Deficiency of CuZn Superoxide Dismutase Promotes Inflammation and Alters Medial Structure Following Vascular Injury

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Aim: The antioxidant enzyme copper/zinc superoxide dismutase (CuZnSOD) metabolizes superoxide anion (O₂⁻) in vascular cells. However, the role of CuZnSOD in vascular injury remains poorly understood.

Methods: Using CuZnSOD-deficient (CuZnSOD⁻/⁻) mice and wild-type (WT) mice, we investigated morphometric changes and the role of O₂⁻ in vascular remodeling after femoral artery injury induced by an external vascular cuff model.

Results: Three days post-injury, inflammatory cell infiltration increased significantly. Moreover, the percent positive area of tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in media were higher in CuZnSOD⁻/⁻ mice than in WT mice (TNF-α: 34.8 ± 8.4% versus 18.8 ± 5.6%, p < 0.05, ICAM-1: 29.6 ± 6.5% versus 11.0 ± 2.8%, p < 0.05, VCAM-1: 23.5 ± 7.5% versus 3.7 ± 1.1%, p < 0.05). mRNA expression of iNOS was markedly increased in CuZnSOD⁻/⁻ mice with cuff injury. Dihydroethidine staining revealed increased levels of vascular O₂⁻ in media from CuZnSOD⁻/⁻ mice. Although neointimal formation remained unchanged, 14 days postinjury, we observed degeneration of the media, and the media/vessel wall ratio increased in CuZnSOD⁻/⁻ mice (40.4 ± 2.1% versus 26.8 ± 1.4%, p < 0.05).

Furthermore, SMemb/MHC-B-stained lesions increased markedly in CuZnSOD⁻/⁻ mice.

Conclusions: CuZnSOD-deficiency promoted inflammation, expressed adhesion molecules, and altered the structure of the media post-injury. Our results suggest that O₂⁻ participates importantly in the progression of early stage vascular inflammation, resulting in vascular remodeling in media but not neointimal formation, post-injury.


Key words; Inflammation, Copper/zinc superoxide dismutase, Cuff injury, Smooth muscle cell, Proliferation

Introduction

Oxidative stress and inflammation contribute to the development of atherosclerosis, and closely interact with each other¹. Various sources, including NADPH oxidases (NOX), mitochondrial electron transport chains, xanthine oxidase, and nitric oxide synthase (NOS), increase the generation of superoxide anion (O₂⁻) by one electron reduction of oxygen with a variety of risk factors. O₂⁻ also generated reactive oxygen and nitrogen species (ROS; H₂O₂, HO·, HOCl etc, RNS; NO₂⁻, NO⁻, peroxynitrite etc). When production of ROS/RNS exceeds the antioxidant capacity of vascular walls, oxidative stress is induced, which results in atherosclerosis progression and plaque rupture, followed by endothelial dysfunction and the proliferation/migration of vascular smooth muscle cells (VSMC)²-³. An earlier study implicated altered VSMC phenotypes in vascular remodeling, which is induced by ROS. Additionally,
ROS can stimulate neointimal formation following vascular injury.\(^4\)

Vascular tissues possess many antioxidant enzymes that suppress ROS, including superoxide dismutases (SODs), catalase, and peroxiredoxin. These enzymes also contribute to the cellular defense system against vascular injury\(^2\), and SODs dismutate \(\mathrm{O}_2^-\) to \(\mathrm{H}_2\mathrm{O}_2\) in all vascular cells. SODs are characterized by three isoforms: copper/zinc SOD (CuZnSOD, or SOD-1), which localizes in cytosol; manganese SOD (MnSOD, or SOD-2), which localizes in mitochondria; and extracellular SOD (ecSOD, or SOD-3). Among these isoforms, CuZnSOD expression predominantly occurs in the vessel wall, and CuZnSOD activity accounts for 50-80% of total SOD activity.\(^9\) Furthermore, another study reported that CuZnSOD dismutates \(\mathrm{O}_2^-\) derived from endothelial NOS (eNOS) to \(\mathrm{H}_2\mathrm{O}_2\), which accounts for endothelium-derived hyperpolarization factor.\(^9\)

