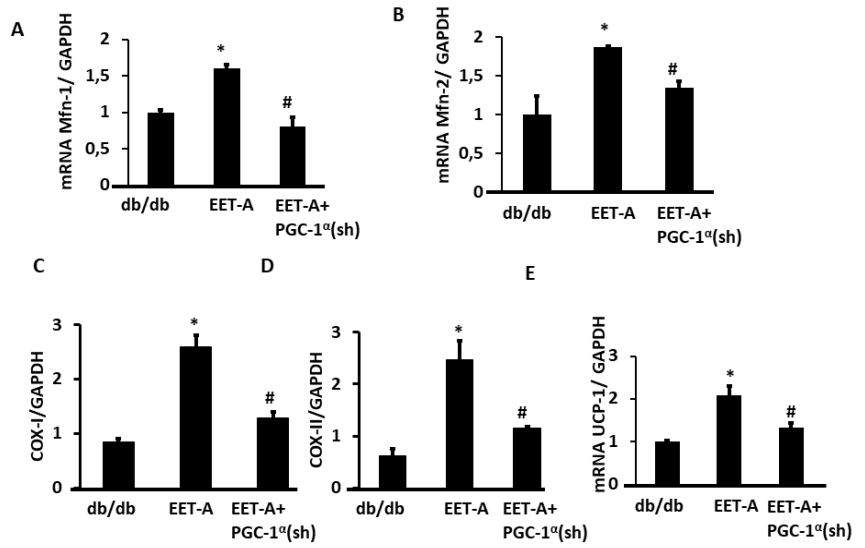

Figure 4. EET-levels increase mitochondrial fusion and thermogenic gene in liver. mRNA expression of Mfn-1 (A), Mfn-2 (B), COX1 (C), COX2 (D) and UCP-1 (E), of db/db control mice, db/db mice treated with EET-agonist, and PGC-1α-deficient db/db mice treated with EET-agonist. Results are mean ± SE, n=6, *p < 0.05 vs. db/db control, #p < 0.05 vs. db/db mice treated with EET-agonist.

Effect of EET on mRNA Expression of MFN1, MFN2, COX1, COX2 and UCP1 in the Liver of db/db Mice

Mfn1 and Mfn2 mRNA expression levels were increased by EET-A ($p < 0.05$), an effect that was reversed by Ln- PGC-1α ($p < 0.05$) (Figures 4A and B, respectively). EET-A increased the COX1 and COX2 mRNA expression compared to db/db mice (Figures 4-C and D, respectively), this effect was reversed by Ln-PGC-1α (sh). EET-A increased the expression of UCP1 by ~2-fold (Figure 4E); this was prevented by Ln-PGC-1α.

Figure 5

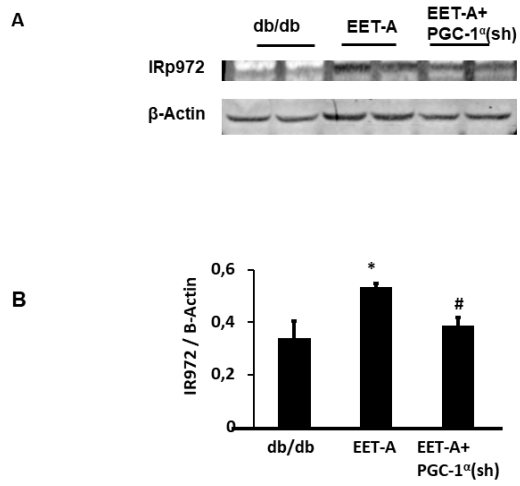

Figure 5. Effect of EET-A treatment on insulin receptor phosphorylation at Tyr-972. (A) Db/db control, db/db treated with EET-A, and PGC-1α-deficient db/db mice treated with EET-A. (A) Representative western blots; (B) and densitometry analysis of insulin receptor IR972. Results are mean ± SE, n=6, *$p < 0.05$ vs db/db control, #$p < 0.05$ vs db/db mice treated with EET-A.

Effect of EET-A on Insulin Receptor Phosphorylation at Tyr-972 in the Liver of db/db Mice

Insulin receptor phosphorylation is known to increase insulin sensitivity and decrease NASH. Our results suggested that induction of EET-A significantly increased insulin receptor phosphorylation on Tyr-972 ($p < 0.05$) and an abrogation of this effect by Ln-PGC-1α (Figure 5A-B). These findings support a role of PGC-1α expression in mediating the beneficial effects of EET-A.

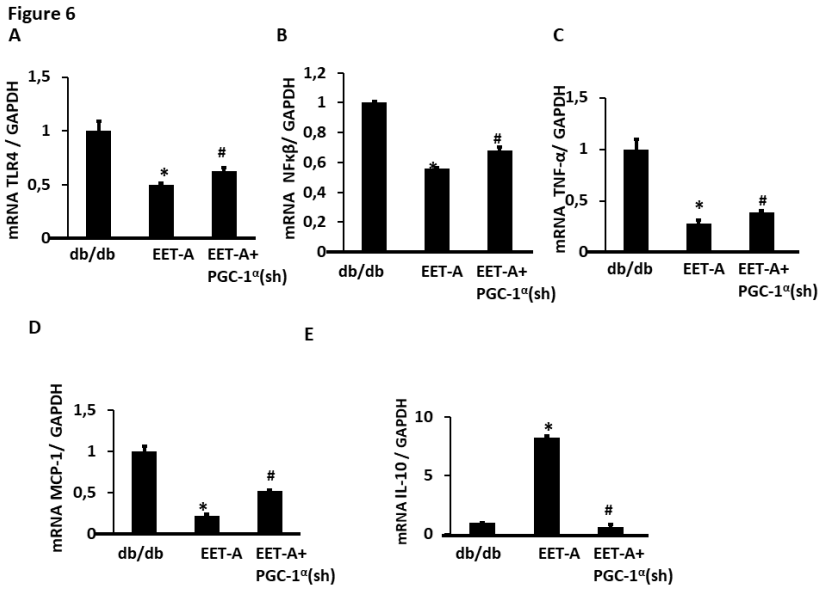

Figure 6. Effect of EET-agonist treatment on inflammatory molecules and NOV and MMP-2 protein levels in liver tissue of db/db mice. mRNA expression of TLR-4 (A), NFκβ (B), TNF-α (C), MCP-1 (D) and IL-10 (E) of db/db control mice, db/db mice treated with EET-agonist, and PGC-1α-deficient db/db mice treated with EET-agonist. Representative western blotting for NOV protein levels (F); densitometry analysis of NOV (G). Results are mean ± SE, n=6, *$p < 0.05$ vs. db/db control, #$p < 0.05$ vs. db/db mice treated with EET-agonist.

EET-agonist on Pro- and Anti-inflammatory Cytokines mRNA Expression and NOV Protein Levels

TLR4 and NF-κB mRNA expression levels were decreased by EET-A ($p < 0.05$), an effect that was significantly reversed by Ln- PGC-1α ($p < 0.05$) (Figure 6A, B). As expected, expression of the cytokines TNFα and MCP-1 were also decreased in mice treated with EET-A, an effect that was prevented by silencing expression of PGC-1α (Figure 6C, D). The anti-inflammatory cytokine IL-10 showed the opposite trend, with a marked increase following EET-A reduction to almost control levels upon suppression of PGC-1α (Figure 6E). The NOV protein levels were decreased by EET-A treatment mice group as compared to db/db mice (Figure6 F and H, respectively), however the effect was reversed by Ln-PGC-1α (sh).

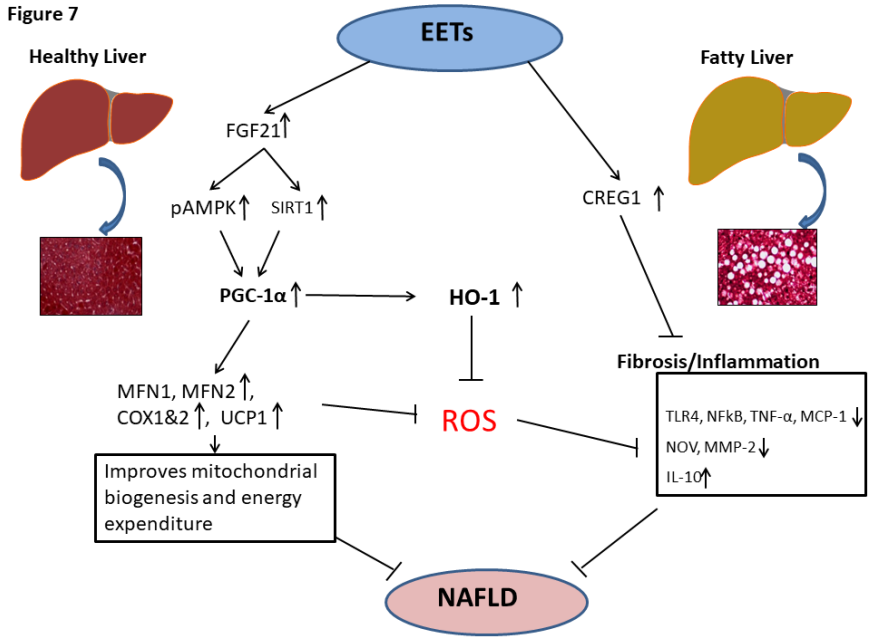

Figure 7. Schematic description of the positive EET effects on NAFLD. EETs increases expression of key regulators signaling, PGC1α-HO-1, FGF21, SIRT1 and pAMPK, leading to improve energy expenditure, increased mitochondrial fusion; Mfn1/2 and function; COX1&2, leading to reduction in NOV and fibrosis and inflammation in liver. These beneficial effects by administration of EET-agonist lead to an improvement in NAFLD in a PGC-1α dependent manner.

DISCUSSION

The present studies show that EET-A-mediated induction of HO-1 improves hyperglycaemia, reduces blood pressure, and provides protection against accumulation of fatty acids in the liver of obese mice, with a clear beneficial impact on NAFLD. This is supported by four main experimental findings. First, EET-A drastically reduced hepatic lipid droplet size, ballooning, and fibrosis in livers of obese mice, with a significant reduction in the NAS score. These effects of EET-A were prevented by suppressing expression of PGC-1α, indicating that the benefit of EET-A is dependent on HO-1-PGC1 activity as an obligatory requirement. These data demonstrate that EET-A functions as a lipid meditator in the reduction of hepatic lipid uptake and fibrosis.

Second, EET-A increased the expression of the key signaling regulator proteins PGC-1α, CREG1, AMPK and SIRT1 in obese mice. PGC-1α is a master regulator of energy metabolism (Lin et al, 2005), and has a role in preventing the development of metabolic

syndrome and type 2 diabetes (Singh et al, 2016). The effect of EET-A on PGC-1α−HO-1-adiponectin signaling leads to improved mitochondrial integrity and amelioration of obesity-induced renal dysfunction by improving sodium excretion (Schragenheim et al, 2018). The present results show improvement in metabolic parameters in fatty liver following EET-A treatment, presumably through the PGC-1α-HO-1 axis, and extend our knowledge of the systemic actions of EETs in obesity as well as their impact on the pathology of NAFLD.

The cellular repressor E1A-stimulated genes 1 (CREG1) is a small glycoprotein, the physiological function of which remains poorly understood. CREG was found mainly in the cytoplasm of hepatocytes and dramatically reduced in livers of NAFLD patients compared to normal subjects (Zhang et al, 2017). Recent studies in mice demonstrate that overexpression of CREG1 is correlated in a positive manner with AMPK and ACC phosphorylation. Following exposure to HFD, overexpression of hepatic CREG1 is associated with decreased expression of genes linked to fatty acid synthesis, and an increased level of genes associated with fatty acid oxidation/anti-inflammation. By contrast, livers from CREG-KO mice exhibit the opposite results (Tian et al, 2017; Zhang et al, 2017). Our results show an increase in CREG1 expression when mice are treated with EET-A, an effect that was prevented by lentiviral-mediated suppression of PGC-1α expression.

Interestingly, we found that EET-A increased the phosphorylation status of AMPK in a PGC-1α-dependent manner. In this context, our present findings confirm the beneficial effect of EET-A on lipid metabolism through a direct effect on the liver, in part due to enhancement of AMPK activation, which is upstream of hepatic fatty acid β-oxidation. Further, EET-A administration increases energy seTQr genes such SIRT1.

Third, EET-A leads to a notable improvement in mitochondrial function as assessed by the increased in expression of mitofusion 1 and 2 (Mfn1 and Mfn2), and cytochrome oxidase complex 1 and 2. Mitochondrial dysfunction increases the production of superoxide anion and other reactive oxygen species (ROS) that play a key role in the pathogenesis of NAFLD, mainly due to increased lipid peroxidation and oxidative damage to proteins and DNA (Armutcu et al, 2005; Chalasani et al, 2004). In addition, mitochondrial dysfunction leads to reduced beta oxidation in the liver, a permissive condition for fat accumulation thus leading to “fatty liver” (Nassir and Ibdah, 2014). Notably, EET-A dependent FGF21

levels are seen to increase. Elevation in hepatic FGF21 is known to reduce adiposity and enhance glycogen storage, resulting in a decrease in oil droplets in hepatic tissue on obese mice (Hinds, Jr. et al, 2014; von Holstein-Rathlou et al, 2016).

