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Nanoformulated Copper/Zinc Superoxide Dismutase Attenuates Vascular Cell Activation and Aortic Inflammation in Obesity

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Abstract

Objective—Endothelial cell (EC) oxidative stress can lead to vascular dysfunction which is an underlying event in the development of cardiovascular disease (CVD). The lack of a potent and bioavailable anti-oxidant enzyme is a major challenge in studies on antioxidant therapy. The objective of this study is to determine whether copper/zinc superoxide dismutase (CuZnSOD or SOD1) after nanoformulation (nanoSOD) can effectively reduce EC oxidative stress and/or vascular inflammation in obesity.

Methods—Human aortic endothelial cells (HAECs) were treated with native- or nanoSOD for 6 h followed by treatment with linoleic acid (LA), a free fatty acid, for 6–24 h. To determine the *in vivo* relevance, the effectiveness of nanoSOD in reducing vascular cell activation was studied in a mouse model of diet-induced obesity.

Results—We noted that nanoSOD was more effectively taken up by ECs than native SOD. Western blot analysis further confirmed that the intracellular accumulation of SOD1 protein was greatly increased upon nanoSOD treatment. Importantly, nanoSOD pretreatment led to a significant decrease in LA-induced oxidative stress in ECs which was associated with a marked

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Disclosure

The other authors have no conflict of interest.

increase in SOD enzyme activity in ECs. *In vivo* studies showed a significant decrease in markers of EC/vascular cell activation and/or inflammation in visceral adipose tissue (VAT), thoracic aorta, and heart collected from nanoSOD-treated mice compared to obese control mice. Interestingly, the expression of metallothionein 2, an antioxidant gene was significantly increased in nanoSOD-treated mice.

Conclusion—Our data show that nanoSOD is very effective in delivering active SOD to ECs and in reducing EC oxidative stress. Our data also demonstrate that nanoSOD will be a useful tool to reduce vascular cell activation in VAT and aorta in obesity which, in turn, can protect against obesity-associated CVD, in particular, hypertension.

Keywords

nanoSOD; superoxide dismutase; oxidative stress; free fatty acid; obesity; vascular inflammation

Introduction

Obesity predisposes individuals to type 2 diabetes and cardiovascular disease (CVD). Hypertension is clearly a risk factor for CVD including heart failure, stroke, and thrombosis [1–3], and is closely associated with obesity (reviewed in [4, 5]). Moreover, obesity-associated hypertension is resistant to treatment with anti-hypertensive drugs [6, 7]. It should be noted that oxidative stress and endothelial/vascular dysfunction, both of which are processes involved in the pathogenesis of hypertension, are increased in obesity. However, it is still unclear whether antioxidant therapy will be effective in ameliorating vascular inflammation in obesity which, in turn, can reduce systemic hypertension.

Several pharmacological and dietary agents with known antioxidant properties are used in the prevention and treatment of human diseases including ischemic stroke, diabetic neuropathy, and osteoarthritis (reviewed in [8–10]). Moreover, compelling evidence in support of the effectiveness of antioxidants is available from human epidemiological studies and from *in vitro* and animal studies. However, the enthusiasm towards antioxidant therapy began to decline due to the failure in clinical trials that addressed the benefits of using antioxidant vitamins in treating CVD [11], (reviewed in [12]). Therefore, attention was focused on exploring the therapeutic benefit of antioxidant enzymes including CuZnSOD, which specifically scavenges superoxide yielding hydrogen peroxide and oxygen. This approach was effective to some extent in ameliorating several inflammatory disorders including arthritis and skin inflammation (reviewed in [13]), [14, 15]. However, the administration of SOD in other conditions like burn injury did not show any protective effects, likely due to its low half-life *in vivo* [16]. The lack of potent and highly bioavailable antioxidants is a major challenge in antioxidant therapy.