Previous studies have investigated the physiological and pathophysiological roles of CuZnSOD in the vasculature. CuZnSOD levels increase in human coronary artery plaques, suggesting its participation in acute coronary syndromes.\(^7,\,8\) Adenovirus-mediated gene transfer of CuZnSOD improves endothelial function in diabetic rabbit aorta.\(^9\) Overexpression of bosh CuZnSOD and catalase inhibits VSMC proliferation\(^10\) and reduces the lesion area of aortic atherosclerosis in apolipoprotein E-deficient mice.\(^11\)

In addition, CuZnSOD-deficient (CuZnSOD\(^{-/-}\)) mice show impaired endothelium-dependent relaxation;\(^12\) however, the role of CuZnSOD in post-injury vascular inflammation is not yet well-elucidated. To determine the functional role of CuZnSOD in vascular remodeling in vivo, the present study subjected femoral arteries of CuZnSOD\(^{-/-}\) mice to cuff-induced injury.

**Materials and Methods**

**Animals**

We obtained 8- to 12-week-old CuZnSOD\(^{-/-}\) mice from Jackson Laboratory (Bar Harbor, ME, USA) and backcrossed them to C57BL/6J strain mice for 8 generations.\(^13\) We then interbred heterozygous CuZnSOD\(^{+/+}\) mice to obtain wild-type (WT) mice and homozygous CuZnSOD\(^{-/-}\) mice within the same litter. Throughout the experiment, the mice consumed a normal diet containing 4.6% crude fat with < 0.02% cholesterol (CLEA Japan, Inc.). The studies were approved by the National Defense Medical College Board for Studies in Experimental Animals.

**Femoral Artery Injury**

The mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and the left femoral artery was exposed under sterile conditions, as previously described.\(^14\)-16. Vascular injury was inflicted by placing a nonocclusive polyethylene cuff (length 2 mm; internal diameter 0.56 mm; Becton Dickinson, Mountain View, CA, USA) around the femoral artery.

**Tissue Preparation and Histology**

After measuring tail-cuff systolic blood pressure, mice were euthanized and perfused with 0.9% saline, followed by 4% paraformaldehyde. The femoral arteries were fixed in 10% formalin for 48 hours, embedded in paraffin, and sectioned (4-μm thickness). All samples were routinely stained with hematoxylin-eosin (H&E), elastic Masson, and elastica van Gieson and also by immunohistochemistry. VSMCs were visualized with \(\alpha\)-smooth muscle cell (SMC) actin (\(\alpha\)-SMA) staining (DAKO, Kyoto, Japan) and the embryonic form of smooth muscle myosin heavy chain (SMemb/MHC-B) stain (Funakoshi, Osaka, Japan).

Frozen sections (16-μm thickness) were used for CuZnSOD staining. CuZnSOD was visualized with an anti-human CuZnSOD polyclonal antibody (SOD100; Stressgen, Victoria, BC, Canada).

Frozen sections (16-μm thickness) were also used for other immunostaining, and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) (Abcam, Cambridge, MA, USA), intercellular adhesion molecule-1 (ICAM-1) (R&D Systems, Minneapolis, MN, USA), and vascular cell adhesion molecule-1 (VCAM-1) (CD106, Chemicon, Temecula, CA, USA) for each immunostaining. Sections were visualized using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) with DAB as the substrate.