Perhaps of greatest interest, we observed that EET-A administration reduced the levels of inflammatory molecules and fibrosis markers such as (TNF-α, MCP1, NOV, TLR-4 and NF-kB, and MMP-2), whereas expression of the anti-inflammatory cytokine, interleukin-10, was increased. These findings strongly support the notion that CYP-derived EETs have potent anti-inflammatory effects mediated in part by attenuation of NF-κB signaling and by having pro-survival and anti-apoptotic effects (Imig, 2012b; Liu et al, 2018; Node et al, 1999). In addition, increased levels of HO-1 in the current study led to a reduction in NOV protein expression, with alleviation of inflammation and steatosis in a PGC-1α dependent manner. NOV expression appears specifically linked to the inflammatory and fibrotic response, affecting hepatic insulin resistance in obese mice (Sacerdoti et al, 2018). In $NOV^{-/-}$ mice on HFD, there is a reduction in body weight compared to wild type and a reduction in expression of pro-inflammatory molecules associated with increases in PGC-1α and UCP1 levels (Martinerie et al, 2016).

In summary, we suggest that EET-agonists offer a multifactorial clinical approach to prevent the development of NAFLD and the concomitant metabolic imbalances (Figure 7). This study demonstrates that EET intervention attenuates fatty liver syndrome by the upregulation of PGC-1α and HO-1, which prevents expression of inflammatory markers through a number of mechanisms. The combination of these effects results in a clear alleviation of NAFLD and a significant shutdown in the progression to NASH. Our findings support the finding that pharmacological induction of EETs offers a potential therapeutic approach to prevent fibrosis, mitochondrial dysfunction, and the development and/or progression of NAFLD.

Table 1. Physiological parameters of treated db/db mice on HFD			
	CTRL n=6	**EET-A n=6**	**EET-A+ PGC-1α(sh) n=6**
Body weight (g)	62.7 ± 1.4	46.1 ± 1.7*	54.3 ± 4.1#
Blood Pressure (mmHg)	160 ± 5	115 ± 9*	135 ± 11#
Glucose (mg/dL)	340 ± 19	112 ± 5*	245 ± 7#

Effects of EET-A treatment on body weight, blood pressure and fasting blood glucose levels. Results are mean ± SE, n=6, *p < 0.05 vs. db/db control, #p < 0.05 vs. db/db mice treated with EET-agonist.

Table 2- Liver NAS score - Degree of steatosis (grade 0 ≤ 5%; 1 = 5-33%; grade 2 = 34%-66%; grade 3 ≥ 66%), lobular inflammation (0: no foci; 1 < 2 foci per 200x field; 2: 2 to 4 foci per 200x field; 3: foci per 200x field), hepatocyte ballooning (0: none; 1: rare or few; 2: many), and fibrosis (0: no fibrosis; 1: perisinusoidal or periportal fibrosis; 2: perisinusoidal and portal/perioral fibrosis; 3: bridging fibrosis; 4: cirrhosis).

NAS pathological score factors	**Db/db CTRL**	**EET-A**	**EET-A+PGC-1α(sh)**
Steatosis	2 (39.44%)	1 (6.83%)	2 (37.4%)
Inflammation	2 (2-4 foci/field)	1 (<2 foci/field)	2 (2-4 foci/field)
Ballooning	2 (many)	1 (rare)	2 (many)
Fibrosis	3 (bridging fibrosis)	0 (no fibrosis)	2 (perisinusoidal and portal fibrosis)
NAS value	**9 NASH**	**3**	**8 NASH**

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Chapter 8

Ablation of Soluble Epoxide Hydrolase expression increases beige fat over white fat through an increase in mitochondrial integrity, HO-1-UCP-adiponectin in vitro and in vivo.

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Abstract

We have shown before that EETs, specifically 11, 12 and 14, 15 EETs, reduce adipogenesis in human mesenchymal stem cells and mouse preadipocytes (3T-3L1). In this is advancement study, we explore the effects of sEH deletion on various aspects of adipocyte-function, including programing for white vs. beige-like fat, and mitochondrial and thermogenic gene-expressions. We further hypothesize that EETs and heme-oxygenase 1 (HO-1) form a synergistic, functional module whose effects on adipocyte and vascular function is greater than the effects of sEH deletion alone. For *in vitro* studies, we examined the effect of sEH inhibitors on hMSC-derived adipocyte. MSC-derived adipocytes exposed to AUDA, an inhibitor of sEH, exhibit an increased number of small and healthy adipocytes, an effect reproduced by siRNA for sEH. *In vivo* studies show that sEH deletion results in a significant decrease in adipocyte size, inflammatory adipokines NOV, TNFα, while increasing adiponectin (P<.0.05). These findings are associated with a decrease in body weight ($p<0.05$), and visceral fat ($p<0.05$). Importantly, sEH deletion was associated with significant increase in Mfn1, COX 1, and thermogenic genes UCP1 and adiponectin ($p<0.03$). sEH deletion was manifested by a significant increase in EETs isomers 5, 6- 8, 9- 11, 12- and 14, 15- EETs and an increased EET/DHETES ratio. Notably, activation of HO-1 gene expression further increased the levels of EETs, suggesting that the antioxidant HO-1 system protects EETs from degradation by ROS. These results provide novel finding that

sEH deletion while increasing EET levels, resulted in the reprograming of white fat to express mitochondrial and thermogenic genes, a phenotype characteristic of beige-fat. Thus, EETs agonist(s) and sEH inhibitors may have therapeutic potential in the treatment of metabolic syndrome and obesity.

Introduction

Metabolic disorders, such as obesity, diabetes, hypertension and cardiovascular disease, are becoming more of an epidemic in recent years. Obesity is characterized by a pathological increase in white fat, associated with high level of inflammation, insulin resistance and mitochondrial dysfunction, contributing to diabetes, hypertension and even heart failure (1). Several studies indicated that browning of white fat can ameliorate obesity and associated metabolic disorders (2). Beige and brown fat, which contains small adipocytes, dissipate energy as heat, resulting in the burning of calories and a potential weight loss in mice and human (3, 4). Therefore, increasing thermogenic genes and mitochondrial function could result in the transformation of white-fat to beige-fat.

Adipose tissue function is controlled by several processes including mitochondrial biogenesis and adaptive thermogenesis, highlighting the essential role of mitochondrial functions in adipocytes. Adipocyte mitochondria are deficient in insulin-resistant individuals and correlate with systemic lipid metabolism, inflammation and insulin sensitivity (7). The function of mitochondrial network depends on quality control, referring to fusion and fission. Mitofusin 1 and 2 (Mfn1 and Mfn2) facilitate the mitochondrial fusion process (8) while COX-1 is related to the mitochondrial oxidative phosphorylation (OXPHOS) (9). Brown adipose tissue is specialized to expend energy as heat by uncoupling respiration with its unique mitochondrial membrane embedded protein UCP1, a process known as non-shivering thermogenesis (10), which increases heat production through an uncoupling oxidative metabolism from ATP production (11).

Epoxyeicosatrienoic acids (EETs) are arachidonic acid derived lipid mediators with beneficial effects on metabolic and cardiovascular health (17). EETs are derived from arachidonic acid by CYP pathway, main being mammalian CYP epoxygenases, which can bend an oxygen atom on double bond, so there are four separate regioisomers of EETs (18). EETs are rapidly degraded by soluble epoxide hydrolase (sEH), which exist in mammalian tissues, including the myocardium, kidney, liver, adipose and blood vessels (19). sEH is

known to play a pro-inflammatory role during inflammation by metabolizing EETs to Pro-diols (20). Our recent research indicated that EETs upregulates the heme-HO system (3, 12, 13), mitochondrial function, as well as thermogenesis which have a profound effect on improving adipocyte function (14). The HO system represents an important protective role in the cardiovascular and metabolic system(s) against the harmful effects of oxidative stress (15, 16).

Clinical studies have shown that sEH gene polymorphism is associated with insulin resistance (IR) (21), and IR is the one of the key feature in obesity. IR is accompanied by increased ROS in adipocyte, release of series of redox and inflammatory mediators, and large, dysfunctional adipocyte (22). Overexpression of sEH in non-diabetic mice resulted in similar vessel abnormalities as diabetic mice with retinopathy (23); whereas, inhibition of sEH has been known to suppress inflammation in cardiovascular disease (24). Recently, we have shown that upregulation of EETs can induce HO-1 expression; leading to a reduction in fatty acid synthesis, convert large adipocytes to small adipocyte and a decrease in body weight (25).

In our present study, we explored the relationship of sEH-inhibition and mitochondrial gene-expression with regard to genes involved in thermogenesis. We have also examined the functional EET-HO-1 module and its effects, if any, on EET production and phenotype of sEH knockout mice. Our results show that sEH knockdown augments pro-thermogenic profile of adipose tissue mitochondria with significantly reduced adipose tissue burden; effects further accentuated by HO-1-induction.

Methods:

Differentiation of human bone marrow-derived MSCs into adipocytes

Frozen bone marrow mononuclear cells were purchased from Allcells (Allcells, Emeryville, CA, USA). After thawing, mononuclear cells were re-suspended in a-minimal essential medium (a-MEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and 1% antibiotic/antimycotic solution (Invitrogen). The cells were plated at a density of 1 to 5 × 10^6 cells per 100-cm2 dish. The medium was replaced with adipogenic medium, and the cells were cultured for an additional 14 days as described previously (27). The cells were treated with 11, 12-EET and 14, 15-EET (1 μM) alone and with sEH inhibitor (AUDA) (1 μM), or with sEH siRNA using an N-TER kit (Sigma-Aldrich, St. Louis, MO) according to manufacturer's protocol.

Oil Red O staining

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

Animal protocols

Male sEH null mice were provided by Dr. Darryl C. Zeldin at age of 12 weeks. Aged-matched B6129SF2/J mice served as controls. Mice were fed a normal chow diet and had free access to water, no difference in food intake was observed between different groups. All mice were divided in to 3 groups: 1) Control; 2) sEH KO male mice; 3) sEH KO + CoPP male mice. Cobalt protoporphyrin IX (CoPP) (3mg/kg once a week) was administered i. P. for 8 weeks. Measurement of body weight was made during the course of the study. At sacrifice, aorta and adipocyte tissues were immediately collected, weighed and stored at -80°C until use. The Animal Care and Use Committee of New York Medical College approved all experiments.

RNA, protein signaling, and EET measurement

After collecting all the samples, we use RT-PCR to measure gene expression in both cells and tissue. Total RNA was isolated using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions (25, 28). Protein signals were measured as previously described (12). EETs were extracted using solid phase C18-ODS AccuBond II 500-mg cartridges (13).

Measurement of superoxide in aorta tissues

Samples were placed in scintillation mini vials containing 5mm of lucigenin and 1 ml of Krebs solution buffered (pH 7.4). Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000IC; Beckman Instruments, San Diego, CA) at 37°C; data are reported as counts/min/mg protein after background subtraction.

Statistical analysis

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

Results:

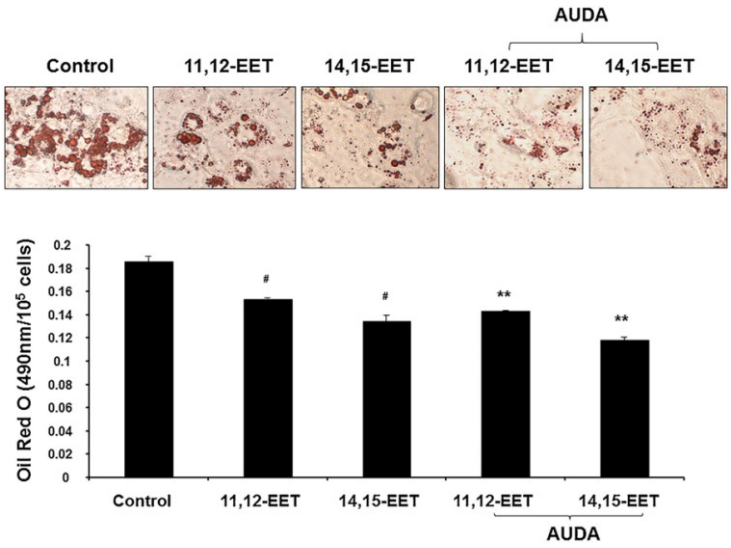

Fig. 1. Effect of EETs and sEH inhibition on oil droplets formation in MSCs derived adipocytes. We measured the effect of EETs administration and suppression of sEH on adipogenesis. Daily supplementation of 11, 12-, and 14, 15-EETs was effective on adipogenesis suppression at 14 days. Inhibition of sEH with AUDA, showed a significant ($p < 0.05$) and synergistic reduction of lipid droplets formation in MSC-derived adipocytes. ($n = 4$), #$p < 0.05$ vs. control, ** $p < 0.05$ vs. control.

Effect of EETs and sEH inhibition on oil droplets formation in MSCs derived adipocytes

To elucidate the role of EETs in the regulation of adipogenesis during MSCs differentiation to adipocyte lineage, we measured the effect of EETs administration and suppression of sEH on adipogenesis. Daily supplementation of 11, 12-, and 14, 15-EETs was effective on adipogenesis suppression at 14 days. Inhibition of sEH with AUDA, showed a significant ($p<0.05$) and synergistic reduction of lipid droplets formation in MSC-derived adipocytes (Figure 1).

