

University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

U.S. Department of Veterans Affairs Staff
Publications

U.S. Department of Veterans Affairs

2016

Nanoformulated Copper/Zinc Superoxide Dismutase Reduces Adipose Inflammation in Obesity

Curtis Perriotte-Olson
UNMC, c.perriotteolson@unmc.edu

Nikhil Adi
Veterans Administration Nebraska-Western Iowa Health Care System

Devika S. Manickam
University of North Carolina at Chapel Hill

Rachel A. Westwood
Veterans Administration Nebraska-Western Iowa Health Care System

Cyrus V. Desouza
University of Nebraska Medical Center, cdesouza@unmc.edu

See next page for additional authors

Follow this and additional works at: <http://digitalcommons.unl.edu/veterans>

Perriotte-Olson, Curtis; Adi, Nikhil; Manickam, Devika S.; Westwood, Rachel A.; Desouza, Cyrus V.; Natarajan, Gopalakrishnan; Crook, Alexandra; Kabanov, Alexander V.; and Saraswathi, Viswanathan, "Nanoformulated Copper/Zinc Superoxide Dismutase Reduces Adipose Inflammation in Obesity" (2016). *U.S. Department of Veterans Affairs Staff Publications*. 118.
<http://digitalcommons.unl.edu/veterans/118>

This Article is brought to you for free and open access by the U.S. Department of Veterans Affairs at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in U.S. Department of Veterans Affairs Staff Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Curtis Perriotte-Olson, Nikhil Adi, Devika S. Manickam, Rachel A. Westwood, Cyrus V. Desouza, Gopalakrishnan Natarajan, Alexandra Crook, Alexander V. Kabanov, and Viswanathan Saraswathi

Nanoformulated Copper/Zinc Superoxide Dismutase Reduces Adipose Inflammation in Obesity

Curtis Perriotte-Olson^{1,2}, Nikhil Adi^{1,2}, Devika S. Manickam³, Rachel A. Westwood^{1,2}, Cyrus V. Desouza^{1,2}, Gopalakrishnan Natarajan^{1,2}, Alexandra Crook^{1,2}, Alexander V. Kabanov³, and Viswanathan Saraswathi^{1,2}

Objective: An intimate association exists between oxidative stress and inflammation. Because adipose tissue (AT) inflammation is intricately linked to metabolic disorders, it was hypothesized that reducing oxidative stress would be effective in ameliorating AT inflammation in obesity.

Methods: Wild-type mice were fed a high-fat diet (HF) for 8 weeks followed by a 2-week treatment with nanoformulated copper/zinc superoxide dismutase (NanoSOD). The mice were divided into: 1) chow diet, 2) HF, and 3) HF + NanoSOD.

Results: The HF + NanoSOD-treated mice showed a significant decrease in plasma and liver triglycerides when compared with HF-fed mice. Interestingly, NanoSOD reduced the expression of macrophage and inflammatory markers in visceral AT (VAT) and stromal cells derived from VAT. Moreover, the activation of proinflammatory signaling pathways, in particular, the extracellular signal-regulated kinases, was blunted in VAT on NanoSOD treatment. However, markers of oxidative stress were not altered significantly in the HF + NanoSOD group in the experimental conditions. Pretreatment of either macrophages or adipocytes significantly reduced the inflammatory response invoked in an *in vitro* coculture system, further supporting the role of NanoSOD in inhibiting obesity-linked inflammation.

Conclusions: This data suggest that NanoSOD is effective not only in reducing AT macrophage accumulation and AT inflammation but also in promoting triglyceride metabolism in obesity.

Obesity (2016) **24**, 148–156. doi:10.1002/oby.21348

Introduction

Adipose tissue (AT) inflammation characterized by macrophage accumulation and secretion of inflammatory mediators is an underlying factor leading to obesity-linked metabolic disorders (1,2). Oxidative stress is a prevalent condition in obesity. When obesity persists for a prolonged period of time, antioxidant sources can be depleted, decreasing the activity of enzymes such as superoxide dismutase (SOD) and catalase in erythrocytes (3). The activity of SOD and glutathione peroxidase in individuals with obesity is significantly lower when compared with that in healthy persons (4). In addition to erythrocyte levels, AT levels of the antioxidant enzymes are reduced in obesity (5). The levels of carbonylated proteins, a marker of oxidative

stress, have been shown to be increased in the subcutaneous fat in human subjects with obesity (6). These studies indicate that oxidative stress is closely associated with obesity and that targeting this process may be effective in alleviating obesity-linked metabolic disorders.

Despite the strong association between oxidative stress and obesity, there is a paucity of evidence suggesting the potential of SOD in ameliorating obesity-associated metabolic disorders. Pires et al. (7) have shown that treatment with MnTBAP, a MnSOD mimetic, reduces visceral adiposity and AT inflammation. The effectiveness of antioxidant therapy still remains questionable because of the failure in clinical trials that addressed the benefits of using antioxidant

¹ Department of Internal Medicine/Division of Diabetes, Endocrinology, and Metabolism, University of Nebraska Medical Center, Omaha, Nebraska, USA. Correspondence: Viswanathan Saraswathi (s.viswanathan@unmc.edu) ² Research Service, Veterans Administration Nebraska-Western Iowa Health Care System, Omaha, Nebraska, USA ³ Division of Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA.

Funding agencies: This project was supported by the NIH-Nebraska Center for Nanomedicine COBRE Grant (2P20GM103480). A.V.K. and D.S.M. also acknowledge support of The Carolina Partnership, a strategic partnership between the University of North Carolina at Chapel Hill Eshelman School of Pharmacy and the University Cancer Research Fund through the Lineberger Comprehensive Cancer Center.

Disclosure: A.V.K. is the coinventor of the nanozyme technology at University of Nebraska Medical Center (patent number: WO2008141155A1). The technology and products have been exclusively out-licensed to NeuroNano Pharma, a start-up company located in Chapel Hill, North Carolina. A.V.K. is a cofounder, shareholder, and director of this company. The other authors declared no conflict of interest.

Author contributions: V.S. conceived and designed the experiments and analyzed the data; V.S., C.P.O., N.A., R.A.W., G.N., and A.C. conducted the experiments and analyzed data; V.S. and C.P.O. wrote the manuscript; D.S.M. and A.V.K. provided the NanoSOD. All authors were involved in the review of the manuscript and had final approval of the submitted version.

Received: 23 April 2015; **Accepted:** 26 August 2015; **Published online 27 November 2015.** doi:10.1002/oby.21348

This document is a U.S. government work and is not subject to copyright in the United States.

vitamins in treating human diseases (8,9). The lack of potent and highly bioavailable antioxidants is considered to be a major challenge in antioxidant therapy. With the advent of nanotechnology, research on antioxidant enzyme therapy has gained increased momentum.

