Treatment with a SOD Mimetic Reduces Visceral Adiposity, Adipocyte Death, and Adipose Tissue Inflammation in High Fat-Fed Mice

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Objective: Obesity is associated with enhanced reactive oxygen species (ROS) accumulation in adipose tissue. However, a causal role for ROS in adipose tissue expansion after high fat feeding is not established. The aim of this study is to investigate the effect of the cell permeable superoxide dismutase mimetic and peroxynitrite scavenger Mn(III)tetraakis(4-benzoic acid)porphyrin chloride (MnTBAP) on adipose tissue expansion and remodeling in response to high fat diet (HFD) in mice.

Design and Methods: Male C57BL/6j mice were fed normal chow or high fat diet (HFD) and treated with saline or MnTBAP for 5 weeks. The effects of MnTBAP on body weights, whole body energy expenditure, adipose tissue morphology, and gene expression were determined.

Results: MnTBAP attenuated weight gain and adiposity through a reduction in adipocyte hypertrophy, adipogenesis, and fatty acid uptake in epididymal (eWAT) but not in inguinal (iWAT) white adipose tissue. Furthermore, MnTBAP reduced adipocyte death and inflammation in eWAT and diminished circulating levels of free fatty acids and leptin. Despite these improvements, the development of systemic insulin resistance and diabetes after HFD was not prevented with MnTBAP treatment.

Conclusions: Taken together, these data suggest a causal role for ROS in the development of diet-induced visceral adiposity but not in the development of insulin resistance and type 2 diabetes.

Introduction

Obesity rates are increasing in the United States, estimating more than 35.7% of adults and 16.9% of children and adolescents are considered obese (1). Obesity is a risk factor for insulin resistance, the metabolic syndrome and cardiovascular disease (2). Unfortunately, obesity rates keep increasing because more and more children are becoming overweight or obese due to a sedentary lifestyle characterized by excess consumption of highly processed and energy-dense food. Furthermore, current strategies aimed to limit obesity such as weight loss programs have proven ineffective due to rapid weight regain. Thus, developing new treatments for obesity is urgent.

Obesity is associated with increased systemic and adipose tissue oxidative stress (3). Indeed, Furukawa et al. (4) showed that adipose tissue of genetically obese or diet-induced obese animals produced higher amounts of hydrogen peroxide (H$_2$O$_2$), have reduced antioxidant enzyme activity and enhanced mRNA level of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits. Furthermore, lower levels of glutathione peroxidase 3 (GPx3) and reduced activity were observed in white adipose tissue (WAT) of genetically obese ob/ob and db/db mice (5). These data suggest that reactive oxygen species (ROS) are locally produced in adipose tissue and may play a role in the pathogenesis of obesity.

There has been substantial interest in using natural antioxidants for the treatment of obesity and the associated insulin resistance and the metabolic syndrome. Metalloporphyrins have been shown to mimic the biochemical activity of superoxide dismutase (SOD) (6) and were found to substitute for it in mutant prokaryotes lacking SOD (7). Day et al. (8) showed that MnTBAP was effective in attenuating
Fatty acid oxidation and mitochondrial function

|                      | NC saline | HF saline | NC MnTBAP | HF MnTBAP |
|----------------------|-----------|-----------|------------|-----------|
| PGC1α                | 2.1 ± 0.1 | 1.5 ± 0.2 | 1.8 ± 0.2  | 1.5 ± 0.2 |
| PGC1β                | 1.6 ± 0.2 | 1.3 ± 0.1 | 2 ± 0.2    | 1.5 ± 0.1 |
| CPT2                 | 1.4 ± 0.1 | 1.5 ± 0.2 | 1.5 ± 0.2  | 1.5 ± 0.1 |
| ACSL1                | 1.3 ± 0.1 | 1.2 ± 0.2 | 1.3 ± 0.2  | 1 ± 0.04  |
| ACSL5                | 1.1 ± 0.1 | 1.3 ± 0.2 | 1.5 ± 0.2  | 1.2 ± 0.1 |
| Fatty acid synthesis |           |           |            |           |
| HSL1/2               | 0.9 ± 0.06 | 0.9 ± 0.07 | 0.9 ± 0.2 | 0.9 ± 0.04 |
| HSL2                 | 1.3 ± 0.07 | 1.4 ± 0.1 | 1.2 ± 0.2  | 1.3 ± 0.07 |

mRNAs were extracted from epididymal white adipose tissue of mice fed normal chow (NC) or high fat (HF) diet and treated with either saline or MnTBAP for 5 weeks starting at 10 weeks of age. mRNA expression was normalized to 16s ribosomal RNA expression which was not significantly different among the groups. NC: normal Chow; HF: high fat. Data are expressed as mean ± SEM; n = 7-8 mice per group.