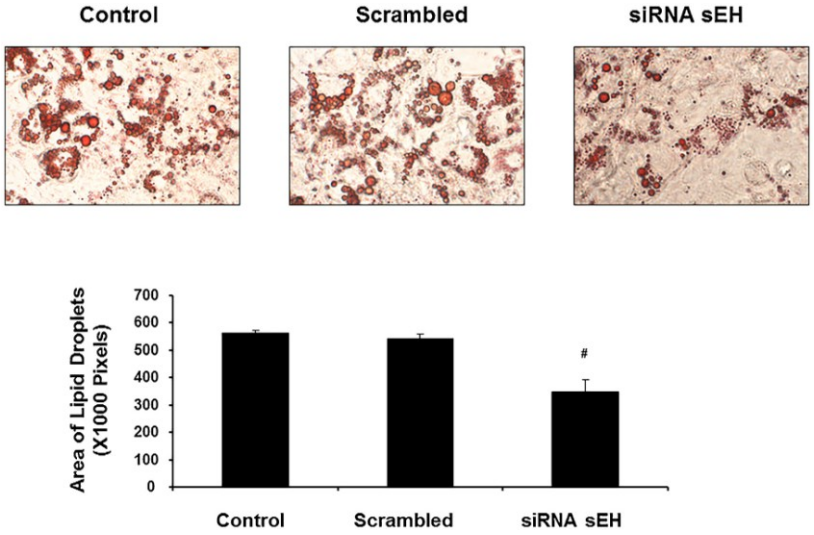

Fig. 2. Effect of sEH deletion on oil droplet formation. Ablation of sEH significantly ($p < 0.05$) decreased lipid droplets size in MSC derived adipocytes. The percentage of cells with morphological lipid droplets decreased following silencing sEH. The effect of siRNA on adipogenesis was determined by counting cells with lipid droplets in the cytoplasm and cells positive for the lipid-specific dye. The adipocyte cell size in the absence of siRNA was ± pixels compared with ± pixels in the presence of siRNA. ($n = 4$), #$p < 0.05$ vs. control.

Effect of sEH deletion on oil droplet formation

As shown in Figure 2, ablation of sEH significantly ($p<0.05$) decreased lipid droplets size in MSC derived adipocytes. The percentage of cells with morphological lipid droplets decreased following silencing sEH. The effect of siRNA on adipogenesis was determined by counting cells with lipid droplets in the cytoplasm and cells positive for the lipid-specific dye. The adipocyte cell size in the absence of siRNA was ± pixels compared with ± pixels in the presence of siRNA (Figure 2).

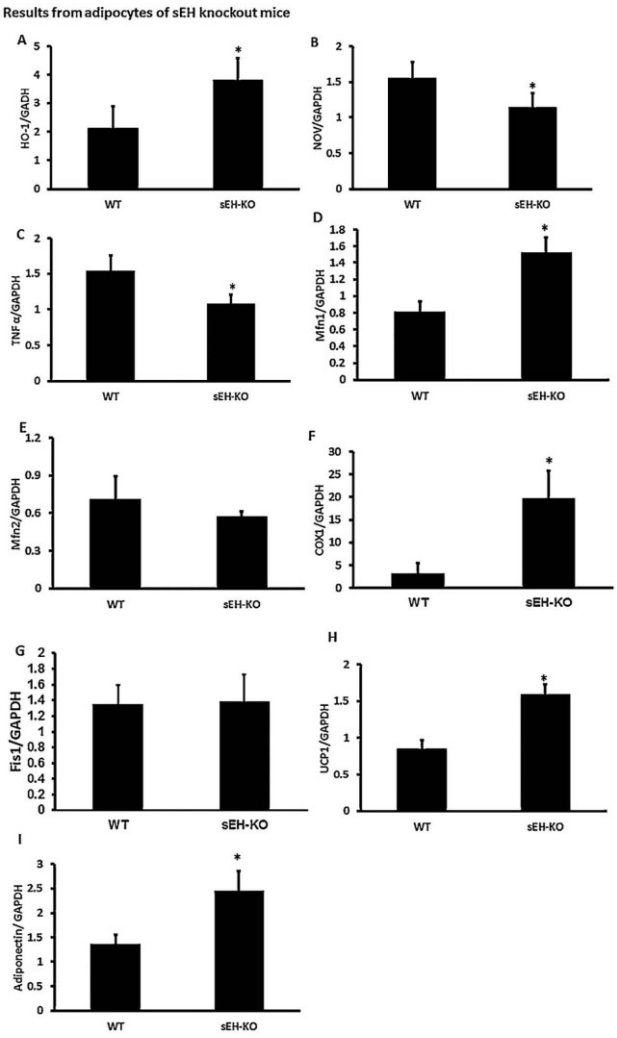

Fig. 3. Effect of sEH knockout on Inflammatory Cytokine levels in adipose tissue and mitochondria. RT_PCR analysis showed significant ($p < 0.05$) increase in HO-1 mRNA expression in adipose tissue of sEH KO mice as compared to WT control mice (Fig. 3A), the mRNA expression of inflammatory cytokines such as NOV and TNFα were significantly lower in sEH mice compared to control (Fig. 3B and 3C). These results indicate that inhibition of sEH leads to HO-1 induction resulting in a decrease of inflammation. We measured Mfn1 and 2 levels in adipose tissue of sEH KO mice. The results showed a significant ($p < 0.05$) increase in Mfn1 mRNA expression in sEH KO mice compared to control. However, there is no significant change in Mfn2 expression (Fig. 3D and E). Furthermore, levels of cytochrome c oxidase subunit I (COX-I) were significantly higher in KO mice (Fig. 3F), and expression of FIS1 was not significantly different between groups (Fig. 3G). Moreover, adiponectin mRNA expression significantly increased in sEH KO mice (Fig. 3H and I). ($n = 4$), * $p < 0.05$ vs.WT.

Effect of sEH knockout on Inflammatory Cytokine levels in adipose tissue

RT_PCR analysis showed significant ($p<0.05$) increase in HO-1 mRNA expression in adipose tissue of sEH KO mice as compared to WT control mice (Figure 3A), the mRNA expression of inflammatory cytokines such as NOV and TNFα were significantly ($p<0.05$)

lower in sEH KO mice compared to control (Figure 3B and 3C), These results indicate that inhibition of sEH leads to HO-1 induction resulting in a decrease of inflammation.

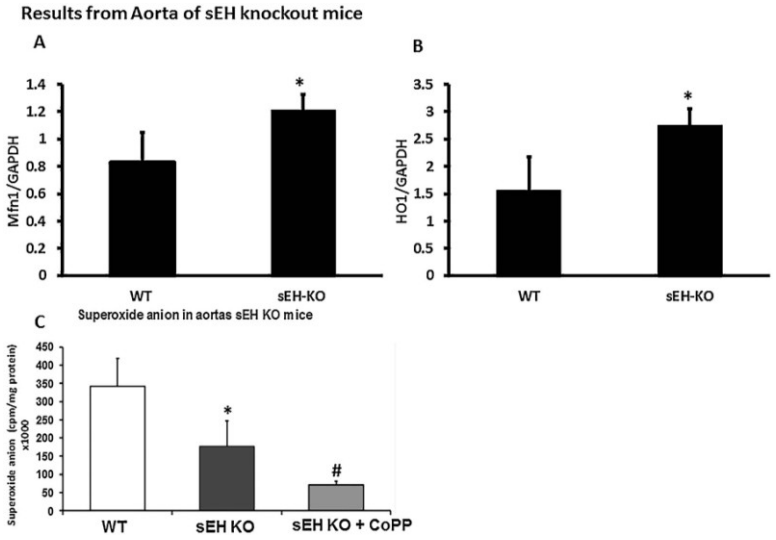

Fig. 4. Effect of sEH knockout on Mitochondrial Function in aorta. In agreement with the results from the fat tissue, we observed a significant ($p < 0.05$) increase in HO-1 (Fig. 4B) as well as in Mfn1 (Fig. 4A) expression in the aortas of sEH KO mice as compared to control. These results indicate that inhibition of sEH resulted in an increase in mitochondrial function. Ablation of sEH and HO-1 induction significantly reduced superoxide anion formation in aortas of mice compared to both KO sEH and WT control (Fig. 4C). (n = 4), * $p < 0.05$ vs.WT, #$p < 0.05$ vs sEH KO.

Effect of sEH knockout on Mitochondrial Function and Thermogenic Genes

Mitochondrial fusion is essential for mitochondrial function, therefore, to examine whether sEH can modulate the mitochondrial fusion-to-fission ratio, we measured Mfn1 and 2 levels in adipose tissue of sEH KO mice. The results showed a significant ($p<0.05$) increase in Mfn1 mRNA expression in sEH KO mice compared to control. However, there is no significant change in Mfn2 expression (Figure 3D and E). Furthermore, levels of cytochrome c oxidase subunit I (COX-I) were significantly ($p<0.05$) higher in KO mice (Figure 3F), and expression of FIS1 was not significantly different between groups (Figure 3G). Moreover, adiponectin mRNA expression significantly increased in sEH KO mice (Figure 3H and I). In agreement with the results from the fat tissue, we observed a significant ($p<0.05$) increase in Mfn1 (Figure 4A) as well as in HO-1 (Figure 4B) expression in the aortas of sEH KO mice as compared to control. These results indicate that inhibition of sEH resulted in an increase in mitochondrial function. To investigate the effect of sEH deletion on lipid metabolism, we analyzed the expression of thermogenic markers

which characterize brown adipocytes. Our analysis demonstrated that UCP1 mRNA expression levels were significantly increased in sEH KO mice compared to control mice.

Effect of sEH ablation and HO-1 induction on Superoxide Formation

Ablation of sEH and HO-1 induction significantly reduced superoxide anion formation in aortas of mice compared to both KO sEH and WT control (Figure 4C).

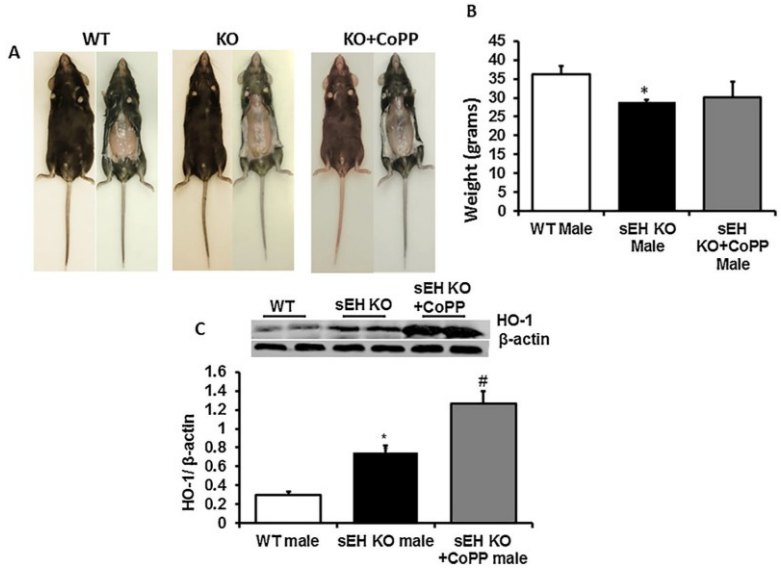

Fig. 5. Effect of sEH ablation with and without HO-1 Inducer on Adiposity. Deletion of sEH decreased body weight in male null mice compared to control. Picture of mice indicated that sEH KO mice are leaner than control group (Fig. 5A). Body weight of KO mice were 28.8 ± 7.28 g compared with 36.17 ± 0.77 g of control mice (Fig. 5B). To further increase the antioxidant functions of EETs, we treated sEH KO mice with a well-known HO-1 inducer (CoPP). Western blot analysis showed significant ($p < 0.05$) increase in HO-1 protein expression in adipose tissue of sEH KO mice as compared to WT control mice, this increase was even more significant when CoPP treatment was given to sEH KO mice (Fig. 5C).The body weight in sEH null mice was similar to sEH + CoPP group. (n = 4), * $p < 0.05$ vs.WT, #$p < 0.05$ vs sEH KO.

Effect of sEH ablation with and without HO-1 Inducer on Adiposity

As seen in Figure 5, deletion of sEH decreased body weight in male null mice compared to control. Pictures of mice indicated that sEH KO mice are leaner than control group (Figure 5A). Body weight of KO mice were 28.8±7.28 g compared with 36.17±0.77 g of control mice (Figure 5B). visceral fat accumulations of KO mice were significantly reduced compared to control mice (Figure 5C). To further increase the antioxidant functions of EETs, we treated sEH KO mice with a well-known HO-1 inducer (CoPP). Western blot analysis showed significant ($p<0.05$) increase in HO-1 protein expression in adipose tissue of sEH KO mice as compared to WT control mice , this increase was even more significant

when CoPP treatment was given to sEH KO mice (Figure 5C). The body weight in sEH null mice was similar to sEH + CoPP group.