Several types of nanoformulated antioxidant enzymes, in particular, SOD1, have been designed and studied for their efficacy in ameliorating various diseases. For example, liposome-encapsulated Cu/ZnSOD (SOD1) reduced angiotensin II (Ang II)-induced hypertension (10). Rosebaugh et al. (11) have reported the benefits of poly(ethyleneimine)-poly(ethylene glycol) (PEI-PEG)-conjugated Cu/ZnSOD nanozyme in attenuating Ang II-induced pressor response in rabbits. Recently, a novel formulation of Cu/ZnSOD was developed wherein SOD1 is covalently stabilized and crosslinked to improve the stability and efficacy of SOD1 *in vivo*. Manickam et al. (12) have reported that this formulation of Cu/ZnSOD (hereafter referred to as NanoSOD) is very effective in scavenging superoxide in cultured endothelial and neuronal cells *in vitro* and in reducing ischemic brain injury in rats. In the current study, we sought to determine the effectiveness of NanoSOD in reducing AT inflammation in obesity. Because oxidative stress is prevalent in obesity and because agents with antioxidant properties can reduce inflammation, we hypothesized that NanoSOD will be effective in reducing AT inflammation and systemic metabolic homeostasis in a model of diet-induced obesity.

Methods

Mice and diet

Male wild-type C57BL/6 mice were purchased from Jackson Laboratory and fed a chow diet (CD; #5001, Purina Laboratory Diet) or a high-fat diet (HF; #D12451, Research Diets Inc.) for 8 weeks. The diet provides 20% protein, 35% carbohydrates, and 45% fat in terms of calories. Lard was used as a fat source.

NanoSOD preparation

NanoSOD was synthesized and characterized as reported earlier (12). Briefly, native bovine Cu/ZnSOD protein (Sigma-Aldrich) was mixed with poly(L-lysine)-poly(ethylene glycol) copolymer (PLL-PEG). The Cu/ZnSOD and PLL-PEG complex was covalently stabilized using a reducible crosslinker, 3,3'-dithiobis(sulfosuccinimidyl propionate) (Thermo Fisher Scientific). The size of NanoSOD was estimated to be ~35 nm.

NanoSOD treatment

Eight weeks post-diet, the mice were divided into three groups: 1) CD, 2) HF, and 3) HF diet + NanoSOD (HF + NanoSOD). The HF-fed mice were weight-matched before the treatment. A cohort of HF diet-fed mice were injected intraperitoneally (i.p.) with NanoSOD once every other day for a period of 15 days (eight injections total). The mice were continued on respective diets until sacrifice. Lean and fat mass were measured by EchoMRI before sacrifice. Twenty-four hours post-injection of the last dose of NanoSOD, the mice were sacrificed after 5-h fasting. All animal care procedures were carried out with approval from the Institutional Animal Care and Use Committee of VA Nebraska-Western Iowa Health Care System.

Metabolic variables

Blood glucose was measured using the Accucheck Aviva glucometer. Plasma free fatty acids (FFAs) were measured using a kit (Wako). Plasma total cholesterol and triglycerides (TGs) were measured

using kits from Raichem. Plasma insulin was measured using kits from Mercodia.

In vivo metabolic studies

A separate set of mice were used for energy expenditure and intra-peritoneal insulin (ITT) and glucose tolerance tests (GTT). Because of the short duration of the treatment, we measured energy expenditure after two or four injections, ITT after five injections, and GTT after seven injections of NanoSOD. For energy expenditure studies, mice were acclimatized for 2 days and housed individually in Promethion cages (Sable Systems International). We recorded VO₂, VCO₂, and food intake for 36 h. Measurements for 12-h light and 12-h dark cycles are reported separately. For ITT and GTT, mice were fasted for 5 h and injected (i.p.) with insulin (0.75 U/kg body weight) and glucose (1 g/kg body weight), respectively. The changes in blood glucose levels were recorded at various time points.

Isolation of stromal vascular cells

Stromal vascular cells (SVCs) were isolated from visceral adipose tissue (VAT) as we described earlier (13).

Real-time PCR analysis

Real-time PCR analysis was carried out using primers from Applied Biosystems. A $\Delta\Delta$ CT method was used to calculate gene expression, and the values were normalized to 18S.

Western blot analysis

Antibodies against ERK and phospho-ERK were purchased from Cell Signaling Technologies. Antibodies for SOD1, MCP-1, and GAPDH were obtained from Santa Cruz Biotechnology. IRDye 680 and 800 conjugated secondary antibodies were obtained from LI-COR Biosciences.

Histology

Liver sections were stained with hematoxylin and eosin (H&E). For immunofluorescence, the VAT sections were incubated with anti-F4/80 primary antibody (AbD Serotec) followed by incubation with Alexa Fluor 568 conjugated secondary antibody (Life Technologies). The sections were mounted with antifade gold containing 4',6-diamidino-2-phenylindole (Invitrogen). The pictures were taken at 10 \times or 20 \times magnification using a Nikon eclipse 80i inverted fluorescence microscope.

Antioxidant status

Antioxidants in VAT such as SOD (activity) and total glutathione were determined using kits from Cayman Chemical. Ascorbic acid was determined using a kit from ABCAM.

Macrophage and adipocyte coculture

Thioglycollate-elicited mouse primary peritoneal macrophages were collected as we described previously (14). Differentiation of 3T3-L1 cells to adipocytes was performed as we reported earlier with minor modifications (15). We obtained hypertrophic adipocytes (adipocytes with large lipid droplets) by maintaining them in culture for 4–6 weeks before they were used for the coculture experiments. In Experiment 1, macrophages were pretreated with NanoSOD for 3 h followed by rinsing in PBS. Macrophages were then added onto adipocytes at 40,000 cells per well in 12-well culture dishes. We collected media after 24 h

TABLE 1 Effect of NanoSOD on metabolic parameters

Measurement	n	CD	HF	HF + Nano
Body weight (g)	20–22	26.3 ± 0.5	34.9 ± 0.8***	34.2 ± 0.8***
Body weight gain (g)	20–22	5.3 ± 0.3	12.5 ± 0.7***	12.6 ± 0.6***
Fat mass (g)	14–16	2.35 ± 0.13	9.69 ± 0.6***	9.48 ± 0.59***
Lean mass (g)	14–16	22.5 ± 0.7	24.2 ± 0.7	23.5 ± 0.5
Liver weight (g)	20–22	1.12 ± 0.04	1.17 ± 0.06	1.13 ± 0.03
VAT weight (g)	20–22	0.39 ± 0.02	1.94 ± 0.08***	1.73 ± 0.12***
Subcutaneous adipose tissue weight (g)	6–8	0.08 ± 0.01	0.40 ± 0.07*	0.43 ± 0.05**
Perirenal adipose tissue weight (g)	6–8	0.08 ± 0.02	0.55 ± 0.09*	0.78 ± 0.10**

* $P < 0.01$.** $P < 0.001$.*** $P < 0.0001$ vs. CD.