*P < 0.05 vs. NC of the same treatment.

Oxidative stress detection

Superoxide production was monitored by the superoxide detector MitoSOX Red (Life Technologies, Grand Island, NY). Freshly isolated epididymal white adipose tissue (eWAT) was sectioned and incubated with MitoSOX Red (50 μmol L⁻¹) for 30 min at 37°C. Fluorescence (expressed as % red) was quantified in three low-power field images from five animals in each group. Reduced and oxidized glutathione content in eWAT homogenates was determined using a spectrophotometric assay kit (EMD Millipore, Billerica, MA).

Body composition

Mice were anesthetized by a single intraperitoneal injection of 400 mg chloral hydrate/kg body weight. Fat mass and lean mass were determined using dual energy X-ray absorptiometry (DEXA) (Norland Medical Systems, Fort Atkinson, WI).

Indirect calorimetry

Mice were housed for 72 h in a four-chamber Oxymax system (CLAMS; Columbus Instruments, Columbus, OH) as previously described (11). O₂ and CO₂ content of the exhaust air from each chamber was compared with ambient air O₂ and CO₂ content. Food consumption was monitored by electronic scales, water by electronic sipper tubes, and movement by XY/Z laser beam interruption. Respiratory exchange ratio (RER) was calculated as VCO₂/VO₂.

Adipocyte diameter

Adipocyte diameter was measured using live tissue imaging as described by Nishimura et al. (12). Briefly, eWAT was removed (~3 mm each), washed and incubated with 5 μmol mL⁻¹ horon-dipyrrromethene BODIPY (Life Technologies, Grand Island, NY). Nuclei were counterstained with 40 μmol L⁻¹ 4',6-diamidino-2-phenylindole (Hoechst 33342, Sigma-Aldrich, St. Louis, MO). Images were acquired with a confocal laser-scanning microscope (FV1000-XY- Olympus IX81). Each resulting image was created from the average of ten frames and processed to produce a surface-rendered three-dimension picture. Ten low-power field images were acquired at regular intervals from five animals in each group. The major and minor adipocyte diameters (μm) were measured to determine the mean diameter using the Image Pro-Plus software (Image-Pro Plus version 7.0., Media Cybernetics, Silver Spring, MD).

Adipocyte death

Sections of eWAT were coincubated with the green-fluorescent probe YO-PRO-1 (0.2 μmol L⁻¹, Life Technologies, Grand Island, NY) and the red dye propidium iodide (PI), (0.3 μg, Life Technologies, Grand Island, NY). Nuclei were costained with 40 μmol L⁻¹ Hoechst (Sigma–Aldrich, St. Louis, MO). Apoptotic cells show green fluorescence, and dead cells show primarily red fluorescence. At least 1,000 nuclei were counted per animal for three animals per group at randomly chosen sections.

Crown-like structures quantification

Tissue samples from eWAT were fixed in formaldehyde (4% w/v in 0.1 mol L⁻¹ phosphate buffer pH 7.2) for 48 h, dehydrated, embedded in paraffin plus (Sigma–Aldrich, St. Louis, MO), sectioned at 5 μm and stained with hematoxylin and eosin (Sigma–Aldrich, St. Louis, MO). Five consecutive microscopic fields were analyzed per animal for a total of four animals per group on a light microscope (Olympus IX71 Olympus, Japan).