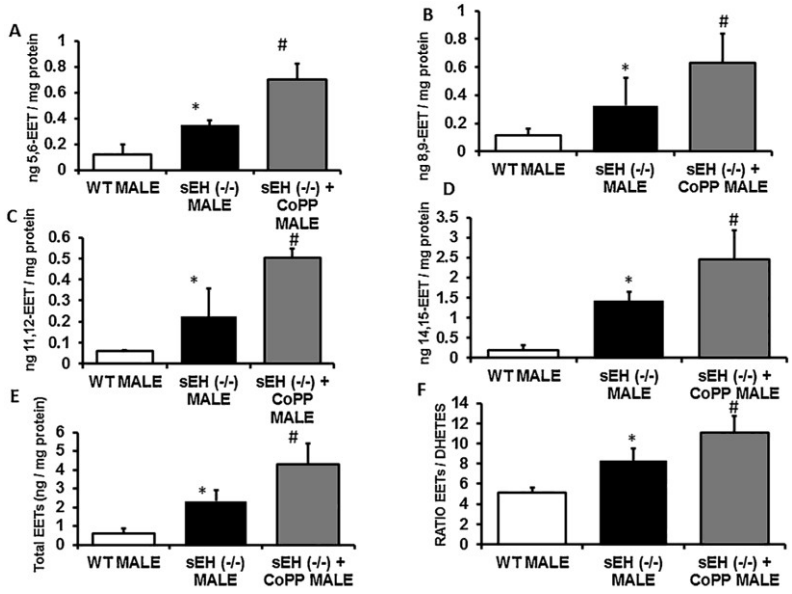

Fig. 6. Effect of sEH ablation and HO-1 induction on EETs levels. The levels of isoforms of EETs (5, 6-EET, 8, 9-EET, 11, 12-EET, 14, 15-EET) and the total EETs (2.32 ± 0.22 mg vs. 0.61 ± 0.06 mg) levels were significantly ($p < 0.05$) increased in sEH KO mice compared to control mice. Since not all EETs are released as functional EETs, we decided to inject CoPP, a potent HO-1 inducer, to KO mice. As a result, we observed that administration of CoPP lead to a significant increase in all forms of EETs (5, 6-EET, 8, 9-EET, 11, 12-EET, 14, 15-EET) as well as total EETs levels compared to control mice and sEH KO without CoPP (Fig. 6A–F) (n = 4), * $p < 0.05$ vs.WT male, #$p < 0.05$ vs sEH (−/−) male.

Effect of sEH ablation and HO-1 induction on EETs levels

The levels of isoforms of EETs (5, 6-EET, 8, 9-EET, 11, 12-EET, 14, 15-EET) and the total EETs (2.32±0.22mg vs. 0.61±0.06mg) levels were significantly ($p<0.05$) increased in sEH KO mice compared to control mice. Since not all EETs are released as functional EETs, we decided to inject CoPP, a potent HO-1 inducer, to KO mice. As a result, we observed that administration of CoPP lead to a significant increase in all forms of EETs (5, 6-EET, 8, 9-EET, 11, 12-EET, 14, 15-EET) as well as total EETs levels compared to control mice and sEH KO without CoPP (Figure 6 A-F)

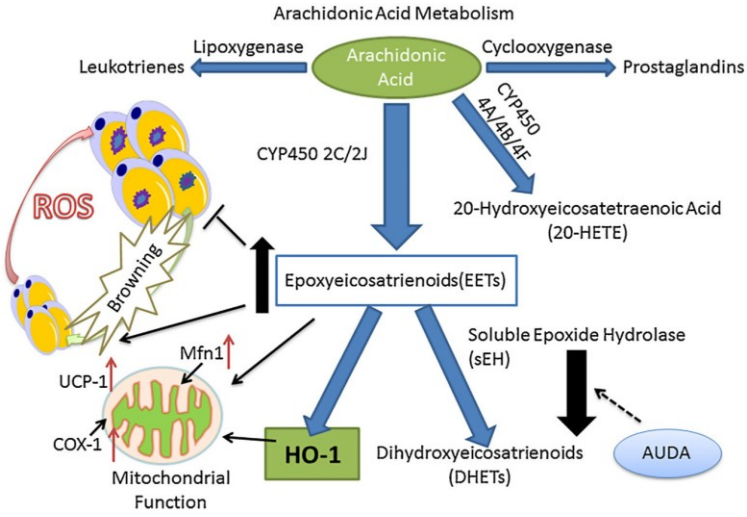

Fig. 7. Schematic Representation of the Effect of EET and HO-1 on ROS, mitochondrial function and thermogenesis. AA-derived epoxides (Epoxyeicosatrienoic acid, EET) through CYP pathway could rapidly degrade EET into less active DHET by soluble epoxide hydrolase (sEH), and inhibitions of sEH could upregulate EET concentration and induce HO-1 activity. Stem cell derived small adipocytes turned into large adipocyte with the upregulated level of inflammation and ROS with mitochondrial dysfunction, while EET-HO correlated with decreased inflammation and reduce ROS, paralleled by the size decrease of adipocytes to prevent adipocyte differentiation. EET and HO-1 could also improve mitochondrial function by upregulating Mfn1 and COX-1, associated with an adaptive thermogenesis. All these benefits will result in to ameliorate adipocyte associated diseases such as obesity and cardiovascular diseases.

Discussion:

This study demonstrates that EETs improve adipocyte structure and function via upregulation of mitochondrial thermogenic genes, and restore redox and inflammatory balance in adipose tissues. We also establish the operational nature of the HO-EET synergistic module by demonstrating elevated EET levels in sEH KO mice treated with CoPP and by improved phenotypic outlook in these mice.

The first key finding of the study is the rescue of adipocyte function by sEH inhibitors and knockdowns. our results show that the production of pro-inflammatory adipokine, NOV, is attenuated by sEH deletion as well as by HO-1 induction. We also found that the level of Mfn1 increased in sEH KO mice. Furthermore, there was an increase of COX-1 in the adipose tissue of KO mice indicating that sEH deletion plays a crucial role in mitochondrial integrity. Previous studies indicate that HO-1 induction decreases superoxide formation as well as controls adiposity in transgenic mice (29). In our current study we showed that

increasing of EETs through inhibition of sEH leads to diminished MSC-derived adipocyte terminal differentiation, decreased lipid droplet formation and inhibition of adipogenesis. In agreement with our previous results, inhibition of sEH in adipocyte can turn large unhealthy adipocytes into small healthy adipocyte. This is further strengthened by the fact that inhibition of sEH increases UCP1 expression, suggesting a transformation of white fat to beige or brown fat.

The second key finding of this study derives from our in vivo experiments where we found that ablation of sEH decreased the degradation of EETs leading to antioxidants, anti-inflammatory and lipid lowering effects, as well as the body size and weight reduction of sEH KO mice, both with and without treatment with a HO-1 inducer. Obesity and metabolic syndrome, characterized by body weight gain, low level inflammation and oxidative stress overexpression, is associated with hyperlipidemia, insulin resistance and cardiovascular diseases (1). Recent research found that white fat increased in obesity and is accompanied with inflammation (1), and the amelioration of renal inflammation will reduce hypertension and type II diabetes (31). A chronic high fat diet increased expression of adipose sEH protein in mice (32), which could be translated to humans because adipose sEH expression is increased in obesity. A high fat diet (for 20 weeks) does not change sEH expression, but elevates sEH activity (33). Inhibition of sEH, which decreased the degradation of EETs, decreased inflammatory and lower lipid accumulation (34). Our results also suggested that sEH knockout mice have a significant body weight reduction associated to an increase of EETs concentration. We can also observe a decrease of inflammatory cytokines NOV and TNF-α compared to WT mice. Taking all of the above information along with the decrease in adipogenesis, paralleled by the size decrease of adipocyte, sEH could have a favorable clinical outcome for the treatment of obesity and associated metabolic disorders.

sEH is upregulated in obesity and diabetes, associated with a decrease of EETs levels (38). Increasing level of EETs was associated with a reduction adipocytes size in visceral adipose tissue, and it can improve glucose tolerance and insulin sensitivity, attenuate hypertension (25, 39, 40). Furthermore, EETs has previously been shown to increase expression of HO-1 in adipose tissue. Previously, it has been shown that HO-1-mediated antioxidant mechanisms can decrease levels of ROS through the increase of adiponectin (41). The secretion of adiponectin is linked to increased mitochondrial function, increase in thermogenic genes, and an inhibition of adipogenesis (42).

Our previous studies indicated that upregulation of EETs can induce HO-1 expression and HO activity (14, 27, 30). As CoPP-mediated induction of HO-1 attenuated inflammatory markers and hypertension, and decreased circulating free fatty acids and C-reactive protein and increased adiponectin through the activation of the AMPK-P13K- eNOS pathway (43). Furthermore, based on our study, sEH KO with CoPP administration resulted in higher EETs levels, which illustrates the connection between HO-1 and EETs. HO-1, which is an antioxidant, decreased superoxide anions in both aorta and adipocyte tissue. It should be noted that HO-1-mediated antioxidant mechanism decreased levels of ROS with increased levels of adiponectin, which when secreted from adipocytes, can improve insulin action and reduce atherosclerotic processes (44). The secretion of adiponectin is linked to mitochondrial function, and exerts anti-hyperglycemic effect (42). In this study, we show that the level of adiponectin in sEH KO mice increased, which indicates that there is an increase of brown fat, associated with a decrease of oxidative stress and inflammation.

The third key finding is that inhibition of sEH could prevent adipocyte differentiation, which can moderately decrease ROS and inflammation and lead to an increase in mitochondrial function, however when coupled with an HO-1 inducer (CoPP), ROS and inflammation are fully attenuated. ROS are mostly generated from the mitochondrial respiratory chain, and reduce molecular oxygen to form the superoxide anion (45), which decreased dramatically in sEH KO mice and more dramatically after administration of CoPP. We also found that the level of Mfn1 increased while Mfn2 was unchanged in sEH KO mice. Furthermore, there was an increase of COX-1 in the adipose tissue of KO mice, which marks an increase in mitochondrial function. Adaptive thermogenesis and mitochondrial function are correlated to adipocyte differentiation, and UCP-1 was also significantly upregulated in sEH KO mice. All the results in our study demonstrated the effect of sEH inhibition in reduction of inflammatory markers, and decrease in ROS, which in return could redirect more white fat into brown fat.

From a clinical perspective, ablation of soluble epoxide hydrolase expression increases beige fat over white fat through an increase in mitochondrial integrity and HO-1-adiponectin. Our study suggests that sEH inhibition can suppress adipogenesis in adipose tissue, prevent adipogenic lineage, furthermore the EETs-HO system participates in the regulation of the level of inflammation and ROS formation, so as to ameliorate associated diseases such as obesity, diabetes, cardiovascular diseases. Hence, inhibition of sEH could

be a novel pharmacologic therapeutic approach to prevent and even reverse obesity and associated metabolic disorders.

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Chapter 9

Beneficial Effects of Thymoquinone on Metabolic Function and Fatty Liver in a Murine Model of Obesity

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Abstract

Aim: Nigella Sativa seeds contain a high amount of Thymoquinone (TQ), an antioxidant.. We therefore hypothesized that Nigella Sativa oil would, through the antioxidant properties of Black seed oil cold press with 3% TQ , ameliorate obesity-induced hyperglycemia, and decrease blood pressure and OX-LDL in obese mice. ***Methods:*** Commencing at eight weeks of age, C57B16 male mice were fed a high fat diet (HF) for 20 weeks. Mice were divided into four treatment groups of five animals each as follows: group 1) Lean, group 2) HF diet, group 3) HF diet treated for the last 8 weeks with normal vegetable oil (N-Oil), and group 4) with TQ. Blood biomarkers, antioxidant biomarkers, mitochondrial function and tissue fat accumulation were determined. ***Results:*** TQ treatment resulted in an increase of energy-dependent genes and oxygen consumption ($P<0.05$) as compared to N-Oil and untreated (CA) oil. TQ decreased fasting glucose levels and blood pressure ($P<0.05$) compared to CA oil. TQ increased the levels of hepatic HO-1, mitochondrial Mfn2, insulin receptor phosphorylation and decreased OX-LDL ($P<0.05$) and haptic apoptosis. ***Conclusions:*** These results suggest that TQ attenuated the obesity-mediated decrease of oxygen consumption, improved mitochondrial biogenesis and through increased levels of HO-1 ablated HF-induced OX-LDL. Our findings indicate a potential clinical role of TQ in the prevention of obesity-related metabolic disease.

Introduction

According to the World Health Organization, annually over 2 million people die worldwide from the complications of excessive body fat. An altered adipose tissue function is characterized by an impaired lipid buffering capacity and subsequently by a systemic lipid over flow and ectopic lipid accumulation in several insulin sensitive peripheral tissues such as skeletal muscle, liver, pancreas, heart and kidneys [1,2]. This obesity trend is followed in men and women, both having a similar pattern, being around 40-45% obese in middle age around 35% obese when younger [3]. The ectopic deposition of triglycerides triggers a series of cardiometabolic perturbations, which are grouped into a diagnosis of metabolic syndrome (MetS). This disorder is not only associated with a higher risk of appearance of type 2 diabetes and cardiovascular events but impacts the liver [4]. Recent data suggest that nonalcoholic fatty liver disease (NAFLD), considered the hepatic manifestation of the MetS, precedes the development of MetS [5]. NAFLD is associated with a number of metabolic diseases including diabetes mellitus, obesity and hypertension. In a five-year retrospective review, individuals with NAFLD had a higher incidence of impaired fasting glucose and type 2 diabetes mellitus (T2DM) compared with NAFLD-free controls [6]. In the last few decades, a higher frequency of obesity, T2DM, and MetS have occurred as a result of various dietary changes [7]. Furthermore, individuals with NAFLD have a higher probability of liver failure and, eventually, cirrhosis [8-10]. Epidemiological results suggest that insulin resistance is a common pathogenic factor for all these obesity-related conditions and that it can be both reversed and prevented by a healthy lifestyle and a wholesome diet [11]. In this regard, beneficial effects have been reported for curcumin and Resveratrol [12] which increase the antioxidant gene and heme oxygenase-1 (HO-1). Resveratrol upregulates HO-1 expression, NAD(P)H, quinone oxidoreductase 1, through activation of nuclear factor (erythroid-derived)-like 2 (Nrf2) target genes. Resveratrol as well as other HO-1 inducers prevent CVD [13]. Importantly, HO-1 induction is regulated by levels of glucose; while glucose deprivation induces HO-1 gene expression [14], elevated levels of glucose suppress HO-1 gene expression [15,16]. HO-1 levels is affected by obesity and glucose levels (reviewed in [3,17]). Thymoquinone, present in *Nigella sativa* (NS), has been proposed, based on its anti-oxidant properties, as a protective factor against several metabolic diseases. *Nigella sativa Linn.* (family Ranunculaceae), commonly known as black seed or black cumin, is an herbal plant that has been cultivated for thousands of years in the Middle and South East Asia. Black cumin seed is composed of fixed (stable) and essential

(volatile). The Essential oil extracted from black cumin contains a rich volatile fraction comprising Thymoquinone (TQ) and Thymohydroquinone (THQ) [18,19]. Thymoquinone (TQ) is the main pharmacologically active compound of NS and is thought responsible for many therapeutic properties, including anti-inflammatory, antioxidant and anti-hyperglycemic effects. The protective effect of TQ is related to its ability to scavenge reactive oxygen species (ROS), including superoxide and hydroxyl free radicals [20], to block lipid peroxidation and to enhance levels of antioxidant enzymes [21,22]. The aim of this study was to demonstrate the effects of black seed oil, with a high content of TQ, on the metabolic profile, including adipose-mediated release in inflammatory adipokines such as NOV, mitochondrial biogenesis, LDL, Ox-HDL and hepatic steatosis in a murine model of obesity.