It has become evident that encapsulating antioxidant enzymes in nano-carriers will increase their half-life and thus the efficacy of these enzymes. Our group has developed various types of nanoformulated CuZnSOD and studied their effectiveness in ameliorating hypertension and cerebral ischemia [17, 18]. Of note, we have recently reported that nanoSOD can reduce obesity-linked adipose inflammation in a mouse model of diet-induced obesity [19]. However, the effect of the nanoSOD in reducing obesity-associated EC activation and/or

vascular inflammation has not been studied. Because EC oxidative stress is an underlying event leading to vascular inflammation and dysfunction which, in turn, can lead to CVD including atherosclerosis and systemic hypertension, we hypothesize that nanoSOD will be effective in reducing EC oxidative stress and also in ameliorating vascular inflammation in obese mice.

Materials and Methods

NanoSOD preparation

NanoSOD was synthesized, purified, and characterized as reported previously [18]. Briefly, native bovine CuZnSOD protein (Sigma-Aldrich) and polymer stock solutions were prepared in 10 mM HEPES (pH 7.4). The CuZnSOD was mixed with poly L-lysine–polyethylene glycol copolymer (PLL–PEG). The CuZnSOD and PLL–PEG complex was covalently stabilized using a reducible cross-linker, 3,3'-dithiobis (sulfosuccinimidylpropionate) (DTSSP; Thermo Fisher Scientific). The molar ratio of DTSSP/PLL50 was 0.5 and the crosslinked-nanoSOD was purified using size exclusion chromatography (small/intermediate scale) or centrifugal filtration (large scale). The size of NanoSOD was estimated to be ~35 nm.

Cell culture

Human aortic endothelial cells (HAEC) were purchased from Lonza. HAECs were grown in endothelial growth medium (EGM-2) supplemented with 10% fetal bovine serum (FBS), growth factors, and heparin (Lonza).

Immunocytochemistry

HAECs cultured in chamber slides were treated with Alexafluor 647-conjugated native or nanoSOD for 6 h. The cells were washed and incubated in fresh medium for another 72 h and pictures were taken using Nikon Eclipse 80i microscope and digital camera.

DHE Fluorescence

HAECs were seeded into 24-well plates and pretreated with native or nanoSOD for 6 h. The pretreated cells were treated with 90µM LA as we reported earlier [20, 21] in EGM-2 (5% FBS) for 6 h. The cells were stained with 10µM dihydroethidine (DHE) or dichlorodihydrofluorescein (DCF) diacetate and incubated for 30 min at room temperature in dark. DHE fluorescence was measured with a fluorescent plate reader at an excitation wavelength of 530 nm and emission wavelength of 615 nm. DCF fluorescence was read with a fluorescent plate reader at an excitation wavelength of 492 nm and emission wavelength of 517 nm.

Western Blotting Analysis

HAECs were seeded in 6-well plates and treated with native or nanoSOD for 6 h followed by incubation in EGM-2 media for 24 h. Cell lysates were analyzed by western blot analysis using anti-SOD1 primary antibody from Santa Cruz Biotechnology.

SOD activity

HAECs were seeded in 6-well plates and pretreated with native or nanoSOD for 6 h followed by treatment with LA for 24h. Cell lysates were collected by sonication and analyzed for SOD enzyme activity using the SOD Assay Kit-WST (Dojindo Molecular Technologies Inc).

In vivo studies

Eight-wk old wild type C57BL/6 mice (Jackson Lab) were fed a high fat (45% fat calories) diet (Research Diets Inc.) for a period of 8 wk. A cohort of HF diet-fed mice were injected intraperitoneally (*i.p.*) with nanoSOD once every other day for a period of 15 days (8 injections total). The mice were continued on respective diets and sacrificed 24 h post-injection of the last dose of nanoSOD after 5 h fasting. The VAT, thoracic aorta, and heart samples were collected. 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.

Isolation of stromal vascular cells (SVCs)

SVCs comprised of various cells including the endothelial cells, endothelial progenitor cells, preadipocytes, and immune cells like macrophages were collected from VAT as described earlier [22].

Tissue processing and real-time PCR Analysis

RNA was isolated from SVCs, VAT, thoracic aorta and heart. The aorta samples were placed in RNAlater and any fat or other tissues from the thoracic aorta samples were carefully removed before extracting RNA. We isolated ventricles from the heart samples and proceeded with RNA extraction. Real-time PCR analysis was carried out using primers from Applied Biosystems. A $\Delta\Delta C_T$ method was used to calculate gene expression and the values were normalized to 18S.