Methods

Animals, diet, and treatment

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Utah. Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were fed high fat diet (HFD) or normal chow (NC) for 5 weeks. At the start of the diet, mice received an intraperitoneal injection of saline (0.9% w/v NaCl) or MnTBAP (20 mg kg⁻¹ body weight) three times a week for 5 weeks. As detailed in Supporting Information Table 1, the % kcal for the NC diet is 34% carbohydrates, 53% proteins, and 13% fat (soybean oil) whereas and for the HFD is 20% carbohydrates, 35% proteins, and 45% fat (soybean oil and lard).
TABLE 2 Serum metabolites and hormones level

| Metabolite                  | NC saline | HF saline | NC MnTBAP | HF MnTBAP |
|-----------------------------|-----------|-----------|-----------|-----------|
| Triglycerides (mg dl⁻¹)     | 7.4 ± 1.1 | 16.7 ± 2.6a | 10.8 ± 1.8 | 12.1 ± 2.2 |
| Free Fatty Acids (mM)       | 0.4 ± 0.04 | 0.7 ± 0.1 | 0.5 ± 0.09 | 0.5 ± 0.03a |
| Leptin (ng ml⁻¹)            | 3 ± 0.2 | 9.9 ± 1.7b | 3.9 ± 0.9 | 6.6 ± 1ba |
| Adiponectin (µg ml⁻¹)       | 5.4 ± 0.6 | 5.1 ± 0.6 | 3.4 ± 0.4a | 4.9 ± 0.4 |

Serum metabolites and hormone levels were measured after a 6-h fast. All metabolites and hormones were determined on blood samples extracted before glucose injection prior to the glucose tolerance tests. NC: normal chow; HF: high fat. Data are expressed as mean ± SEM; n = 4-12 mice per group.

MnTBAP reduced HFD-induced adiposity
HFD (45% in fat content) resulted in a modest 11% (P < 0.05 vs. NC saline) increase in body weight that was completely prevented by MnTBAP treatment (Figure 2a). Despite a modest weight gain, body composition was significantly altered by HFD as evidenced by a two-fold increase in fat mass whereas lean mass was unchanged (Figure 2b,c). MnTBAP treatment significantly attenuated adiposity in HF-fed mice as evidenced by reduced fat mass (Figure 2c), an effect that persisted even when lean and fat mass were expressed as percentage of body weights (data not shown). It should be noted that MnTBAP-treated HF-fed animals exhibited a slight but significant increase in lean mass relative to saline-treated mice fed the same diet (Figure 2b). Next, we sought to determine whether MnTBAP reduced fat pads weight and found that HFD-induced expansion of brown adipose tissue (BAT) and eWAT was attenuated with MnTBAP treatment (Figure 2d,f). Interestingly, the effect of MnTBAP is depot-specific as inguinal and perirenal weights, which were increased by HFD were unaffected by MnTBAP (Figure 2e,g).

MnTBAP effects on adiposity are independent of food intake, substrate oxidation or physical activity
The reduction in weight gain with MnTBAP could be due to either a decrease in energy intake or an increase in energy expenditure. To address that, we measured oxygen consumption (VO₂), carbon dioxide release (VCO₂), food intake and movement. VO₂ was not statistically different between the groups (Figure 3a), whereas VCO₂ was reduced in HF-fed saline-treated group but not MnTBAP-treated group (Figure 3b). Upon HFD, oxidation of fatty acids increased as evidenced by the reduction of RER. Despite no alterations in VCO₂ with MnTBAP treatment, RER was still reduced, suggesting that substrate preference was not altered by the treatment (Figure 3c). However, there was a slight but significant (P < 0.05) increase in RER during the day in the NC-fed animals treated with MnTBAP, suggesting increased carbohydrates utilization (Figure 3c). Furthermore, energy expenditure was unaffected by MnTBAP as heat production was indistinguishable between the groups (Figure 3d). Finally, neither food intake nor physical activity was altered by MnTBAP treatment (Supporting Information Figure 2).