Materials and Methods

Animal protocols

Eight-week-old C57B16 male mice were fed western diets with 51% fat content while control mice fed regular diets, high fat diets (Harlan, Teklad Lab animal diets, Indianapolis, IN) (HFD) for 20 weeks. Mice were divided into three treatment groups of five animals each as follows: group 1) Lean, group 2) HFD, group 3) HFD treated for the last 8 weeks with HFD treated for the last 8 weeks with black seed-cold press oil formulation containing thymoquinone (TQ) between 3-3.1% obtained from TriNutra Israel. Formulation of TQ oil is as follows; TQ 3.14 %, p-Cymene, 1.24%, Carvacrol 0.08%, FFA 1,29%, Oleic Acid 21.53%, palmitic acid 11.31%, linoleic acid 57.44%, other fatty acid 1.98% and TPGS, 0.8%. TQ oil was mixed into the HFD food and made into pellets using a mixer. At the end of the experiment, mice were euthanized, assessed for total body weight, fat content and liver fibrosis. All animal experiments followed the NYMC IACUC institutionally approved protocol in accordance with NIH guidelines.

Fasting blood glucose, glucose tolerance testing

Fasting blood glucose and glucose tolerance were measured from tail blood following a 6 h fast. Blood pressure was measured by the tail-cuff method using the CODA tail-cuff System (Kent Scientific, CT, Torrington) as we previously described [23-25].

Determination of oxygen consumption The C57 mice groups were allowed to acclimatize in the oxygen consumption chambers over a three-week period. Adaptation periods for the three-week duration were executed in two-hour increments, three times a week. The Oxylet gas analyzer and air flow unit (Oxylet, Panlab-Bioseb, Vitrolles, France) were used to determine mouse oxygen consumption (VO2). Each mouse was placed individually in the machine and VO2, VCO2 and respiratory quotient (RQ) was calculated as VCO2/VO2. The data for VO2 are expressed as the consumed oxygen per Kilogram body weight per minute (ml/kg/min) [23-25].

Measurement of HO activity

Liver microsomal HO activity was assayed by the method of Abraham et al. in which liver tissues was homogenates in phosphate buffer, pH 7.8, 0.1 mM EDTA and 1mM PMSF. HO activity was measured in presence of 20 uM heme, glucose 6 phosphate (G-6P), glucose 6 phosphate dehydrogenase (G6PDH), NADPH, at 37°C for 60 minutes. Bilirubin, the product of HO degradation was extracted with chloroform, spin down and leave overnight in the freezer. Samples defrost;

spin the samples for 20 minutes and with pasture pipets remove the lower layer which has chloroform. Bilirubin concentration in chloroform determined spectrophotometrically (Perkin-Elmer (Norwalk, CT) Dual UV/VIS Beam Spectrophotometer) using the difference in absorbance at wavelength from λ 460 to λ 530 nm with an absorption coefficient of 40 mM-1 and cm-1.

Western blot analysis

For protein expression analyses, liver tissues were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (CompleteTM Mini and PhosSTOPTM, Roche Diagnostics, Indianapolis, IN) Frozen mouse adipose tissue was ground under liquid nitrogen and suspended in homogenization buffer (comprising mmol/L :10 phosphate buffer, 250 sucrose, 1.0 EDTA, 0.1 PMSF and 0.1%v/v tergitol, pH 7.5). For *In vitro* Western blot analysis pelleted cells were lysed and HO-1, HO-2, OPA1, MFN1, MFN2 and NOV proteins were measured. Protein detection was carried out using a secondary infrared fluorescent dye conjugated antibody absorbing at both 800 nm and 700 nm. The blots were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor Science Tec) and quantified by densitometric analysis performed after normalization with β-actin. Results were expressed as arbitrary units (AU). Histopathological examination of liver tissue Liver samples from each experimental group were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin wax, and sectioned (6 μm thick). The main liver histopathological features commonly described in NAFLD including steatosis, inflammation, hepatocyte ballooning, and fibrosis were scored according to the NAFLD histologic activity score (NASH) system, and lipid droplet analysis was performed as previously described [26].

Cell culture and adipocyte cell differentiation

3T3-L1 murine pre-adipocytes, were purchased from American Type Culture Collection (Rockville, MD, USA). After thawing, 3T3- L1 cells were resuspended in DMEM, supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic solution (Invitrogen). The medium was replaced with adipogenic medium, and the cells were cultured for an additional 6 days. Differentiating 3T3-L1 pre-adipocytes were treated for 6 days with 3% TQ (2, 4, 6 M).

Oil red O staining

Staining was performed using 0.21% Oil Red O in 100% isopropanol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, adipocytes were fixed in 10% formaldehyde, stained with Oil Red O for 10 minutes, rinsed with 60% isopropanol (Sigma-Aldrich), and the Oil Red O eluted by adding 100% isopropanol for 10 minutes and the optical density (OD) measured at 490 nm, for 0.5 sec reading.

Statistical analysis

Data are expressed as means ± S.E.M. Bonferroni’ s post -test analysis for multiple comparisons was used to calculate the significance of mean value differences using one-way analysis of variance. The null hypothesis was rejected at $p<0.05$.

Results

Figure 1

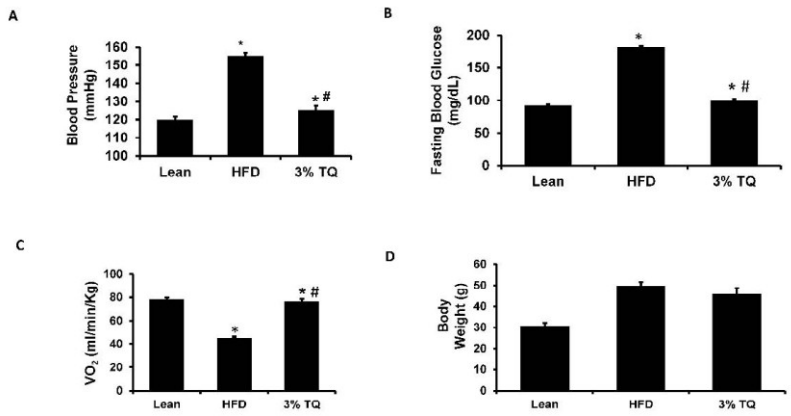

Figure 1: Effect of TQ on blood pressure, blood glucose, oxygen consumption and body weight. Results are mean +/- SE n=6,*p<0.05 *vs* lean mice, #p<0.05 *vs* HFD mice

Effects of TQ on body weight, blood pressure, fasting blood glucose and oxygen consumption

We examined the effect of TQ in mice fed a HFD for 20 weeks (Figures 1). Blood pressure and fasting blood glucose levels were increased in mice fed a HFD as compared to control animals (Figure 1A and 1B). TQ reduced blood pressure and fasting blood glucose levels in mice fed a HFD. Mice on a HFD displayed a decrease in VO2 consumption. In contrast, TQ produced a significant ($p<0.05$) increase in oxygen consumption (Figure 1C). As shown in Figure 1D, weight of the HFD group was increased ($p<0.05$) compared to Lean, but no difference occurred between the HFD and TQ groups.

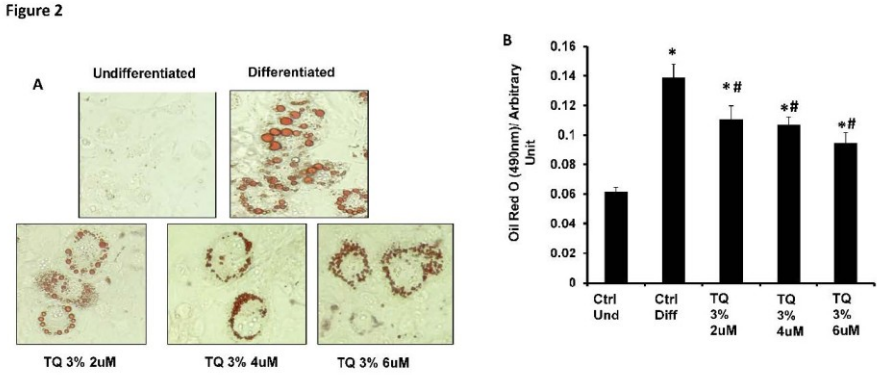

Figure 2: Effect of TQ on oil droplets formation in 3T3 adipocytes. We measured the effect of 3% TQ administration on adipogenesis. Daily supplementation of TQ was effective on adipogenesis suppression at 6 days. TQ treatment showed a significant ($p<0.05$) reduction of lipid droplets formation in 3T3 adipocytes. (n=4), #$p<0.05$ *vs.* control, ** $p<0.05$ *vs*. control.

Effect of TQ on adipogenesis *in vitro*

TQ decreased large lipid droplet content in differentiated adipocytes compared with differentiated control cells ($p< 0.05$) (Figure 2A and 2B). Furthermore, TQ3 3% decreases oil lipid accumulation seen clearly between differentiation cell and cells treatment with TQ 3% at 6 µM, suggesting that TQ decreased adipocyte terminal differentiation preventing the conversion of small "healthy" adipocytes to large adipocytes. TQ decreased lipid content in a dose-dependent manner (Figure 2B). Sardana and Kappas reported that the increase in HO-1mRNA and protein are several orders of magnitude higher than the increase in liver HO activity [27], therefore, we measured the consequence of TQ treatment on liver HO activity and generation of bilirubin anti-oxidant effect. Since HO-1 converts heme to equimolar amounts of CO and bilirubin (20), we measured HO activity by formation of bilirubin. HO activity in control liver tissues was 0.81 ± 0.16 nmol bilirubin formed/mg protein/hour and decreased to 0.49 ± 0.12 nmol bilirubin formed/mg protein/hour in high fat liver ($p<0.05$) (Figure 2B-3F). The stimulatory effect of 3%TQ on HO-1 protein was associated with an increase in HO activity (Figure 3G) to 0.78 ± 0.12 nmol bilirubin/mg/hour ($p<0.05$).

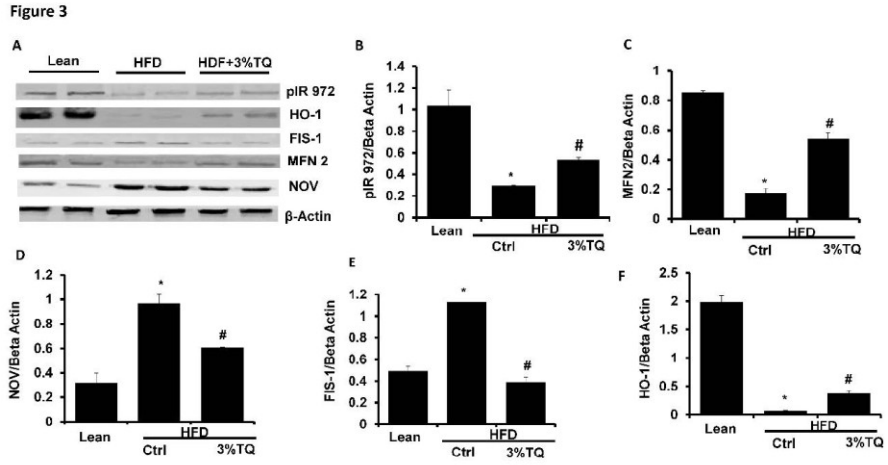

Figure 3: Effect of TQ administration on levels of HO-1, Fis1, MFN2, NOV and pIR972 in adipose tissue on Lean, HFD and HFD +3% TQ. Representative western blots; (A) and densitometry analysis of (B) pIR972, (C) MFN2, (D) NOV, (E) Fis1, (F) HO-1 of Lean, HFD and HFD +3% TQ. Results are mean ± SE, n=6, *p<0.05 *vs*. Lean, #p<0.05 *vs.* HFD.