Values are mean ± SEM for each group.

of coculture and cell lysates after 48 h. In Experiment 2, adipocytes were pretreated with NanoSOD for 6 h followed by rinsing in PBS. Untreated control macrophages were then added onto adipocytes, and media and cell lysates were collected. The media were analyzed for MCP-1 by enzyme-linked immunosorbent assay (ELISA) and cell lysates for SOD1 content by Western blot analysis.

ELISA

MCP-1 protein in cell culture supernatant was measured by sandwich ELISA. Briefly, diluted cell culture supernatant was added to a 96-well plate that was coated with hamster anti-mouse MCP-1 antibody

(BD Biosciences). After careful washes, biotin-conjugated hamster anti-mouse MCP-1 antibody was added (BD Biosciences). Next, avidin peroxidase was added followed by color-developing reagents.

Gas chromatography

Hepatic TGs were analyzed by gas chromatography at the Lipid Core Laboratory of Vanderbilt University.

Statistical analysis

The results are presented as mean ± SEM. Statistical significance was determined by one-way analysis of variance followed by Bonferroni's *post hoc* analysis. In experiments involving two groups, a one-tailed Student's *t* test was performed. Graph-Pad Prism software was used to determine statistical significance ($P < 0.05$ was considered significant). Outliers were detected using the Grubb's test or box plot analysis (values ± 1.5 times the interquartile range) and were removed from the data set.

Results

Effect of NanoSOD on metabolic variables

The body weight, body weight gain, and fat mass were significantly higher in HF and HF + NanoSOD groups when compared with CD controls (Table 1). The weights of individual fat pads such as VAT, subcutaneous AT, and perirenal AT were significantly higher in HF and HF + NanoSOD groups when compared with CD controls. However, no significant difference was noted between HF and HF + NanoSOD-treated mice. The plasma total cholesterol was increased significantly ($P < 0.0001$) in both HF and HF + Nano groups when compared with CD controls (Figure 1A). The plasma FFAs were not altered, whereas plasma TGs were significantly reduced ($P < 0.01$) in HF + Nano group ($P < 0.05$) when compared with HF-fed mice (Figure 1B,C). The fasting blood glucose level was

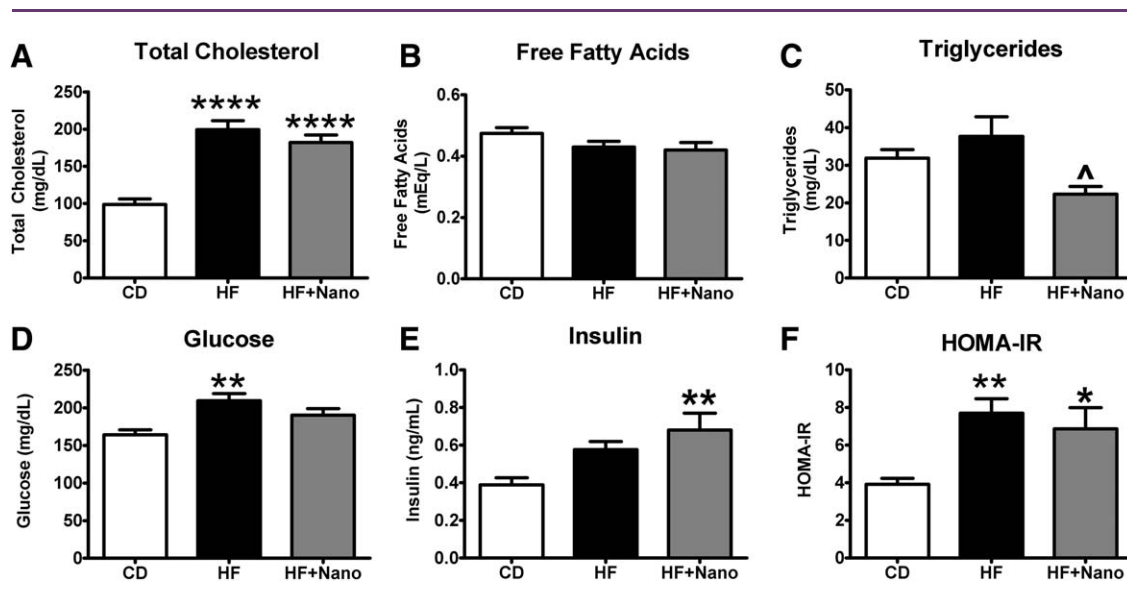


Figure 1 Effect of NanoSOD on metabolic variables in mice exhibiting diet-induced obesity. (A) Total cholesterol, (B) free fatty acids, (C) triglycerides, (D) fasting blood glucose, (E) insulin, and (F) insulin resistance via HOMA-IR were recorded. Values are expressed as mean ± SEM of 12–14 samples in each group. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ vs. CD; * $P < 0.05$ vs. HF. CD, chow diet; HF, high-fat diet; Nano, NanoSOD.

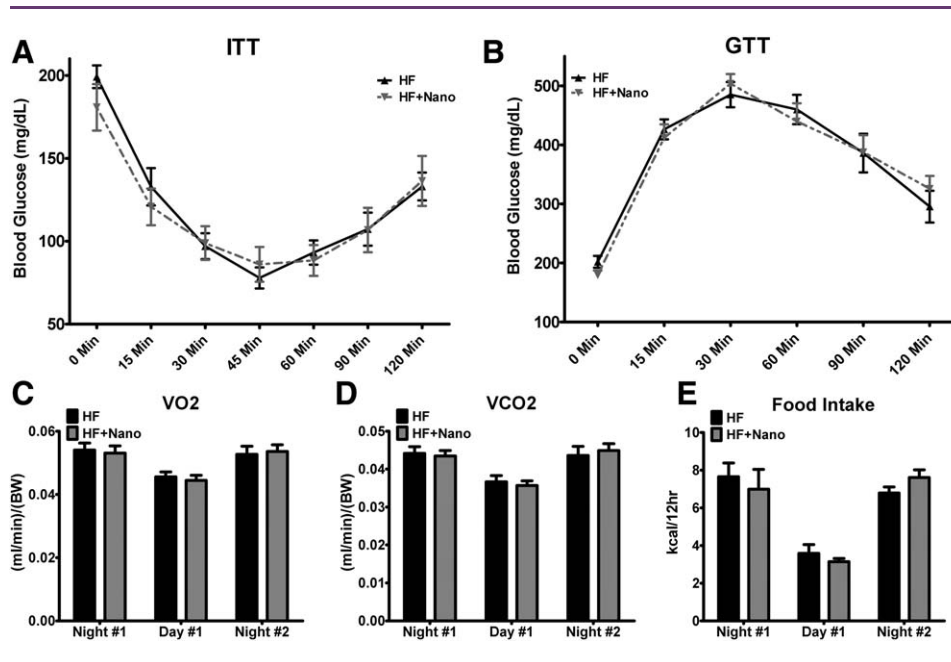


Figure 2 Effect of NanoSOD on insulin and glucose tolerance, energy expenditure, and food intake. Fasted mice were intraperitoneally injected with (A) insulin and (B) glucose, and blood glucose was recorded at select time points. Energy expenditure in the form of (C) VO_2 , and (D) VCO_2 and (E) food intake was measured using a Promethion metabolic system. Energy expenditure was measured after two or four injections, and values are expressed as mean \pm SEM of seven to eight samples in each group. Food intake was measured after four or five injections, and values are mean \pm SEM of four to five samples in each group. HF, high-fat diet; Nano, NanoSOD.