MnTBAP reduced adipocyte hypertrophy, adipogenesis and fatty acid uptake in eWAT
Because there was no change in energy intake or expenditure, other mechanisms were explored to explain the reduction in adiposity following MnTBAP treatment. First, we performed morphometric analysis of eWAT and found that while MnTBAP had no effect on adipocyte number (Data not shown), it significantly affected adipocyte...
FIGURE 1 MnTBAP treatment effectively reduced HFD-induced oxidative stress in epididymal white adipose tissue (eWAT). (a) Freshly isolated eWAT sections stained with the superoxide detector MitoSOX red and (b) the % red fluorescence (arrows) was quantified in three low-power field images from five animals in each group. NC: normal chow; HF: high fat. (c) and (d) mRNA expression of Sod2 (MnSOD) and NADPH oxidase 4 (NOX 4), respectively in eWAT. Data are mean ± SEM; n = 5 mice per group for MitoSOX and n = 5-8 mice per group for mRNA. *P<0.05 vs. NC of the same treatment, #P<0.05 vs. saline under the same feeding condition. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 2 MnTBAP prevented HFD-induced weight gain and visceral adiposity. (a) Body weights, (b) lean mass, (c) fat mass, (d) brown adipose tissue (BAT) weight/body weight (BW), (e) perirenal weight/BW, (f) epididymal weight/BW and (g) inguinal weight/BW. Data are mean ± SEM; n = 15-18 mice per group for (a-d), (f) and (g), n = 8 mice per group for (e). *P<0.05; **P<0.005 vs. NC of the same treatment, #P<0.05; ##P<0.005 vs. saline under the same feeding condition.
distribution and diameter. Thus, short-term HFD resulted in a two fold ($P < 0.005$) increase in the average adipocyte diameter compared to NC-fed saline (75 $\pm$ 3.3 $\mu$m vs. 37 $\pm$ 9.4 $\mu$m) and caused a significant shift toward larger adipocytes (Figure 4a,b). Treatment with MnTBAP prevented this shift, restored the normal distribution of adipocyte size and significantly ($P < 0.005$) reduced the mean diameter. It is worth noting that MnTBAP caused a slight but significant increase in the average adipocyte diameter in NC-fed animals (Figure 4a,b). Next, we examined the effect of MnTBAP on adipogenesis and fat uptake in eWAT and found that consistent with prior reports (14,15), HFD enhanced adipogenesis in eWAT as evidenced by the significant ($P < 0.05$) increase in mRNA expression of key adipogenic genes such as $PPAR\gamma$ and $aP2$ (Figure 4c), an induction that was prevented with MnTBAP. Interestingly, these changes in adipogenesis were not observed in iWAT (Supporting Information Figure 3), suggesting a depot-specific effects of HFD and MnTBAP.

MnTBAP treatment attenuated the effects of HFD on de novo lipogenesis

In addition to adipogenesis and fat uptake, we also assessed the expression of genes involved in fatty acid oxidation, lipolysis, and de novo lipogenesis. HFD was associated with a significant ($P < 0.05$) reduction in $PGC1\alpha$ and $PGC1\beta$ mRNA expression in eWAT and iWAT that was not affected by MnTBAP treatment (Table 1 and Supporting Information Table 3). Furthermore, HFD significantly ($P < 0.05$) reduced mRNA expression of fatty acid synthase (FAS) and enhanced stearoyl-CoA desaturase 1 (SCD 1) mRNA in eWAT, consistent with an inhibition of de novo lipogenesis and increased monounsaturation of saturated fatty acids. Although not significant, there was a small attenuation of these effects with MnTBAP treatment in eWAT (Table 1). The reduction in de novo lipogenesis with HFD was also observed in iWAT but it was insensitive to MnTBAP treatment (Supporting Information Table 3). However, HFD-associated $SCD1$ mRNA expression was unique to eWAT as the expression of this gene was rather reduced with HFD and not affected by MnTBAP treatment in iWAT (Supporting Information Table 3), highlighting depot-specific effects of HFD and MnTBAP.

MnTBAP treatment prevented HFD-induced adipocytes apoptosis and inflammation in eWAT

We next examined cell death and inflammation in eWAT and found that apoptotic and necrotic nuclei were observed only in HF-fed saline-treated mice (Figure 5a, arrows). Thus, the sum of apoptotic and necrotic nuclei = total nuclei was increased by three fold in the HF saline group when compared to NC saline group, an effect that was completely prevented by MnTBAP treatment (Figure 5b). As adipocyte death is often associated with inflammation due to the surrounding of dead cells by macrophages, we measured the number crown-like structures as well as $TNF\alpha$ mRNA expression in eWAT. Crown-like structures were more prevalent in eWAT of HF-fed saline-treated mice but rarely seen in HF-fed MnTBAP-treated animals (Figure 5c). Furthermore, $TNF\alpha$ mRNA was enhanced in eWAT of HF-fed animals treated with saline, an effect attenuated with MnTBAP (Figure 5c).
MnTBAP ameliorated the metabolic milieu but failed to prevent HFD-induced systemic insulin resistance and diabetes