Statistical Analysis

Data were analyzed with Prism Graphpad using one way ANOVA followed by Tukey's post-hoc test unless specified otherwise. In experiments involving 2 groups, a one-tailed Student's *t* test was performed. A statistical probability of $P < 0.05$ was considered significant.

Results

NanoSOD was preferentially taken up by ECs compared to native SOD

We first determined if nanoSOD is preferably taken up and retained by the ECs compared to native SOD. After pre-treatment with native or nanoSOD for 6 h, the cells were rinsed and incubated with regular growth media and micrographic images were taken at 72 h. We noted that the ECs take up and retain the nanozymes for up to 72 h (Figure 1 A–E) as reported previously in cultured neurons [17]. We also noted a dose-dependent increase in fluorescence intensity upon nanoSOD treatment.

NanoSOD increased the delivery of SOD1 protein in ECs

To determine whether nanoSOD treatment can actually increase the protein content of SOD1, we performed a western blot analysis. As shown in Figure 1F, a pronounced increase in SOD1 protein content was noted in nanoSOD treated cells whereas the intracellular SOD1 content was not altered upon native SOD treatment. Of note, the SOD1 in nanoSOD was derived from bovine erythrocytes and migrates more quickly on gel electrophoresis as observed in the western blot.

NanoSOD reduced LA-induced superoxide production in ECs

LA by itself led to a significant increase in DHE fluorescence, a measure of superoxide levels. Pretreatment with native SOD did not reduce the superoxide levels whereas nanoSOD led to a significant decrease in LA-induced DHE fluorescence, indicating a decrease in superoxide levels (Figure 2A).

NanoSOD reduced LA-induced H₂O₂ in ECs

Increased superoxide scavenging can result in an increase in H₂O₂ which is also a marker of oxidative stress. To rule out the possibility that increased SOD activity in nanoSOD treated cells leads to increased H₂O₂, we measured DCF fluorescence in cells treated with LA in the presence or absence of native or nanoSOD (Figure 2B). There was a mild but significant decrease in DCF fluorescence upon native SOD treatment ($P<0.05$). Interestingly, we noted that there was a profound reduction in DCF fluorescence in cells treated with LA+nanoSOD compared to LA alone treated cells ($P<0.001$). These data indicate that nanoSOD was effective in reducing the overall oxidative stress in ECs.

Reduction of LA-induced oxidative stress was accompanied by an increase in SOD activity

To ascertain whether reduction in oxidative stress in nanoSOD treated cells is due to increased SOD activity, we determined SOD activity in ECs treated with LA in the presence or absence of native or nanoSOD. It was interesting to note that the SOD activity was increased to some extent with LA treatment likely due to the response of ECs to LA-induced superoxide generation (Figure 2C). However, we noted a much greater increase in SOD activity in LA+Nano treated ECs showing that increased SOD1 protein upon nanoSOD treatment (as shown in Figure 1) is in fact, accompanied by an increase in enzymatic activity.

NanoSOD reduced AT markers of EC activation

Obesity is associated with oxidative stress and vascular dysfunction. AT is highly vascularized and each adipocyte is surrounded by a capillary. In order to determine the physiological relevance of our findings, we conducted an *in vivo* study in a mouse model of diet-induced obesity. We isolated RNA samples from VAT and SVCs derived from VAT. We noted a significant decrease in E-selectin ($P<0.01$) in VAT of nanoSOD-treated mice compared to controls (Figure 3A) whereas the expression of VCAM-1 and ICAM-1 were not altered. Of note, the mRNA expression of VCAM-1 and ICAM-1 was significantly decreased in SVCs collected from nanoSOD-treated mice compared to controls. These data

further support our hypothesis that nanoSOD will be effective in reducing obesity-linked EC/vascular cell activation.