significantly increased in HF ($P < 0.01$) but not in HF + NanoSOD mice when compared with CD-fed mice. The plasma insulin level was significantly higher ($P < 0.01$) in HF + Nano group when compared with CD controls. HOMA-IR, a measure of insulin resistance, was significantly increased in both HF ($P < 0.01$) and HF + NanoSOD ($P < 0.05$)-treated mice (Figure 1D–F). ITT and GTT revealed that the response of HF + Nano mice to acute insulin or glucose exposure was not altered when compared with HF controls (Figure 2A,B). The analysis of energy expenditure revealed that VO_2 and VCO_2 did not vary between HF and HF + Nano groups after two or four injections with NanoSOD (Figure 2C,D). In addition, food intake was not altered between HF and HF + Nano-treated mice (Figure 2E). Altogether, these data suggest that NanoSOD treatment for 2 weeks resulted in reduced plasma TG without improvements in glucose handling.

Effect of NanoSOD on VAT inflammation

Because macrophages accumulate in AT in obesity and secrete inflammatory mediators, we next analyzed macrophage and inflammatory markers in whole VAT. Immunofluorescence analysis revealed that F4/80, a macrophage marker, is lower in HF + Nano group when compared with HF-fed mice (Figure 3A,B). Real-time PCR analysis of VAT for the mRNA expression of F4/80 showed a profound increase only in HF-fed mice, whereas HF + Nano-treated mice showed a reduction (Figure 3C). The markers of inflammation including MCP1, TNF α , and MMP12 were significantly increased in HF diet-fed mice when compared with CD controls, whereas treatment with NanoSOD greatly reduced the expression of these genes. We next analyzed the mRNA levels of macrophage and/or inflammatory markers in macrophage-rich SVCs collected from VAT. As shown in Figure 3D, the mRNA level of F4/80 was significantly

reduced on treatment with NanoSOD when compared with HF controls. Moreover, we noted that markers of inflammation such as MCP1 α and MMP12 were significantly lower in NanoSOD-treated mice when compared with HF controls.

We also noted that the plasma level of MCP1 was significantly lower in HF + NanoSOD-treated mice when compared with HF group (Figure 3E). We next determined whether reduction in inflammation is associated with an increase in SOD1 protein in HF + Nano-treated mice (Figure 3F–H). Although the inflammatory markers were reduced, the protein level of SOD1 was not altered significantly in VAT, SVCs, and plasma samples of HF + Nano-treated mice. Altogether, these data show that NanoSOD treatment is highly effective in reducing AT and systemic inflammation; however, the protein levels of SOD1 are not altered significantly when mice are sacrificed 24 h post-treatment.

Western blot analysis of VAT for signaling pathways activated by oxidative stress, in particular, ERK1/2, showed that the phosphorylation of this protein was significantly increased in HF when compared with CD controls. On the other hand, NanoSOD treatment leads to a profound decrease in phospho-ERK1/2 when compared with HF diet-fed mice (Figure 4A). However, markers of oxidative stress/antioxidant response such as SOD activity, GSH depletion, and ascorbate levels were not altered significantly (Figure 4B–D).

Pretreatment of macrophages or adipocytes with NanoSOD reduces coculture-induced inflammatory response

To determine the direct effect of NanoSOD in delivering SOD1 to adipocytes and/or macrophages and in reducing their inflammatory

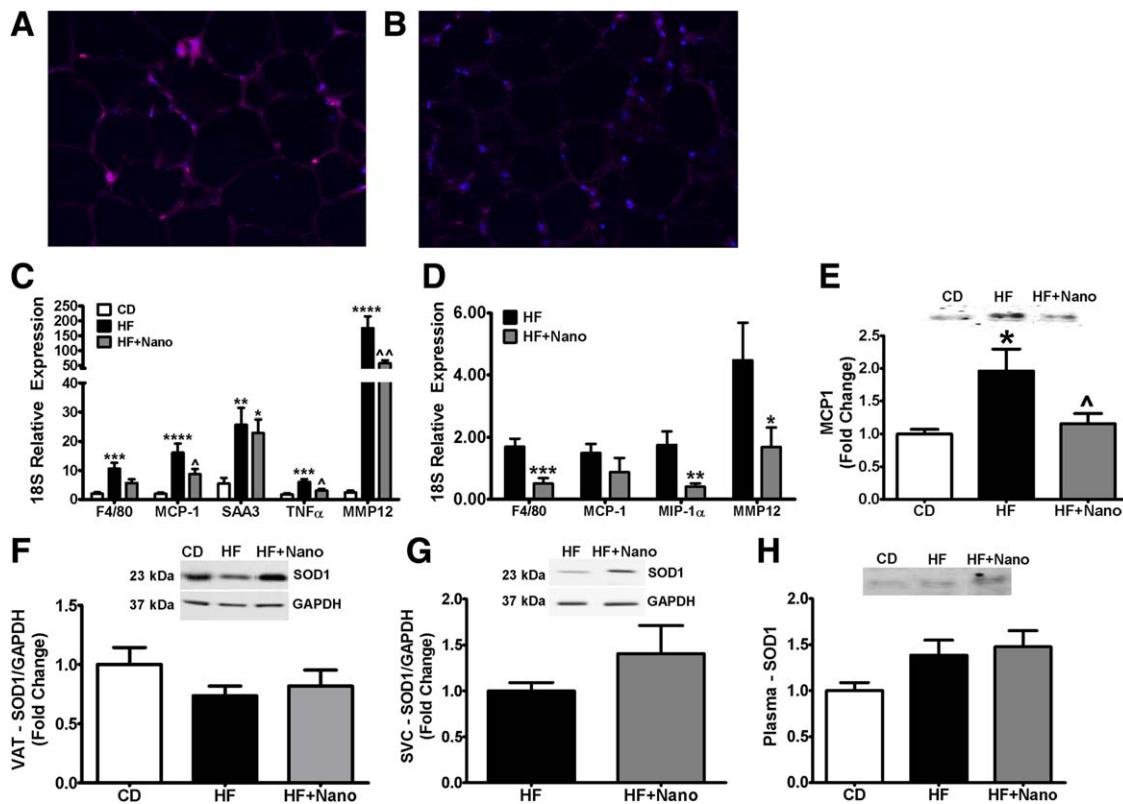


Figure 3 Effect of NanoSOD on VAT and systemic inflammation and SOD1 bioavailability. VAT sections from (A) HF and (B) HF + Nano were assessed for F4/80 expression, and fluorescent images were obtained at $\times 20$ magnification. Markers of inflammation were determined in (C) VAT and (D) SVC via real-time PCR. (E) MCP1 protein levels were also analyzed in the plasma. SOD1 bioavailability was determined in (F) VAT, (G) SVCs, and (H) plasma via Western blot analysis. All mRNA values were normalized to 18S, and protein levels were normalized to GAPDH. Values are expressed as mean \pm SEM of 10–14 samples in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ vs. CD (all but 3D) or HF (3D); $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ vs. HF. CD, chow diet; HF, high-fat diet; Nano, NanoSOD.