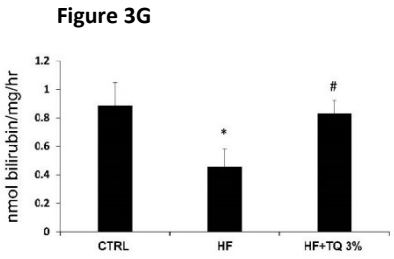

Figure 3G: HO activity in control, HF untreated and HF-treated with 3%TQ treated mice. HO activity was determined and results are mean±SE, n= 3, *p<0.05 *vs.* control, # p<0.05 *vs.* HF mice

Effects of TQ on protein expression in adipose tissue

Western blot analysis of fat tissue showed significant differences in protein expression levels of pIR972, HO-1, Fis-1, Mfn2 and NOV in obese mice compared to control mice. Untreated obese animals exhibited a significant (p<0.05) decrease in insulin receptor phosphorylation levels and HO-1 when compared to age-matched lean mice. TQ increased both pIR972 mitochondrial fusion protein and HO-1 levels in obese mice (Figure 4A-4E). A HFD resulted in a decrease in Mfn2 (p<0.05) and an increase in FIS-1 a fission protein (p<0.05). TQ treatment reversed the negative effect on mitochondrial protein as seen by the

increased in the levels of MFN2 ($p<0.05$) and decreased FIS-1 ($p<0.05$) compared to HF mice (Figure 4B and 4D). As seen in Figure 4D, levels of adipose tissue derived NOV, a pro-inflammatory protein in lean group are significantly ($p<0.05$) lower than in the HFD group. As shown in Figure 4C, TQ treatment decreased NOV protein expression compared to mice fed a HFD alone.

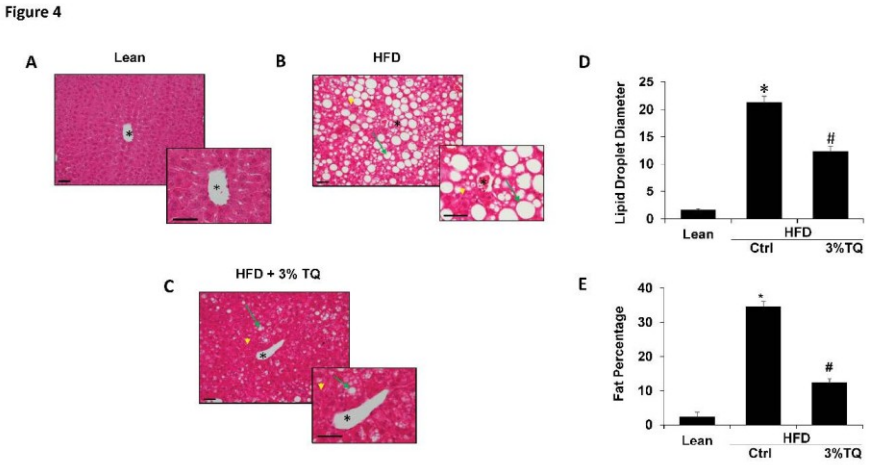

Figure 4: Haematoxylin-eosin staining of liver of lean (A), HFD (B), HFD treated with 1% TQ (C), and HFD treated with 3% TQ (D) mice. Graphs summarize the morphometrical analysis of liver lipid droplet diameter (F) and adipose tissue percentage (G). * $p< 0.05$ versus lean; # $p< 0.05$ versus HFD. Bar 50 μm. Yellow arrowheads show inflammatory cells, green arrows indicate adipose tissue and * denote centrolobular vein.

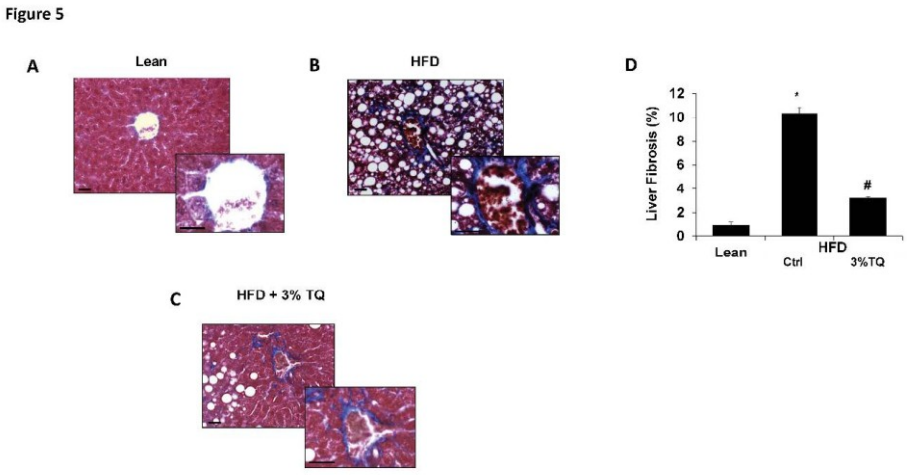

Figure 5: Masson's trichrome staining of liver and presence of fibrosis, lean (A), HF diet (B), HFD treated with 1% TQ (C), and HF diet treated with 3% TQ (D) mice. Graph summarizes the morphometrical analysis of fibrosis percentage (F). *$p<0.05$ *versus* lean; # $p<0.05$ *versus* HFD. Bar 50 μm.

TQ intervention decreased level lipid, steatosis and fibrosis

Liver of lean mice exhibited no significant steatosis, no inflammatory foci and no fibrosis (Figures 4 and 5). Livers of HFD mice had elevated steatosis, moderate lobular inflammatory loci, hepatocyte ballooning, and fibrosis. Lipid content (Figure 4) was significantly increased ($p<0.05$) in mice fed a HFD as compared to control mice. TQ treatment decreased lipid content as compared to mice on a HFD alone. Morphometric analysis of liver lipid droplets showed that TQ decreased lipid droplet diameter compared to the HFD group ($p<0.05$). As seen in Figure 5, non-treated HFD mice display more fibrosis than HFD mice treated with TQ. TQ reduced HFD-induced fibrosis and collagen deposition ($p<0.05$).

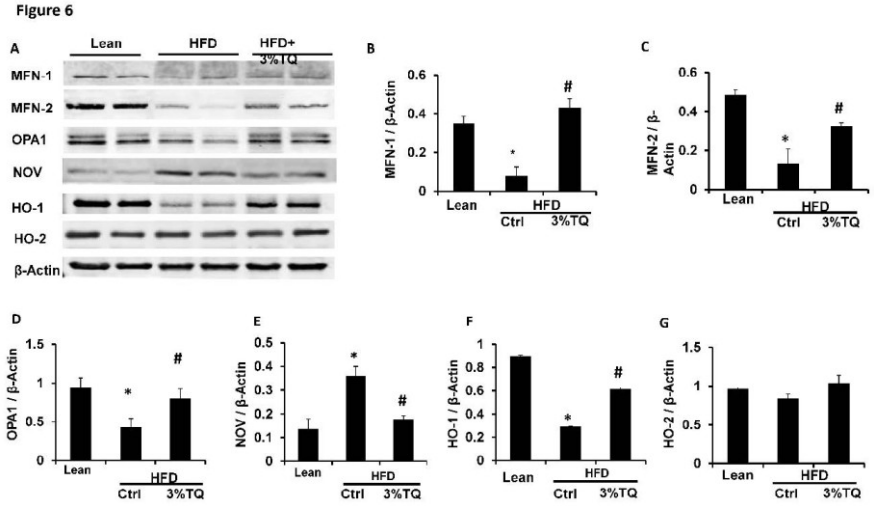

Figure 6: Effect of TQ administration increases mitochondrial function, antioxidant HO-1 and decreases cytokine NOV in liver tissue on obese mice. Representative western blots; (A) and densitometry analysis of (B) MFN1, (C) MFN2, (D) OPA1, (E) NOV, (F) HO-1 and (G) HO-2 of Lean, HFD and HFD +3% TQ. Results are mean ± SE, n=6, *$p<0.05$ *vs*. Lean, #$p<0.05$ *vs.* HFD.

Effect of TQ on MFN-1, MFN-2, OPA1, NOV, HO-2 and HO-1 protein expression

Control obese mice exhibited lower hepatic protein expression of MFN-1, MFN-2, OPA1 and HO-1. TQ produced a significant ($p< 0.05$) increase in the hepatic levels of MFN-1, MFN-2, OPA1 and HO-1 (Figure 6). TQ prevented the HFD-mediated increase in NOV expression (Figure 6). No significant changes were observed on HO-2 among the different groups.

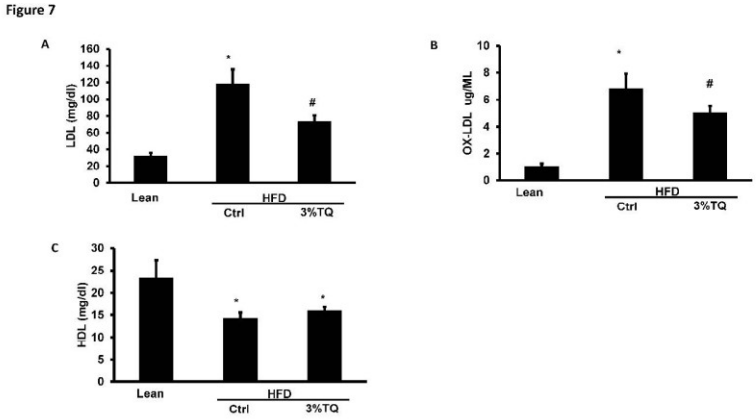

Figure 7: Analysis on plasma levels of (A) LDL, (B) OX-LDL and (C) HDL in Lean, HFD and HFD + 3% TQ mice respectively. Results are mean ± SE, n=6, *p<0.05 *vs*. Lean, #p<0.05 *vs.* HFD diet.

Effect of TQ on serum levels of Oxidized LDL, OX-LDL and HDL

Plasma from obese mice displayed an increase in LDL and OXLDL and a decrease in HDL levels. TQ reduced the levels of LDL and oxidized LDL ($p<0.05$), HDL levels were unaffected (Figure 7).

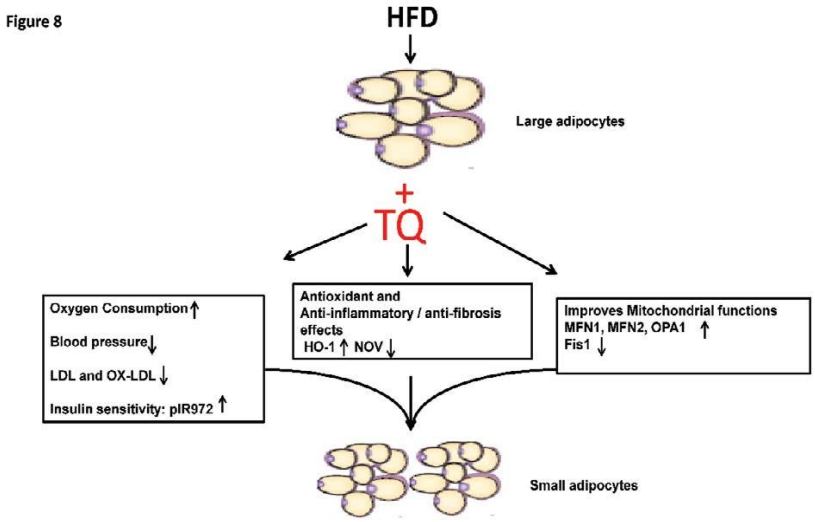

Figure 8: Schematic representation of TQ effects on reprogram adipocyte phenotype from naïve state to healthy adipocyte that increase activity of HO-1, mitochondrial proteins and insulin receptor phosphorylation. TQ treatment change the quality of fat from sick fat, adiposopathy, to healthy fat, i.e., metabolically active, that express more mitochondrial signaling, increase oxygen consumption and increase insulin receptor phosphorylation.

Discussion

TQ is an active component of TriNutra' s™ Nigella seed oil and is considered responsible for most of the latter therapeutic potential. The plant *Nigella sativa (N. sativa)* has been

used throughout the world in various traditional systems of medicine as a therapy for many different ailments and conditions. The key finding of the present study highlights the hepato-protective effects of TQ in a rodent model of NAFLD. TQ administration for 8-weeks reduced hepatic fat accumulation preventing the development of NASH and liver fibrosis in 36-week study of obese mice. NAFLD affects ~ 25% of the adult population and is the most common cause of chronic liver disease in the Western World. Concomitantly it is associated with obesity, type II diabetes and hyperlipidemia, and may serve as a marker of increased morbidity and mortality from cardiovascular disease. While the mechanism of NAFLD has not been elucidated, it is manifest as tissue injury as a result of fat accumulation. In this process oxidative stress results in mitochondrial damage [28,29] and tissue dysfunction manifest as hepatocellular oxidative damage leading to hepatic inflammation (non-alcoholic steatohepatitis). Hepatic dysfunction leads to fibrosis followed by cirrhosis, liver failure and hepatocellular carcinoma. Treatment of obese mice with TQ improves mitochondrial function in adipose and hepatic steatosis by decreasing levels of adipocyte derived NOV, and increasing, an antioxidant gene, HO-1 expression, resulting in increased mitochondrial biogenesis, function, and fusion potential, leading to an improvement in oxidative stress and inflammation in obese mice. The following key findings substantiate this conclusion. A HFD increased the expression of the genes regulating mitochondrial fission in mice, while concomitantly reducing the expression of the genes responsible for mitochondrial quality control and fusion processes in adipose and hepatic tissue. We further investigated whether TQ treatment positively affect signaling proteins. TQ positively increased HO-1 and insulin receptor phosphorylation in liver adipose tissue (Figures 7 and 8). Similar effect is seen in heart and kidney signaling protein (data not shown). A HFD enhanced FFA generation and increased mitochondrial dysfunction and ROS levels [30,31]. Mitochondrial dysfunction results in a decrease in beta oxidation in the liver allowing fat to accumulate resulting in a “fatty liver” [26,32,33]. TQ reduced mitochondrial fission potential and normalized an enhanced expression of mitochondrial fusion-associated genes in mice fed a HFD. TQ is a natural antioxidant and hypoglycemic compound that may prove advantageous therapeutically when compared to the high cost and the adverse effects of pharmacological drugs. NOV expression in obese mice was increased when compared to lean mice, the levels of NOV in HFD-fed mice treated with TQ were lower than mice fed a HFD alone. Increased NOV levels are linked to increased levels of inflammatory cytokines which deleteriously affect insulin signaling,