**Morphometry**

Ten equally spaced cross sections were used to quantify intimal and medial lesions for each mouse, and intimal and medial areas were measured using the NIH Image 1.55 (National Institutes of Health, public domain software). The area was quantified, expressed as a percentage of the total cross-sectional vessel wall area. SMC accumulation was quantified in the lesion by calculating the percentage of \(\alpha\)-SMA and SMemb/MHC-B positive area to the total cross-sectional vessel wall area. Similarly, TNF-\(\alpha\), ICAM-1, and VCAM-1 positive areas were calculated in the intima or media and expressed as a percentage of the total area.
Analysis of Gene Expression by Real-Time Quantitative Polymerase Chain Reaction

Injured femoral arteries were collected 3 days after cuff placement. Total RNA was extracted from the aortas using TRIReagent (Sigma, St. Louis, MO, USA) and the quantity was determined by measuring the absorbance at 260 nm, as previously described[17]. Reverse-transcription was performed with AMV Reverse Transcriptase XL (Takara Biochemicals, Japan). Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA) using SYBR Green technology. The following oligonucleotide primer pairs were used: NOX2 sense, 5'- CCC TTT GGT ACA GCC AGT GAA GAT-3'; antisense, 5'- CAA TCC CAG CTC CCA CTA ACA TCA-3'; inducible NOS (iNOS) sense, 5'- CAG AAG CAG AAT GTG ACC ATC-3'; antisense, 5'- CTT CTG GTC GAT GTC ATG A-3'; GAPDH sense, 5'- GTC ATT GAG AGC AAT GCC AG-3'; antisense, 5'- GTG TTC CTA CCC CCA ATG TG-3'. The optimum number of cycles was set for each gene product with uniform amplification. Each mRNA level was expressed as the ratio to GAPDH RNA expression.

Detection of Superoxide

Femoral arteries were stained with dihydroethidium (DHE; Invitrogen Molecular Probes, Eugene, OR, USA) to detect superoxide, as previously described[18]. Frozen sections of arteries were immediately cut into 16-μm-thick sections and placed on glass slides. Samples were then incubated at room temperature for 30 min with DHE (2×10⁻⁶ mol/L) and protected from light. Images were obtained with a microscopic system (BZ-8000; Keyence, Japan) with an excitation wavelength of 540 nm and an emission wavelength of 605 nm.

Statistical Analysis

The results are shown as the mean ± SEM. The two groups were compared using Student's t test. P<0.05 was regarded as significant.

Results

Blood Pressure and Baseline Characteristics

Systolic blood pressure was similar between groups (WT mice: 87.1±8.7 mmHg [n=5] versus CuZnSOD⁻/- mice: 84.8±3.8 mmHg [n=5]). We observed no neointimal formations in the contralateral control femoral arteries of either CuZnSOD⁻/- or WT mice, and the media showed no structural differences (Fig.1A), suggesting that vessel growth was normal in CuZnSOD⁻/- mice up to the age of 8-12 weeks. To confirm CuZnSOD localization, immunohistochemical staining for CuZnSOD was performed but detected no CuZnSOD protein in CuZnSOD⁻/- mice (Fig.1B).

Expression of Inflammatory Cytokine

To assess the role of CuZnSOD following vascular injury, the mice in both groups were euthanized 3 days following cuff placement. At 3 days, inflammatory cell infiltration had increased markedly in arteries from CuZnSOD⁻/- mice (Fig.2A). We used immunohistochemistry to further examine the expression of the proinflammatory cytokine TNF-α, which increased in CuZnSOD⁻/- mice compared with WT mice (Fig.2B). Fig.2C shows quantification of the area that expressed TNF-α. In both the intima and media, the percent positive area of TNF-α expression in CuZnSOD⁻/- mice tended to be much larger than in WT mice (intima: WT mice: 18.8±5.6% [n=5] versus CuZnSOD⁻/- mice: 34.8±8.4% [n=5], NS, media: WT mice: 8.5±1.4% [n=5] versus CuZnSOD⁻/- mice: 33.8±8.5% [n=5], p<0.05). Our findings indicate that CuZnSOD might suppress medial inflammation following vascular injury.
Adhesion Molecule Expression