Because MnTBAP treatment attenuated diet-induced visceral adiposity, we hypothesized that it would ameliorate the metabolic milieu and the hormonal profile and improve insulin sensitivity. Indeed, MnTBAP significantly \( (P < 0.05) \) reduced serum levels of free fatty acids (FFAs) and triglycerides (TGs) \( (P < 0.05) \) (Table 2). Consistent with reduced adiposity, leptin levels, which were increased by threefold with HFD, were reduced by 34\% \( (P < 0.05) \) with MnTBAP (Table 2). Our short-term HFD did not influence adiponectin levels, but MnTBAP treatment of NC animals caused a slight but significant reduction in adiponectin levels. Despite these improvements in serum FFAs, TGs and leptin levels, MnTBAP treatment failed to reduce fasting glucose and insulin levels (Figure 6a,b). Furthermore, MnTBAP treatment did not prevent glucose intolerance and insulin resistance as evidenced by increased area under the curve for glucose and insulin tolerance tests compared to saline-treated HF-fed group (Supporting Information Figure 5a,b).

MnTBAP treatment failed to rescue HFD-induced mitochondrial dysfunction in skeletal muscle

To explore the mechanisms for persistent insulin resistance with MnTBAP, we examined mitochondrial function in skeletal muscle and showed that MnTBAP treatment failed to rescue the reduction in maximal respiration (VADP) caused by HFD (Figure 6c). Furthermore, ATP/O ratios trended lower in the HF-MnTBAP group (Figure 6d) that was associated with enhanced UCP3 protein content (Supporting Information Figure 4a,b).

Discussion

The present study was designed to test whether reducing oxidative stress was able to prevent diet-induced adiposity, insulin resistance and diabetes in mice. The major findings of this study are: (1) MnTBAP diminished HFD-induced oxidative stress in eWAT; (2) attenuated weight gain and adiposity; (3) prevented diet-induced adipocyte death and adipose tissue inflammation; (4) improved lipid profile and reduced circulating leptin levels; (5) but failed to prevent hyperglycemia and hyperinsulinemia.
Mechanisms of attenuated visceral adiposity with MnTBAP treatment

While low levels of ROS are important for cell signaling, their increase is associated with many diseases. Genetic and nutritional obesity are characterized by increased systemic and adipose tissue oxidative stress but a causal role for ROS in the development of this condition has not been established. Furthermore, ROS are involved in many metabolic alterations associated with obesity such as insulin resistance, diabetes, hepatic steatosis and adipose tissue inflammation (16–19), thus providing a rational for using antioxidant molecules to prevent these conditions. Here we showed that superoxide production increased in eWAT with short-term HF feeding consistent with prior reports (4,20). The mechanisms for increased adipose superoxide generation is less well understood but could involve mitochondrial dysfunction (21), reduced antioxidant defense or enhanced NADPH oxidase activity (4). While no defect in mRNA expression of NOX4, Sod1 or GPX4 was observed, a post-transcriptional regulation affecting their enzymatic activity is possible. In contrast, we observed a compensatory increase in mRNA level of Sod2 (MnSOD) with HF feeding, suggesting mitochondria as the primary source for superoxide, that could diffuse to the cytosol in the form of hydrogen peroxide. Nonetheless, superoxide generation as well as the induction of Sod2 gene by HFD was prevented by MnTBAP treatment. In addition to reducing oxidative stress, we showed for the first time that MnTBAP reduced body weights and adiposity. Consistent with our results, Laurent et al. (22) showed that MnTBAP treatment of genetically obese ob=ob mice resulted in a modest but significant decrease in weight gain but the underlying mechanisms were not investigated. The effect of MnTBAP on body weight and adiposity in our study was confirmed by the decrease in fat mass, brown adipose tissue (BAT) and eWAT weights and circulating leptin levels. MnTBAP specifically targeted BAT and eWAT which could be due to their specific response to HFD. Indeed, both BAT and eWAT are known to expand by hypertrophy through increased lipid accumulation, whereas other depots such as perirenal and iWAT increased mainly by hyperplasia after HFD (23–25). To investigate the mechanisms by which MnTBAP attenuated HFD-induced obesity, several possibilities were examined. First, MnTBAP had no effect on caloric intake, energy expenditure or physical activity. Second, although we did not assess the effect of MnTBAP