resulting in insulin resistance and eventually obesity [34,35]. In contrast, downregulation of NOV is associated with a reduction in adipose tissue deposition and inflammatory cytokines, as well as enhanced insulin sensitivity in obese mice [23,26]. Figures 4 and 5 showed that TQ improved hepatic steatosis, fibrosis and metabolic balance in obese mice. More importantly, ingestion of TQ in HF mice led to a reversal of this trend and a resultant increase in both the level and activity of HO-1, which strongly suggests a role for HO-1 and HO activity in the antioxidant and anti-inflammatory effect of TQ. Other report in agreement with our finding that induction of HO-1 suppresses adiposity and diabetes [36,37]. Further, sex-depends effect of HO-1 in adipose is well described [38], in which expression of HO-1 in adipose tissue may have a greater protective role in female as compared to male [38]. Further, HO-1 is considered a novel target for the treatment of hypertension and obesity [39]. More importantly beneficial effect of HO-1 is seen in human liver transplant biopsies; subjects with higher HO-1 levels showed decreased hepatocellular damage and improved outcomes [40]. Additionally, it appears that the increase of HO-1 levels, decrease in pro-inflammatory NOV expression and the normalization of mitochondrial function rescue liver function in obese mice. The beneficial effects of TQ on hepatic protein expression suggest an antisteatosis effect that prevents disease progression to steatohepatitis in our animal model support the effect on decrease fasting glucose and oxygen consumption. It appears that TQ was capable of reprogramming the adipocyte phenotype by regulating energy gene and mitochondrial function and HO-1 expression, leading to an increase in “healthy”, i.e. small, adipocytes and a decrease in large adipocyte qualitatively and in terminal differentiation as evidence suggesting that increase in activity as evidence of increase in oxygen consumption, may maintain healthier adipocytes in obese mice. This occurred without body weight change, further, TQ improved the metabolic profile of obese mice by lowering fasting glucose, BP and hypertension, and increasing oxygen consumption compared to non-treated obese mice. One plausible explanation for body weight remaining unchanged could be the direct effect of TQ on adipocyte hyperplasia. This supports the hypothesis that the expansion of adipocytes may lead to an increased number of adipocytes of smaller size; smaller adipocytes are considered to be “healthy”, insulin-sensitive adipocyte cells that are capable of producing adiponectin [41,42]. There is a tight link exists between adipocyte hypertrophy and inflammation; followed by a reduction in adipocyte size leading to amelioration of metabolic functions [43-46]. In our current study we show that TQ decreased lipid content.

In agreement with our *in vivo* results and previously published reports, the increase of HO-1 levels in adipocytes turns large unhealthy adipocytes into small healthy insulin-sensitive adipocytes [47]. In addition, the decrease in pro-inflammatory adipocyte NOV expression, the increase of HO-1 levels and the increased levels of insulin receptor phosphorylation in adipose tissue lead to the normalization of mitochondrial function and a reversal of adipocyte phenotype from an inflammatory to a healthy functional status. Together, these results clearly indicate that activation of the HO-1 antioxidant response is crucial to the beneficial effects of TQ on mitochondrial biogenesis and on the reduction of fission and increase of fusion-associated processes in both adipose and hepatic tissue. Importantly, in obesity, it is well established that there is association of elevation in HDL and OxHDL in obese animals. Obese mice treated with TQ demonstrated a significant decrease in LDL and OX-LDL levels. LDL oxidation, as well as HDL oxidation, is critical in the development of atherosclerosis and NAFLD has many features in common with cardiovascular disease, including lipid accumulation, macrophage activation and infiltration, and inflammation [48-50]. The activation of Kupffer cells by OX-LDL leads to a rapid release of various inflammatory mediators and signaling molecules such as cytokines, ROS, proteases, and lipid mediators that contribute to hepatic inflammation [51]. Fundamentally, TQ ingestion that result in decrease in OX-LDL and inflammatory molecule, NOV and increase in mitochondrial biogenesis and attenuates liver steatosis and NASH will contribute to an increase in insulin sensitivity and organ protection, indicates the potential of this nutraceutical approach to prevent disease progression in an animal model of metabolic syndrome. TQ intervention that contributes to lower blood pressure, fasting glucose may be beneficial to obese and non-obese subjects, may involve the increase of HO-1. HO-1 induction shown to lower blood pressure in hypertensive and obese animal models [52-55]. The beneficial effects of TQ in the pathogenesis of NAFLD in a murine model of obesity offer a portal into therapeutic approaches to the treatment of this and other obesity-related diseases.

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Chapter 10

Effect of Adipose tissue cross-talk on NAFLD: Role of Heme Oxygenase

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Background: Non-alcoholic fatty liver disease (NAFLD) is a inflammatory disorder associated with obesity and Metabolic Syndrome, characterized by fatty acid accumulation, oxidative stress, and lipotoxicity. We have previously reported that HO-1 induction by epoxyeicosatrienoic acid-agonist (EET-A) decrease liver inflammation and fibrosis in a PGC1-a dependent manner. We hypothesized that a selective adipose tissue HO-1 and PGC1-a over-expression, would attenuates obesity-induced steatosis and hepatic fibrosis in NAFLD, through a cross-talk between adipose tissue and liver. **Methods**: For this study we create two model of transgenic mice with selective adipose tissue HO-1 or HO-1 and PGC1-a overexpression. The mice were fed a high fat diet (HF) for 21 weeks as obesity model. **Results**: Control mice fed HF showed an impaired mitochondrial function with a decreases of mitochondrial proteins expression mitofusin protein 2 (Mfn 2) and mitochondrial dynamin like GTPase (OPA1) and a decrease of anti-obesity Fibroblast growth factor 21 (FGF21), Cellular Repressor of E1A-Stimulated Genes 1 (CREG1) and Sirtuin 1 (SIRT1) protein levels. Moreover, the HF group showed an increased Fatty acid syntetase (FAS) and cytokine Nephroblastoma-Overexpressed (NOV) protein levels. Over expression of HO-1 reversed the metabolic impairment and reduced liver steatosis and inflammation. Mice with OE of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and HO-1 showed the same trend of HO-1-OE.

INTRODUCTION

The increasing burden of non-alcoholic fatty liver disease (NAFLD) is a major health concern. The NAFLD worldwide prevalence shows an upward trend over time and has reached “pandemic” proportions. In the general population it is estimated to be 20%-30% in Western countries and 5%-18% in Asia and is associated with an increased prevalence of obesity, insulin resistance, metabolic syndrome and diabetes, which are often paired to NAFLD [1]. Indeed in at risk patients, such as patients with diabetes mellitus, the prevalence of NAFLD increases up to 40%-70% [2]. In addition, NAFLD can run a unfavourable course, given the possible evolution to cirrhosis and hepatocellular carcinoma and can constitute an indication for liver transplantation [3]. NAFLD and more specifically non-alcoholic steatohepatitis (NASH) are closely related to metabolic impairment, such as visceral adiposity, hyperinsulinaemia or diabetes, dyslipidaemia and arterial hypertension, which define the metabolic syndrome. NAFLD and NASH are considered the hepatic manifestation of the metabolic syndrome [4]. Moreover patients with NAFLD or NASH, are at higher risk of developing diabetes mellitus and are at increased risk of morbidity and mortality related to cardiovascular diseases [5]. These considerations arise the need of understanding the complex mechanisms underlying the onset of NASH. At the basis of a wide clinical spectrum of NAFLD that includes metabolic impairment at different levels, there is a complex interaction between different organs at the pathogenetic level. This is conceptualized in the “multiple parallel hit hypothesis” [6] and has been substantiated by further research. The liver damage, driven by insulin resistance, iron accumulation, oxidative stress and hepatocyte death, can be triggered by an imbalance in anti- and pro-inflammatory factors originating from the liver itself or from extra hepatic sites that cross talk with the liver, particularly the adipose tissue [7].

Obesity is characterized by an increase in the mass of adipose tissue. Fat tissue plays an important role in the genesis of insulin-resistance and liver complications. Trunk fat, evaluated by dual-energy X-ray absorptiometry on 11,821 adults without viral hepatitis, was a major body composition that was indicative of an increase in ALT. This supported the hypothesis that liver injury can be induced by metabolically active intra-abdominal fat [8]. Under conditions of over nutrition adipocytes are pushed to the limit of their ability to store lipids and to regulate nutrient metabolism. Obesity is then accompanied by an increase in inflammatory marker expression [9, 10]. The increased pro-inflammatory cytokines impairs insulin signalling in adipocytes, leading to increased lipolysis and release of free fatty acids

into the circulation, which in turn promotes insulin-resistance in liver and muscles, and ultimately leads to type 2 diabetes and liver complications. Adipokines secreted by visceral adipose tissue also act directly on the liver via the portal vein and could regulate hepatic inflammation, glucose and lipid homeostasis, and fibrosis with an axis adipose tissue-liver that play an important role in the development of NAFLD [11, 12].

Heme Oxygenase (HO) exists in two forms, HO-1, the inducible form, and HO-2, the constitutive form. Both isozymes degrade heme in an identical stereospecific manner to biliverdin with the concurrent release of CO and iron [13]. HO-1 can be induced by an extraordinarily wide variety of drugs and chemical agents including statins, aspirin, niacin, certain prostaglandins, eicosanoids such as epoxyeicosatrienoic (EETs) and free and complexed metals [13]. Iron, bilirubin and CO, the three degradation products of the HO reaction, may have important regulatory functions in cells.

Several studies showed that a chronic HO-1 induction increased oxygen consumption, and lowered body weight in obese melanocortin-4 receptor deficient mice with an improvement in vascular function [13, 14]. Induction of HO-1 by many inducers such L-4F, CoPP, heme, or by gene transfer [15-17] is associated with an increased number of healthy adipocytes, a concomitant increase of plasma adiponectin levels, improved insulin sensitivity and a decrease in inflammatory adipokines and blood pressure [18-21]. This effect of HO-1 induction on adipocyte morphology was confirmed in Zucker diabetic rats [21] and extended to ob/ob diabetic mice, where increased levels of HO-1 and HO activity prevented weight gain and decreased visceral and subcutaneous fat levels.

We hypothesize that the development of NAFD, and its progress to NASH, is the result of the combination of increased inflammation and a decrease of HO-1 in adipose and hepatic tissues leading to the impairment of mitochondrial function. We further propose that induction of HO-1 in adipose tissues will have positive impact on hepatic tissue, decreasing NASH scores and increasing mitochondrial integrity and function.

Materials and Methods

Animal protocols

All experimental protocols were performed following an institutionally (NYMC) approved protocol in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. We used four-week-old lean mice from Jackson Laboratories (Bar Harbor, ME) fed a HF (western diets, containing 58% fat, 25.6%carbohydrate and 16.4% protein with total calories of 23.4 kJ/g (Harlan, Teklad Lab Animal Diets, Indianapolis, IN) for an additional 21-weeks. HF used to expedite development substantial hepatic lipid uptake and fibrosis. Mice were divided into 4 treatment groups as follows: Group 1) Lean Group 2) HF Control mice were injected intraperitoneally with water and lentivirus vector Group 3) Mice with selective adipose tissue overexpression of HO-1 (HO-1-OE) Group 4) Mice with selective adipose tissue overexpression of HO-1 and PGC1-a. A the end of the experiment, mice were euthanized with ketamine (100 mg/kg)/xylazine owed by cervical dislocation.

Western blot analysis

Frozen liver tissues were ground under liquid nitrogen and resuspended in RIPA lysis buffer that included protease and phosphatase inhibitors (Complete™ Mini and PhosSTOP™). Homogenates were cen- trifuged at 12000 x g for 12 min at 4 °C, supernatants were collected and protein concentrations measured. Protein detection was performed using a secondary infrared fluorescent dye conjugated antibody. The blots were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor Science Tec) and quantified by densitometry analysis performed after normalization with actin. Results were expressed as arbitrary units (AU).

Results

Figure 1

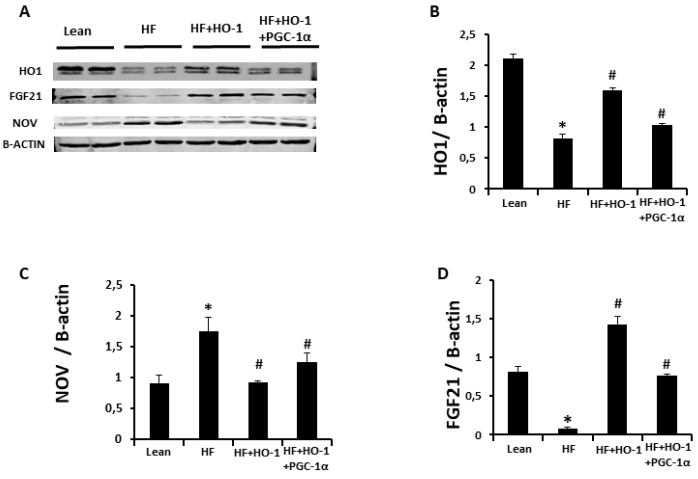

Figure 1. HO-1 Overexpression increases HO-1 and FGF21 and decrease inflammatory cytokine NOV. A) Representative western blots and densitometry analysis of (B) HO-1, (C) NOV and (D) FGF21. Results are mean ± SD, n=4, ∗p<0.05 versus lean mice, #p<0.05 versus HF fed mice.