response, we performed an *in vitro* coculture study (16). In this experiment, we determined the impact of preloading macrophages versus adipocytes with NanoSOD in modulating the inflammatory response provoked on their interaction. Our data show that pretreatment of either macrophages or adipocytes with NanoSOD attenuates coculture-induced inflammatory response as detected by MCP-1 secretion (Figure 5A,B). Our data also show that pretreatment of macrophages did not increase cellular SOD1 content (Figure 5C) likely due to the smaller number of macrophages added to the coculture system (40,000 cells per well). However, pretreatment of adipocytes with NanoSOD leads to a significant increase in intracellular SOD1 protein (Figure 5D). These data suggest that nanoformulation of SOD1 actually increases the delivery of SOD1 protein to cells.

Effect of NanoSOD on hepatic lipid accumulation

Obesity is associated with excess lipid accumulation in liver leading to hepatic steatosis. The mRNA expression of carnitine palmitoyl transferase-1, an enzyme involved in fatty acid metabolism, was increased only in HF diet-fed mice but not in HF + NanoSOD-treated mice (Figure 6A). The mRNA expression of *ACOX-1*, an enzyme involved in peroxisomal fatty acid metabolism, and, *SREBF1*, a gene promoting lipogenesis, was not altered among different groups. Interestingly, the expression of fatty acid synthase

(*FASN*), another lipogenic enzyme involved in *de novo* fatty acid synthesis, was reduced in HF + NanoSOD-treated mice when compared with HF-fed mice. H&E staining of liver sections showed that the lipid accumulation was greatly increased in HF group when compared with CD controls. We noted a marked reduction in lipid droplets in HF + NanoSOD-treated mice (Figure 6B–D). Moreover, the levels of hepatic TGs were significantly reduced in HF + Nano when compared with HF-fed mice (Figure 6E). Altogether, these data show that NanoSOD reduces hepatic lipid accumulation and suggest that inhibition of *de novo* lipogenesis in liver may be one mechanism for reduced plasma and liver TGs in these mice.

Discussion

In the current study, we have demonstrated that treating obese mice with NanoSOD for only 2 weeks results in attenuation of AT inflammation characterized by reduction in macrophage and inflammatory markers in VAT as well as in VAT stromal cells. This was associated with a reduction in ERK1/2 activation, a signaling pathway activated by both oxidative stress and inflammation. Furthermore, we provide evidence that pretreatment of macrophages or adipocytes with NanoSOD can attenuate coculture-induced inflammatory response *in vitro*.

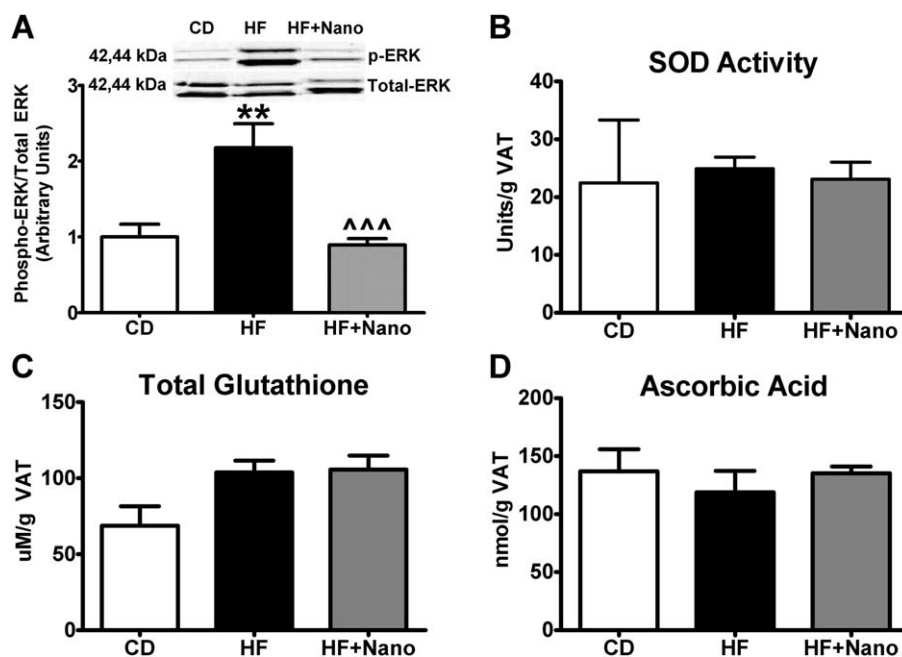


Figure 4 Effect of NanoSOD on VAT oxidative stress. (A) Phosphorylation of ERK1/2, as well as markers of antioxidant defense such as (B) superoxide dismutase activity, (C) total glutathione, and (D) ascorbic acid, were analyzed. P-ERK was normalized to total ERK. Values are expressed as mean \pm SEM of 6–14 samples in each group. ** $P < 0.01$ vs. CD; *** $P < 0.001$ vs. HF. CD, chow diet; HF, high-fat diet; Nano, NanoSOD.

Our data also demonstrate that plasma and liver TG levels are reduced on NanoSOD treatment indicating improvements in lipid homeostasis. However, NanoSOD treatment did not alter markers of oxidative stress in VAT in our experimental condition. Moreover, systemic glucose homeostasis was not altered between HF and HF + NanoSOD-treated mice. Altogether, our data suggest that NanoSOD treatment can reduce AT inflammation and systemic and liver TG, which, in turn, could reduce obesity-linked lipid disorders including hypertriglyceridemia and nonalcoholic fatty liver disease.

Evidence from animal and human studies suggest that oxidative stress in AT may contribute to the development of inflammation (17,18) and insulin resistance in obesity (19–21). An important finding of our study is that NanoSOD treatment leads to a profound reduction in macrophage and inflammatory markers in AT. The *in vitro* coculture studies provide further evidence for the direct role of NanoSOD in delivering SOD1 to adipocytes and/or macrophages and reducing macrophage and/or adipocyte inflammation, which, in turn, can reduce AT inflammation in obesity. The reduced AT macrophage accumulation and AT inflammation may also be due to improved lipid homeostasis (reduced plasma and liver TGs). This notion is supported by a previous report showing that increased local and circulating FFAs in obesity was associated with increased AT macrophage accumulation (22). Therefore, it is very likely that an improvement in systemic and local fatty acid/TG homeostasis can also reduce AT macrophage accumulation and inflammation.