**FIGURE 5** MnTBAP treatment attenuated adipocyte death and reduced inflammation in eWAT. (a) Representative images of eWAT sections stained with apoptotic nuclei stain YO-PRO-1 (green) and the cell death stain propidium iodide (PI) (red). The overlap between green and red is represented (arrows). (b) The sum of dead nuclei positive for both YO-PRO-1 and PI over total nuclei. At least 1,000 nuclei were counted per animal from randomly chosen sections. (c) Representative eWAT sections stained with Hematoxylin and Eosin and showing crown-like structures (arrow). (d) Crown-like structures quantification per area on at least five images per mouse. (d) TNFα mRNA expression in eWAT. Data are mean ± SEM; n = 5-7 mice per group for (a-d), n = 3-6 mice per group for (d). *P<0.05 vs. NC of the same treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
treatment on fat absorption, both NC and HF-fed animals treated with this compound showed no clinical evidence of malabsorption as would be seen with induced diarrheal disorders. Furthermore NC-fed animals treated with MnTBAP did not lose weight as you would expect with malabsorption. Third, decreased adiposity with MnTBAP could be caused by reduced adipogenesis and/or increased lipolysis. To test that, we measured mRNA expression of key adipogenic and lipolytic enzymes in eWAT and iWAT. In accordance with prior studies (26, 27), we showed that short-term HFD increased transcription of PPARγ, the master regulator of adipogenesis, as well as one of its downstream targets; the aP2 gene. Interestingly, HF-induced adipogenesis in eWAT was prevented by MnTBAP treatment, which could have participated in the reduction of adiposity. Although it did not reach significance, we observed a slight reduction in mRNA levels of PPARγ, CEBPα, and aP2 in NC-fed MnTBAP-treated animals, which suggest that scavenging physiological levels of ROS could inhibit adipogenesis in vivo as it was previously reported in vitro (28). In addition to reducing the adipogenic program, MnTBAP diminished HF-induced adipocyte hypertrophy as evidenced by smaller adipocyte diameter. This could be due to increased lipolysis, reduced fat intake or enhanced fat oxidation. Basal lipolysis was unaffected by MnTBAP treatment, as evidenced by normal mRNA level of HSLs and reduced rather than increased circulating FFAs. However, assessing mRNA levels is not sufficient to draw a definite conclusion on lipolysis, thus further studies are needed to address whether catecholamine-stimulated lipolysis is different between saline-treated and MnTBAP-treated mice fed HFD. In contrast to lipolysis, mRNA levels of fatty acid translocase CD36 was enhanced in saline-treated but not in MnTBAP-treated mice fed HFD, suggesting that CD36-facilitated fatty acid (FA) uptake, known to increase with HF feeding (29), was reduced with MnTBAP treatment leading to smaller adipocytes. Despite less FA uptake with MnTAP, we did not observe an increase in circulating levels of FFAs and TGs, if anything they were reduced with the treatment raising the possibility for higher FA utilization. Finally, genes involved in fatty acid oxidation and mitochondrial function were similarly altered by HFD in saline and MnTBAP-treated animals respectively whereas genes involved in de novo lipogenesis decreased only in HF-fed saline treated animals.

Mechanisms of prevented adipocyte death and inflammation in eWAT

Adipose tissue inflammation is often associated with oxidative stress, adipocyte death, and the scavenging of dead adipocytes by proinflammatory macrophages (19). We tested the hypothesis that MnTBAP treatment protects against adipocyte death and inflammation. Indeed, we detected more adipocyte death, more crown-like structures and higher TNFα mRNA expressions in eWAT of HF-fed saline-treated mice but not in HF-fed MnTBAP-treated animals. This is to our knowledge the first report to show that the use of a SOD mimic is capable of alleviating inflammation and reducing
cell death in white adipose tissue. The mechanism by which MnTBAp reduced cell death and adipose tissue inflammation could be related to reduce adipocytes hypertrophy. Indeed, adipocytes hypertrophy creates areas of local adipose tissue hypoxia which in turn could trigger the expression of genes involved in inflammation and matrix remodeling (30). Although we did not detect any difference in HIF1α mRNA (data not shown) the stabilization of this protein was not investigated. Furthermore, HF-induced PAI-1 mRNA expression in eWAT was completely prevented by MnTBAp treatment (data not shown), supporting that MnTBAp reduced adipose tissue remodeling.