NanoSOD reduced vascular inflammation in obesity

Obesity is associated with vascular inflammation and impairments in vascular functions play an important role in the pathogenesis of CVD in particular, atherosclerosis and systemic hypertension. Therefore, we wanted to determine the role of nanoSOD in modulating vascular inflammation. We analyzed the mRNA samples isolated from thoracic aorta for markers of inflammation, EC activation, and oxidative stress. As shown in Figure 4A, the expression of MMP3 and IL-1 β was significantly reduced upon nanoSOD treatment. Moreover, markers of EC activation such as E-selectin and ICAM-1 were reduced significantly. Interestingly, the expression of metallothionein II (MT-2), an antioxidant gene, was significantly increased upon nanoSOD treatment compared to control. Together, these data suggest that delivering SOD in the form of nanoSOD will be effective in reducing vascular inflammation and oxidative stress in obesity.

Effect of nanoSOD on cardiac gene expression

Because we noted a decrease in MMP3 in aorta upon nanoSOD treatment and because MMP3 plays an important role in mediating cardiac dysfunction and hypertension [23], we next analyzed the expression of MMP3 in the ventricles. We noted that the expression of MMP3 in nanoSOD-treated mice was significantly reduced in the ventricles as well (Figure 4B). However, the markers of vascular cell activation were not altered between groups. As we noted in the aorta, the expression of MT2 was significantly increased in hearts of nanoSOD-treated mice.

Discussion

In the current study, we have demonstrated that nanoSOD can 1) effectively deliver active SOD1 to ECs, 2) attenuate EC/vascular cell activation in VAT, 3) reduce EC/vascular cell activation and aortic inflammation in mice, and 4) increase the expression of antioxidant defense genes, in particular, MT-2 in aorta and heart. Our study provides a proof-of-concept for our hypothesis that nanoformulation of SOD will improve the bioavailability of SOD and that nanoSOD can ameliorate systemic hypertension in obesity.

It is becoming clear that obesity is associated with oxidative stress and impairment in EC functions [24–26], processes leading to vascular dysfunction and systemic hypertension. Elevated FFAs are considered to initiate the pathological processes in obesity. A dysfunctional AT characterized by uncontrolled basal lipolysis can lead to excessive release of FFAs and these FFAs can induce oxidative stress and lipotoxicity in a wide variety of cells. We previously reported that FFAs, in particular, LA, can induce EC dysfunction via superoxide production [20]. In the current study, we have demonstrated that LA-induced oxidative stress can be attenuated by nanoSOD. Previously, Manickam *et al.* have shown that nanoSOD was effective in scavenging superoxide generated by hypoxanthine and xanthine oxidase system in cell-free extracts collected from brain microvascular endothelial cells and neuronal cells [18]. In this study, we provide evidence that the actual SOD1 protein and

SOD activity were increased intracellularly upon treatment with nanoSOD. Moreover, we show that nanoSOD was able to scavenge LA-induced superoxide in whole cells. Thus, in addition to demonstrating that nanoSOD is effective in delivering SOD1 protein to ECs, we also show that this enzyme is biologically active within intact cells.

It is widely accepted that obesity-linked diseases arise as a result of inflammation in adipose tissue [27]. The role of immune cells, in particular, macrophages in mediating obesity-associated adipose inflammation has been extensively studied [28]. However, relatively little is known regarding the role of ECs and other vascular cells in AT in modulating inflammation and/or vascular dysfunction in AT. It should be noted that adipose tissue is highly vascularized suggesting that EC activation in AT can alter adipose inflammation in obesity. Moreover, it has been reported that deleting myeloid differentiation primary response 88 (Myd88), an inflammatory gene, specifically in ECs, can protect mice against obesity-linked inflammation [29]. In addition, Nishimura *et al* have shown that markers of EC activation including E-selectin and ICAM-1 are increased in the SVCs collected from adipose tissue of obese mice [30]. In the current study, we provide evidence that markers of EC activation such as E-selectin, VCAM-1, and ICAM-1 are attenuated in AT and/or SVCs collected from nanoSOD-treated mice compared to HF controls. These data suggest that markers of EC activation are reduced upon nanoSOD treatment in AT. It should also be pointed out that some of these adhesion molecules such as ICAM-1 and VCAM-1 are not only expressed by ECs but also by other vascular cells including smooth muscle cells and/or macrophages [31–33]. Therefore, the role of nanoSOD in reducing obesity-linked vascular dysfunction via reducing adhesion molecule expression in other vascular cells cannot be ruled out.