A previous study by Pires et al. (7) has shown that simultaneous exposure of mice to HF diet and MnTBAP, a MnSOD mimetic, reduces AT inflammation and systemic FFA levels. In line with this

study, we provide evidence that NanoSOD treatment led to a reduction in AT inflammation. Our study differs from that of Pires et al. in several ways. First, we used nanoformulated Cu/ZnSOD, whereas Pires et al. used a MnSOD mimetic. Next, we studied the therapeutic effect of NanoSOD by first placing the mice on a HF diet followed by treatment with NanoSOD. In their study, the preventive effect of MnTBAP was assessed. Finally, our data show that in addition to reducing AT inflammation, NanoSOD reduced plasma and liver TG levels. Altogether, these studies provide critical information regarding the role of superoxide in modulating obesity-linked inflammation and lipid metabolism.

It is interesting to note that the plasma and liver TGs were significantly lower in HF + Nano when compared with HF-fed mice suggesting an improved TG metabolism. The link between SOD1 and metabolism is evident from the fact that SOD1 mutant mice with gain of function exhibit hypermetabolism (23,24) characterized by increased TG metabolism in muscle. Moreover, mice lacking SOD1 have been shown to exhibit increased lipid accumulation in liver via increased lipogenesis (25). Our data show that the expression of FASN, a lipogenic enzyme, is significantly reduced in liver on NanoSOD treatment. Therefore, it is possible that similar mechanisms play a role in reducing TG levels in plasma and liver in our study.

Regarding signaling mechanisms, we noted that activation of ERK1/2 was increased in HF-fed mice, whereas NanoSOD attenuated this effect. Evidence suggests that ERK1/2 is activated by FFAs and oxidative stress ((26); reviewed in ref. 27). Moreover, bioflavonoids and triterpenoids with known antioxidant properties have been shown to attenuate inflammation via inhibiting ERK1/2 phosphorylation (28–

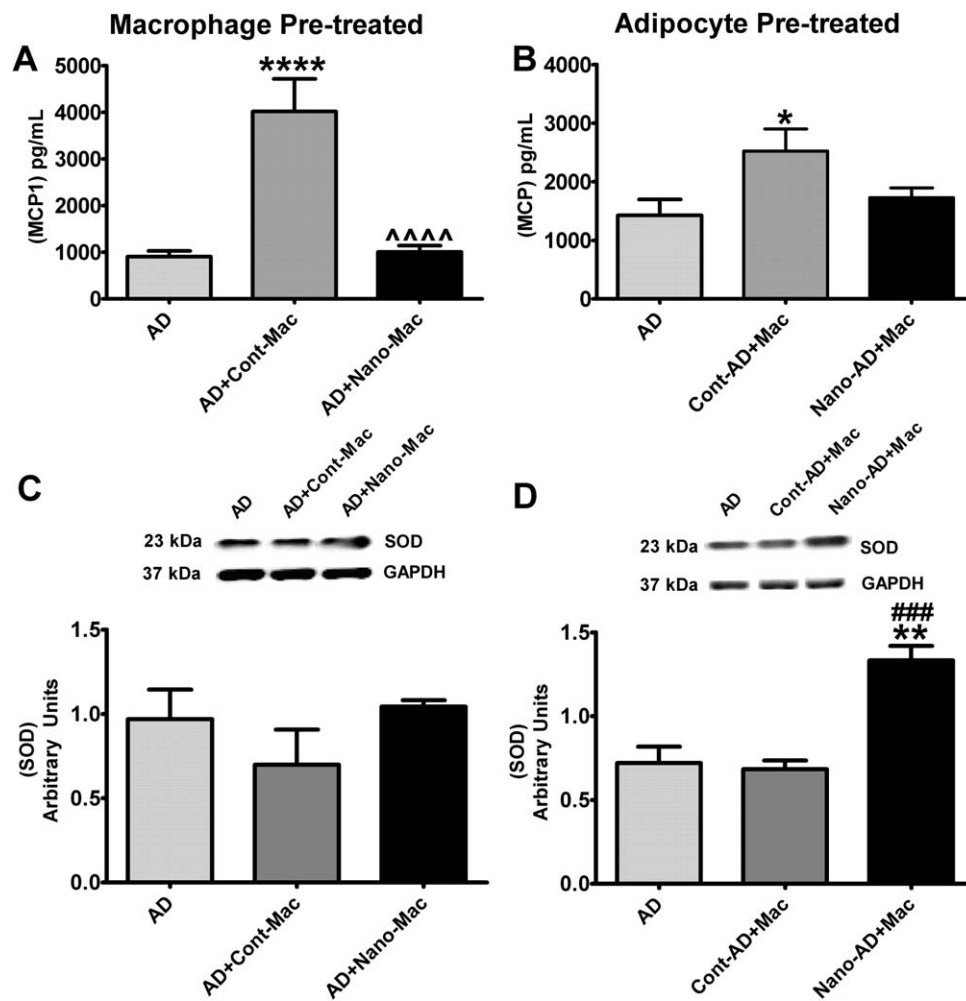


Figure 5 Effect of NanoSOD on inflammatory response in adipocyte and macrophage cocultures. MCP1 secretion by (A) adipocytes cocultured with NanoSOD-pretreated macrophages (Nano-mac) and (B) NanoSOD-pretreated adipocytes (Nano-AD) cocultured with macrophages. MCP-1 levels in coculture media samples at 24 h were analyzed by ELISA. Cell lysates from adipocytes cocultured with (C) NanoSOD-pretreated macrophages and (D) NanoSOD-pretreated adipocytes cocultured with control macrophages for 48 h were analyzed for intracellular SOD1 levels by Western blotting. Values are expressed as mean \pm SEM of 6–12 samples for ELISA (A and B) and 3–6 samples for Western blot (C and D) in each group. Protein was normalized to GAPDH. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ vs. AD; ^^^^^ $P < 0.0001$ vs. AD + Cont-Mac; ### $P < 0.01$ vs. Cont-AD + Mac. AD, adipocytes; AD + Cont-Mac, adipocytes with nontreated macrophages; AD + Nano-Mac, adipocytes with NanoSOD-treated macrophages; Cont-AD + Mac, nontreated adipocytes with macrophages; Nano-AD + Mac, NanoSOD-treated adipocytes with macrophages.

30). ERK1/2 activation can lead to activation of NF- κ B, which, in turn, can activate the inflammatory response. We previously showed that treatment of endothelial cells with linoleic acid, a FFA, can induce oxidative stress and inflammatory response and that this was mediated via ERK1/2 and NF- κ B (26). Therefore, it is very likely that NanoSOD reduces inflammatory response via blunting ERK1/2 activation.