Adipose tissue cross-talk affect the key metabolism regulator proteins in the liver.

Obese mice had decreased expression levels of HO-1, SIRT1, FGF21 ($p<0.05$), as well as levels of CREG1, as compared to control lean mice. In transgenic mice the OE of HO-1 or HO-1+PGC1-a in adipose tissue, reversed the effect of HF on these liver proteins with beneficial effects on lipid and glucose metabolism ($p<0.05$) (Figures 2).

Heme oxygenase Overexpression in adipose tissue reduce inflammation and enhanced HO-1 and FGF21 levels in liver

To further test the possibility of potential crosstalk between adipose and hepatic tissue we examined the effects of HO-1 or HO-1+PGC-1a OE in adipose tissue, on liver proinflammatory adipokine NOV, the antioxidant enzyme system HO-1 and the metabolism key factor FGF21. As seen in Figures 1, obese mice expressed elevated levels of NOV and decreased levels of HO-1 and FGF21 compared to lean mice ($p<0.05$). In the transgenic mice groups, the effect of HF on these proteins levels was reversed ($p<0.05$), suggesting that elevate levels of HO-1 or HO-1+PGC-1a on white adipose tissue, resulting in less inflammation, oxidative stress and an enanched metabolism.

Discussion

The preliminary results of this study show that selective adipose tissue overexpression of HO-1 or HO+PGC-1a was able to enhance the liver metabolism and lower the inflammation and fatty acids accumulation in obese-derived fatty liver with possible beneficial impact on NAFLD. Our data showed an increase of the proinflammatory adipokine NOV and decrease of HO-1 in hepatic tissue of obese mice associated with mitochondrial dysfunction and a decreased levels of lipid and glucose metabolism key regulator protein FGF21.

NOV are increased while adiponectin is decreased in obese mice [22]. Reduction of levels of heme through the pharmacological induction of HO-1 reduces the severity of steatosis, inflammation, and fibrosis through the improvement of hepatic mitochondrial function. Others studies have shown that heme levels are elevated in lipid laden, unhealthy, terminal differentiated adipocyte, and increase of HO-1; i.e., increase of heme degradation decreases adiposity [23, 24]. Diminishing HO-1 levels are seen in maturing inflamed adipocyte in hepatic tissues [24-26].

Increased levels of HO-1 shown to play a critical role in the amelioration of oxidative stress, and, in both humans and mice, low levels of HO-1 lead to organ damage [27, 28].

Moreover, overexpression of HO-1 lowers levels of the inflammatory mediators TNF-α and IL-6 in the liver of mice [29]. A decrease in HO activity exacerbates mitochondrial lipid peroxidation and mitochondrial dysfunction, while induction of HO-1 upregulates mitochondrial transcription factor [30], all of which support the hypothesis that a reduction in HO activity results in mitochondrial dysfunction and increased insulin resistance [31, 32]. Mitochondrial dysfunction leads to a decrease in beta oxidation in the liver which allows fat to accumulate resulting in a "fatty liver" [33, 34].

The adipose tissue is a key organ in the pathogenesis of NASH and the associated metabolic impairment. Obese adipose tissue secretes various inflammatory cytokines, such as IL-6 and TNF-α [35], and dysregulated production of these proinflammatory mediators over the antiinflammatory adipokine (e.g., adiponectin) is thought to be a central mechanism underlying adverse metabolic and cardiovascular consequences.

HO-1-mediated antioxidant mechanism were able to affect the dysfunctional adipose tissue decreasing levels of ROS and increasing levels of adiponectin, which when secreted from adipocytes, can improve insulin action and reduce inflammation and fibrotic processes in liver [36].

Mice with HO-1 OE showed elevated levels of FGF21, that is known to reduce adiposity and enhance glycogen storage, resulting in a decrease in oil droplets in hepatic tissue on obese mice [25, 37] in according with our finding that have shown a reduced levels of FAS protein.

In conclusion this preliminary results suggest that HO-1 and PGC-1a have a crucial role in adipose tissue endocrine function, that affect the fatty liver through a cross-talk action with a beneficial effects on lipid metabolism regulation and inflammation.

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Chapter 11

Summarizing discussion and Conclusion

Although the prevalence of MetS in the United States has slightly decreased in the last decade from 26% to 23% based on the National Health and Nutrition Examination Survey (NHANES) data from 1999 to 2010, the prevalence of some of its constituents
such as abdominal obesity has increased from 45% to 56% [254]. Genetics and lifestyle factors play an important role, IR is the major driving force for MetS and its risk factors [255]. Because visceral adiposity is a marker of IR [256], it is not surprising that the mechanism of development of MetS starts in the adipose tissue where adipokines and free fatty acids (FFA) play a central role in the pathogenesis of MetS [257]. Similarly, FFA accumulation with subsequent TG accumulation leads to hepatic steatosis in IR individuals [258]. Hepatic accumulation of FFA and cholesterol leads to hepatocyte damage, which is mediated by oxidative stress and direct lipotoxicity causing mitochondrial dysfunction [202, 259]. Individuals with IR have mitochondrial dysfunction owing to increased FFA uptake and their incomplete oxidation [123]. This leads to oxidative stress and production of reactive oxygen species owing to energy depletion, respiratory chain deficiency, and subsequent necrosis or apoptosis [260]. Furthermore, mitochondrial dysfunction causes IR, thus propagating this vicious cycle [261]. All these mechanisms are not independent of each other and work together in this complex pathogenesis [155].

In view of the importance that oxidative stress have in many aspects of this pathologies, this thesis investigated the role of Heme Oxygenase, a main antioxidant enzymatic system, in the different metabolic disease. In the Chapter 2 I used the NAC as natural antioxidant and HO-1 inducer compound, on an *in vitro* model of bone marrow adipose tissue. that demonstrated to increase in mRNA levels of genes responsible for fatty acid oxidation, reduce triglycerides accumulation and inflammatory cytokines levels in bone marrow stromal cells-derived adipocytes, restoring the function of BMAT which may protect skeletal osteoblasts from obese-derived lipotoxicity. In Chapter 3 I investigate the potentially protective effect of phenethyl ester of caffeic acid (CAPE), one of the main components of propolis with a flavonoid-like structure, and of a novel CAPE analogue called VP961, as HO-1 inducers, on a STZ-induced diabetes model rat. At the experimental conditions body weight of CAPE or VP961-treated STZ rats was significantly increased compared to the diabetic control rats. Moreover, treatment of STZ rats with CAPE or VP961 significantly reduced blood glucose levels, increased plasmatic

insulin levels, decreased plasmatic and pancreatic LOOH and nitrite/nitrate levels respect to control STZ rats. The reduction of plasmatic and pancreatic nitrite/nitrate levels may be related to iNOS downregulation in CAPE or VP961 treated STZ rats. These results suggest that the effects of CAPE or VP961 may be due to protecting from damage of the pancreatic tissue. Moreover, the significant increase of plasmatic and pancreatic LOOH induced by low insulin content and consequent hyperglicemia, may be related to downregulation of pancreatic antioxidant defences, both enzymatic than nonenzymatic, such as HO-1, GGCL and RSH. In Chapters 4 and 5 I used *in vitro* model of hepatic cells to investigate the effects of natural and synthetic HO-1 regulator compounds, on hepatic steatosis and fibrosis. In Chapter 4 I used an Olive leaf extract as treatment on HepG2 cells treated with free fatty acids to reproduce steatosis. I found that OLE ameliorated lipid metabolism, with a parallel decrease in number of lipid droplets and a concomitant increase in FABP-4, SIRT-1 and HO-1 expression. Furthermore, OLE treatment induced a significantly reduction of the inflammatory cytokines IL-1β and TNF-α. In Chapter 5 I used two well-known HO-1 synthetic inhibitor and inducer compounds, SnMP and CoPP respectively, on two hepatic cells *in vitro* model, to assess how HO-1 activity affect lipid metabolism and collagen release in pathological condition. I found that hepatocytes treated with free fatty acids showed a lipid metabolism pathway impaired, and SnMP co-treatment significantly exacerbates this state. Contrarily, co-treatment with the HO-1 inducer CoPP reversed this results. Also in the fibrosis *in vitro* model, obtained activating LX-2 cells with TGF-B, I found that HO activity inhibition further increased collagen release compared to cells treated exclusively with TGF-β. Furthermore, I found that increased HO-1 levels by CoPP reversed the effect mediated by SnMP and reduced the levels of soluble collagen released from activated LX2. These results provide additional evidences for a link between liver disorders and HO system.

In Chapters 6 to 10 I focused my studies more on NAFLD related aspects, integrating the previous mechanistic *in vitro* data with functional *in vivo* data using mouse models. In Chapter 6 I investigate the possible correlation between the increase in the inflammatory adipokine NOV and the decreases in HO-1 levels in adipose and hepatic tissue of C57 obese mice with NASH. NASH mice displayed decreased HO-1 activity, increased levels of hepatic heme, NOV, MMP2, hepcidin, and increased NAS scores and hepatic fibrosis. The CoPP treatment showed to decrease NOV, improved hepatic NAS score, ameliorated fibrosis, and increases mitochondrial integrity and insulin receptor phosphorylation.

Similary, in the adipose tissue of obese mice, characterized by an increase in pro-inflammatory molecules such as NOV and a decrease in adiponectin, CoPP was able to increased levels of thermogenic and mitochondrial signaling associated genes. All these beneficial effects by HO-1 increases were abolished when I co-treated the mice with the Ho-1 activity inhibitor SnMP, showing the crucial role of this protein in NASH pathology. In Chapter 7 I explored the effects of sEH deletion on various aspects of adipocyte-function, including programing for white vs. beige-like fat, and mitochondrial and thermogenic gene-expressions. I further hypothesize that EETs and HO-1 form a synergistic, functional module whose effects on adipocyte and vascular function is greater than the effects of sEH deletion alone. For *in vitro* studies, I examined the effect of sEH inhibitors on hMSC-derived adipocyte while for the *in vivo* experiments I used the male sEH null mice with or without CoPP treatment. In both models sEH deletion results in a significant decrease in adipocyte size and inflammatory adipokines. sEH deletion was manifested by a significant increase in EETs isomers, and in the mice model these findings were associated with a decrease in body weight, visceral fat and a significant increase in mitochondrial proteins expression Mfn1, COX1, and thermogenic genes UCP1 and adiponectin. In the group with CoPP, activation of HO-1 gene expression further increased the levels of EETs, suggesting that the antioxidant HO-1 system protects EETs from degradation by ROS. These data provide novel evidences on EETs and HO-1 beneficial effects on adipocytes health. In Chapter 8 based on the findings described above, I analysed the link among EETs, HO-1 and PGC-1a and their role in NAFLD disease. For these experiments I used an others mice model called db/db mice, with leptin deficiency, fed with HF to induce obesity and the correlated fatty liver and then I treated the mice with an EET analogue alone or in combination with a lentivirus for the PGC-1a knockdown. I found that EET-A increased the expression of the key metabolism signaling regulator proteins PGC-1α, CREG1, AMPK, SIRT1 and FGF21 in liver obese mice, with improvement in mitochondrial function as assessed by the increased in expression of mitofusion 1 and 2 (Mfn1 and Mfn2), and cytochrome oxidase complex 1 and 2. Futhermore, I observed that EET-A administration reduced the levels of inflammatory molecules and fibrosis markers such as (TNF-α, MCP1, NOV, TLR-4 and NF-kB, and MMP-2), whereas expression of the anti-inflammatory cytokine, interleukin-10, was increased. The combination of these effects results in a clear alleviation of NAFLD and a significant shutdown in the progression to NASH. In Chapter 9 I studied the effects of

black seed oil, with a high content of Thymoquinone, on the metabolic profile, including adipose-mediated release in inflammatory adipokines such as NOV, mitochondrial biogenesis, LDL, Ox-HDL and hepatic steatosis in a murine model of obesity. I found that the TQ treatment resulted in an increase of energy-dependent genes and oxygen consumption and decreased fasting glucose levels and blood pressure compared to the control group. Moreover, TQ increased the levels of hepatic HO-1, mitochondrial Mfn2, insulin receptor phosphorylation and decreased OX-LDL and haptic apoptosis. These findings indicate a potential clinical role of TQ in the prevention of obesity-related metabolic disease. Finally, the preliminary data presented in the Chapter 10, explored the cross-talk relation between adipose tissue and liver. I used transgenic mice with adipose tissue selective HO-1 or HO-1+PGC-1a overexpression, fed to HF to obtain an obesity and NAFLD state. I found that in the liver of transgenic mice groups, the metabolism key regulator FGF21 protein expression was enanched. Futhermore, HO-1 OE in adipose tissue resulted in increased HO-1 expression and a decrease in the pro-inflammatory cytokines NOV in the liver. More experiments are needed to support these first findings and complete the study.

In conclusion, this PhD thesis provides new evidences that identifies the enzyme Heme Oxygenase as a key protein in metabolic homeostasis, with a pivotal role in preventing systemic lipid overload, insulin resistance and decreasing inflammation. Consequently, increase of HO-1 levels may provide a therapeutic approach to address the metabolic alterations associated with diabetes type 2, obesity and NAFLD.

References *(General Introduction and Summarizing discussion and Conclusion)*

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