While the role of obesity in mediating insulin resistance is well-established, emerging evidence suggest that the crosstalk between adipose tissue and blood vessels is vital to regulating vascular homeostasis [34]. Interestingly, we noted a significant decrease in markers of EC/vascular cell activation and inflammation in thoracic aorta as well. Studies suggest that MMPs play a role in various CVD including aortic aneurysm, and left ventricular dysfunction [23, 35]. We noted a significant decrease in MMP3 gene expression in both aorta and cardiac ventricles. Another interesting observation was that the expression of MT-2, an antioxidant gene was increased upon nanoSOD treatment in these two tissues. MT-2 has been shown to play a role in improving mitochondrial function in obesity and also in the prevention of diabetic nephropathy [36, 37]. Moreover, cardiac-specific overexpression of MT-2 has been shown to protect against myocardial dysfunction in a model of experimental hypertension [38]. These data further support our hypothesis that nanoSOD will be effective in ameliorating obesity-linked CVD.

Because EC activation and vascular inflammation are the underlying events leading to other CVD including systemic hypertension and atherosclerosis, our data suggest that nanoSOD can be a viable therapeutic tool to combat CVD in obesity, in particular, obesity-linked hypertension which is resistant to traditional anti-hypertensive medications. Our future studies will address the effectiveness of nanoSOD in reducing systemic hypertension in animal models of obesity.

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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 out-licensed to NeuroNano Pharma, a start-up company, located in Chapel Hill, NC. A.V.K. is a cofounder, shareholder, and director of this company.

Abbreviations

HAEC	Human aortic endothelial cell
LA	Linoleic acid
NanoSOD	Nanoformulated copper/zinc superoxide dismutase
SVC	Stromal vascular cell

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Highlights

- NanoSOD was effective in delivering active SOD to endothelial cells (ECs).
- NanoSOD was superior to native SOD in reducing free fatty acid-induced EC oxidative stress.
- NanoSOD was effective in reducing EC/vascular cell activation in adipose tissue and aorta in obesity.

Figure 1.

Effect of nanoSOD on the uptake of SOD1 by the HAECs. Cells were treated with Alexafluor 647-conjugated native or nanoSOD at 100 or 200 units/mL for 6h. The cells were then incubated in fresh medium for 72 h and pictures taken at 20×. Control (A), Native, 100 units/mL (B), Native 200 units/mL (C), Nano, 100 units/mL (D), and Nano, 200 units/mL (E). Intracellular accumulation of SOD1 protein was detected by western blot analysis and values were normalized to β-actin (* $P<0.05$ vs control as analyzed by 1-way Anova followed by Dunnett's post-hoc test) (F). Values are mean $\pm$ SEM of 6 samples in each group. Native, native SOD; Nano, nanoSOD.

Figure 2.

HAECs were pretreated with Native or NanoSOD for 6h followed by treatment with LA for 6h. DHE (A) and DCF (B) fluorescence were measured to detect superoxide and H_2O_2 , respectively. HAECs were pretreated with Native or NanoSOD for 6h followed by treatment with LA (60 μ M) for 24h and SOD enzyme activity was measured (C). Values are mean $\pm$ SEM of 4–6 samples in each group. LA, linoleic acid; Native, native SOD; Nano, nanoSOD.

Figure 3.

Effect of NanoSOD on EC/vascular cell activation in visceral adipose tissue. Wild type mice were fed a high fat diet for 8 wk followed by 2 wk treatment with nanoSOD. The markers of EC/vascular cell activation were determined by realtime PCR in VAT (A) and SVCs collected from VAT (B). Values are mean $\pm$ SEM of 6–8 samples in each group. VAT, visceral adipose tissue; SVC, stromal vascular cell; Nano, nanoSOD.

Figure 4.

Effect of NanoSOD on markers of EC/vascular cell activation, inflammation, and oxidative stress in aorta and heart. The markers of EC/vascular cell activation, inflammation, and oxidative stress were determined by realtime PCR in aorta (A) and cardiac left ventricles (B). Values are mean $\pm$ SEM of 5–8 samples in each group. Nano, nanoSOD.