Despite the promising effects seen with regard to AT inflammation and lipid homeostasis in HF + Nano-treated mice, we were not able to show a change in markers of oxidative stress in these mice. One possible reason for this could be our experimental condition. For example, we sacrificed mice 24 h after injecting the last dose of NanoSOD. It is possible that NanoSOD was effective in the initial hours and then removed from the system likely via proteolytic deg-

radation. However, the effectiveness of NanoSOD in scavenging superoxide is evident from previous studies. For example, NanoSOD reduced superoxide-induced oxidative stress in cultured endothelial and neuronal cells *in vitro* (12). Moreover, our *in vitro* data provide evidence that NanoSOD is effective in increasing SOD1 protein in adipocytes. Therefore, it is possible that improvements seen in AT inflammation and metabolic markers in our study could be due to improved superoxide-scavenging effects of NanoSOD. However, further time course studies will be required to confirm this notion *in vivo*.

It should be pointed out that clinical trials in humans did not support the benefit of antioxidant therapy against cardiovascular disease (reviewed in ref. 8). The failure of these trials could be due to the

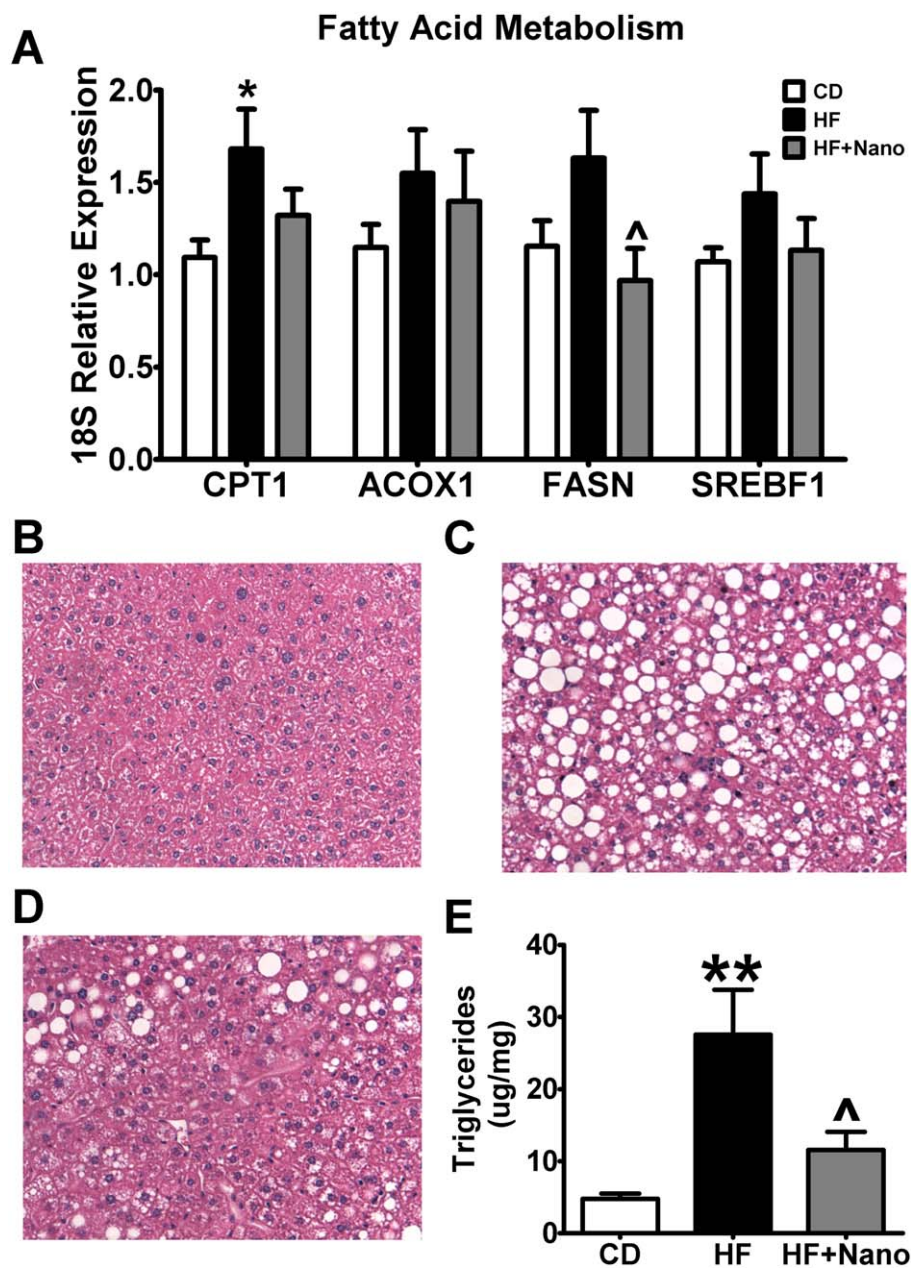


Figure 6 Effect of NanoSOD on genes modulating fatty acid metabolism and hepatic steatosis. (A) Markers of fatty acid metabolism were assessed in liver samples by real-time PCR. Hepatic lipid accumulation in (B) CD, (C) HF, and (D) HF + Nano was analyzed using hematoxylin and eosin staining, whereas (E) hepatic triglycerides were determined using gas chromatography. All mRNA values were normalized to 18S. Values are expressed as mean \pm SEM of 10–14 samples in each group. * $P < 0.05$ and ** $P < 0.01$ vs. CD; [^] $P < 0.05$ vs. HF. CD, chow diet; HF, high-fat diet; Nano, NanoSOD.

choice of the antioxidants and the endpoints. For example, mostly vitamins were used as antioxidants, and vitamin E, in particular, can exert prooxidant effects under certain conditions (31,32). Moreover, although the role of antioxidant vitamins on cardiovascular disease and cancer has been studied in humans, not much is known regarding their effects on metabolic pathways. Our findings are relevant to human diseases in two ways. First, our findings have implications in considering NanoSOD as a novel therapy to target AT inflammation

and lipid disorders in obesity. Second, patients with amyotrophic lateral sclerosis (ALS) with increased SOD activity exhibit hypermetabolism, and a high-caloric diet may improve their energy balance and survival (33,34). Our data suggest the role of reduced *de novo* fatty acid synthesis as a potential mechanism for improved energy balance in patients with ALS on a high-caloric diet. Overall, our study provides a better understanding on the role of NanoSOD in reducing AT inflammation and improving lipid homeostasis in obesity. **O**

Acknowledgements

The authors thank Drs. Tatiana Bronich and Irving Zucker for their support and feedback on this project and in the critical review of this manuscript. Lipid profiles were analyzed at the Lipid Core Laboratory of the Mouse Metabolic Phenotyping Center at Vanderbilt University (DK59637). This study is the result of work conducted with the resources and the facilities at the VA-Nebraska Western Iowa Health Care System, Omaha, Nebraska.