**Short-term treatment with MnTAP did not prevent HFD-induced systemic insulin resistance and diabetes**

Prior studies have associated oxidative stress to the development of insulin resistance. Indeed, it was proposed that ROS are causal in the development of insulin resistance in 3T3-L1 preadipocytes exposed to TNFα or dexamethasone (31) and in L6 myotubes rendered insulin resistant by exposure to palmitate (32). Furthermore, reducing hydrogen peroxide emission through the use of mitochondria-targeted small peptide antioxidant (SS31) attenuated HFD-induced insulin resistance in vivo (33). In addition, chronic treatment of genetically obese and insulin resistant ob/ob mice with MnTBAp improved insulin sensitivity and glucose tolerance without affecting body weights (31) and animals with enhanced antioxidant capacity such as mice over-expressing mitochondria-targeted catalase, MnSOD, or mice with MnSOD gene delivery in skeletal muscle were either completely or partially protected against aging or HFD-induced glucose intolerance and insulin resistance (32,34,35). In contrast to these studies, other investigations were unable to confirm a causal role for oxidative stress in the etiology of insulin resistance specifically under conditions of higher caloric intake. For example, Paglialunga et al. (36) showed that the use of mitochondria-targeted antioxidant SKQ failed to improve HFD-induced insulin resistance despite reduced oxidative stress in skeletal muscle. Furthermore, mice lacking glutathione peroxidase 1 (GPX1) and exhibiting higher hydrogen peroxide levels are protected against HFD-induced insulin resistance (37). In the present study, we extended our previously published studies showing that protection against oxidative stress in FVB mice reduced weight gain but did not prevent insulin resistance after HFD (10) by demonstrating that scavenging ROS by the use of a SOD mimetic attenuated adiposity and weight gain but was not able to prevent insulin resistance and diabetes. Thus, animals treated with MnTBAp and fed HFD still exhibit fasting hyperglycemia, hyperinsulinemia, and increased area under the curve of glucose and insulin tolerance tests, raising the question whether ROS are causal for development of insulin resistance and diabetes. Taken together, these results suggest that while reducing ROS levels could reduce weight gain, adiposity, and adipose tissue remodeling other mechanisms might be involved in the development of glucose intolerance and insulin resistance in response to short-term HF feeding. Some of these mechanisms may include impaired skeletal muscle glucose uptake, irreversible mitochondrial dysfunction, reduced mitochondrial content, altered mitochondrial dynamics or that the treatment was not long enough to reverse glucose intolerance and insulin sensitivity. Although, we did not investigate muscle glucose uptake in the present study, we examined mitochondrial function in saponin-permeabilized soleus fibers and observed a reduction in ADP-stimulated respiration in HF-fed saline-treated group, that was not recovered by MnTBAp treatment, thus the ATP/O ratios trended lower in HF-fed MnTBAp-treated samples, suggesting a slight uncoupling of the electron transport chain that could limit ATP availability for muscle glucose uptake (38). In conclusion, this study highlights a role for ROS in the development of diet-induced visceral adiposity but not insulin resistance and diabetes.

In summary, these results highlight the role of oxidative stress in the etiology of weight gain caused by HF feeding but not in the development of the associated insulin resistance. Furthermore, these studies reveal novel redox-sensitive pathways involved in visceral adipose tissue expansion and hepatic steatosis caused by dietary obesity and that could be targeted by antioxidant treatment.

**Study limitation**

We acknowledge that there are limitations in these studies such as the type and the duration of the diet used. Indeed, the NC and the HFD used in these studies are not isocaloric as the amount of calories obtained some components of the diet (carbohydrates) is not similar and the composition of these components is also different.

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