© 2015 The Obesity Society

References

- Bai Y, Sun Q. Macrophage recruitment in obese adipose tissue. *Obes Rev* 2015;16:127-136.
- Revelo XS, Luck H, Winer S, Winer DA. Morphological and inflammatory changes in visceral adipose tissue during obesity. *Endocr Pathol* 2014;25:93-101.
- Amirkhizi F, Siassi F, Djalali M, Foroushani AR. Assessment of antioxidant enzyme activities in erythrocytes of pre-hypertensive and hypertensive women. *J Res Med Sci* 2010;15:270-278.
- Ozata M, Mergen M, Oktenli C, et al. Increased oxidative stress and hypozincemia in male obesity. *Clin Biochem* 2002;35:627-631.
- Uthus EO, Picklo MJ, Sr. Obesity reduces methionine sulphoxide reductase activity in visceral adipose tissue. *Free Radic Res* 2011;45:1052-1060.
- Frohnert BI, Sinaiko AR, Serrot FJ, et al. Increased adipose protein carbonylation in human obesity. *Obesity (Silver Spring)* 2011;19:1735-1741.
- Pires KM, Ilkun O, Valente M, Boudina S. Treatment with a SOD mimetic reduces visceral adiposity, adipocyte death, and adipose tissue inflammation in high fat-fed mice. *Obesity (Silver Spring)* 2014;22:178-187.
- Steinberg D, Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? *Circulation* 2002;105:2107-2111.
- Pashkow FJ. Oxidative stress and inflammation in heart disease: do antioxidants have a role in treatment and/or prevention? *Int J Inflamm* 2011;2011:514623.
- Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation* 1997;95:588-593.
- Rosenbaugh EG, Roat JW, Gao L, et al. The attenuation of central angiotensin II-dependent pressor response and intra-neuronal signaling by intracarotid injection of nanoformulated copper/zinc superoxide dismutase. *Biomaterials* 2010;31:5218-5226.
- Manickam DS, Brynskikh AM, Kopanic JL, et al. Well-defined cross-linked antioxidant nanozymes for treatment of ischemic brain injury. *J Control Release* 2012;162:636-645.
- Saraswathi V, Ramnanan CJ, Wilks AW, et al. Impact of hematopoietic cyclooxygenase-1 deficiency on obesity-linked adipose tissue inflammation and metabolic disorders in mice. *Metabolism* 2013;62:1673-1685.
- Saraswathi V, Hastay AH. Inhibition of long-chain acyl coenzyme A synthetases during fatty acid loading induces lipotoxicity in macrophages. *Arterioscler Thromb Vasc Biol* 2009;29:1937-1943.
- Murali G, Desouza CV, Clevenger ME, Ramalingam R, Saraswathi V. Differential effects of eicosapentaenoic acid and docosahexaenoic acid in promoting the differentiation of 3T3-L1 preadipocytes. *Prostaglandins Leukot Essent Fatty Acids* 2014;90:13-21.
- Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor- α . *Arterioscler Thromb Vasc Biol* 2005;25:2062-2068.
- Furukawa S, Fujita T, Shimabukuro M, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004;114:1752-1761.
- Bondia-Pons I, Ryan L, Martinez JA. Oxidative stress and inflammation interactions in human obesity. *J Physiol Biochem* 2012;68:701-711.
- Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 2006;440:944-948.
- Grimsrud PA, Picklo MJ, Sr., Griffin TJ, Bernlohr DA. Carbonylation of adipose proteins in obesity and insulin resistance: identification of adipocyte fatty acid-binding protein as a cellular target of 4-hydroxynonenal. *Mol Cell Proteomics* 2007;6:624-637.
- Paglialunga S, Ludzki A, Root-McCaig J, Holloway GP. In adipose tissue, increased mitochondrial emission of reactive oxygen species is important for short-term high-fat diet-induced insulin resistance in mice. *Diabetologia* 2015;58:1071-1080.
- Kosteli A, Sogari E, Haemmerle G, et al. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *J Clin Invest* 2010;120:3466-3479.
- Dupuis L, Oudart H, Rene F, Gonzalez de Aguilar JL, Loeffler JP. Evidence for defective energy homeostasis in amyotrophic lateral sclerosis: benefit of a high-energy diet in a transgenic mouse model. *Proc Natl Acad Sci USA* 2004;101:11159-11164.
- Fergani A, Oudart H, Gonzalez De Aguilar JL, et al. Increased peripheral lipid clearance in an animal model of amyotrophic lateral sclerosis. *J Lipid Res* 2007;48:1571-1580.
- Wang L, Jiang Z, Lei XG. Knockout of SOD1 alters murine hepatic glycolysis, gluconeogenesis, and lipogenesis. *Free Radic Biol Med* 2012;53:1689-1696.
- Hennig B, Lei W, Arzuaga X, Ghosh DD, Saraswathi V, Toborek M. Linoleic acid induces proinflammatory events in vascular endothelial cells via activation of PI3K/Akt and ERK1/2 signaling. *J Nutr Biochem* 2006;17:766-772.
- McCubrey JA, Lahair MM, Franklin RA. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxidants Redox Signal* 2006;8:1775-1789.
- Sahu BD, Kumar JM, Sistla R. Baicalein, a bioflavonoid, prevents cisplatin-induced acute kidney injury by up-regulating antioxidant defenses and down-regulating the MAPKs and NF- κ B pathways. *PLoS One* 2015;10:e0134139.
- Karuppagounder V, Arumugam S, Thandavarayan RA, et al. Naringenin ameliorates daunorubicin induced nephrotoxicity by mitigating AT1R, ERK1/2-NF κ B p65 mediated inflammation. *Int Immunopharmacol* 2015;28:154-159.
- Ma JQ, Ding J, Zhang L, Liu CM. Ursolic acid protects mouse liver against CCl₄-induced oxidative stress and inflammation by the MAPK/NF- κ B pathway. *Environ Toxicol Pharmacol* 2014;37:975-983.
- Ouchi A, Ishikura M, Konishi K, Nagaoka S, Mukai K. Kinetic study of the prooxidant effect of α -tocopherol. Hydrogen abstraction from lipids by α -tocopheroxyl radical. *Lipids* 2009;44:935-943.
- Stocker R. The ambivalence of vitamin E in atherogenesis. *Trends Biochem Sci* 1999;24:219-223.
- Genton L, Viatte V, Janssens JP, Heritier AC, Pichard C. Nutritional state, energy intake and energy expenditure of amyotrophic lateral sclerosis (ALS) patients. *Clin Nutr* 2011;30:553-559.
- Wills AM, Hubbard J, Macklin EA, et al. Hypercaloric enteral nutrition in patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet* 2014;383:2065-2072.