Immunohistochemistry was used to investigate the expression of adhesion molecules ICAM-1 and VCAM-1 (Fig. 3), and detected ICAM-1 expression in the medial artery of CuZnSOD⁻/⁻ mice (Fig. 3A). The intima showed no significant difference in the percent positive area of ICAM-1 expression between groups (WT mice: 44.8 ± 2.4% [n=5] versus CuZnSOD⁻/⁻ mice: 56.8 ± 12.0% [n=5], NS); however, ICAM-1 expression increased significantly in the media of CuZnSOD⁻/⁻ mice (WT mice: 11.0 ± 2.8% [n=5] versus CuZnSOD⁻/⁻ mice: 29.6 ± 6.5% [n=5], p<0.05). Furthermore, we detected VCAM-1 in the medial artery of CuZnSOD⁻/⁻ mice (Fig. 3B). WT mice: 38.6 ± 6.6% [n=5] versus CuZnSOD⁻/⁻ mice: 44.5 ± 5.4% [n=5], NS), but the percent positive area of VCAM-1 increased in the media of CuZnSOD⁻/⁻ mice (Fig. 3C). WT mice: 3.7 ± 1.1% [n=5] versus CuZnSOD⁻/⁻ mice: 23.5 ± 7.5% [n=5], p<0.05), suggesting that CuZnSOD might selectively inhibit adhesion molecule expression in the media.

NOX2 and iNOS Expression

The expression of enzymes for ROS/RNS was tested by real-time PCR (RT-PCR). Because the sample amount was limited, we chose two typical enzymes NOX2, catalytic subunit of NADPH oxidase for ROS, and iNOS for RNS three days after cuff injury. NOX2 expression was increased after cuff injury in both wild-type and CuZnSOD⁻/⁻ mice. Expression of iNOS was markedly increased in CuZnSOD⁻/⁻ mice compared with WT after cuff injury (284 ± 55 times versus 39 ± 21 times) (Fig. 4).

Detection of Superoxide by Dihydroethidine

A DHE assay was used to assess the amount of $\cdot O_2^-$ in the femoral arteries, and observed no significant difference in control femoral arteries from both groups (Fig. 5). Compared with WT mice, however, the arteries of CuZnSOD⁻/⁻ mice 7 days following cuff injury revealed a higher red fluorescent signal that localized primarily in medial smooth muscle.
Neointima Formation and Structural Components of Media Following Injury

To examine post-injury neointima formation, we harvested femoral arteries from both groups 14 days following cuff placement (Fig. 6A). At 14 days post-injury, the percent medial area increased significantly in CuZnSOD\(^{-/-}\) mice compared with WT mice (Fig. 6B, a: WT mice: 26.8\(\pm\)1.4\% \(n=5\) versus CuZnSOD\(^{-/-}\) mice: 40.4\(\pm\)2.1\% \(n=5\), \(p<0.05\)) but the percent intimal area was similar (WT mice: 14.6\(\pm\)2.5\% \(n=5\) versus CuZnSOD\(^{-/-}\) mice: 18.1\(\pm\)3.8\% \(n=5\), NS). We detected no difference in intima/media ratios between groups (CuZnSOD\(^{-/-}\) mice: 43.6\(\pm\)7.5\% versus WT mice: 54.5\(\pm\)10.0\% \(n=5\), NS).

We visualized VSMCs with elastica van Gieson, \(\alpha\)-SMA, and SMemb/MHC-B stain at 14 days post-injury (Fig. 6A). Histological analysis revealed increased numbers of large nuclei in SMCs in the media of CuZnSOD\(^{-/-}\) mice compared to WT mice. In addition, sections stained with elastica van Gieson showed degeneration of the media in CuZnSOD\(^{-/-}\) mice. Furthermore, immunohistochemical analysis of arteries from CuZnSOD\(^{-/-}\) mice revealed significantly increased \(\alpha\)-SMA-stained (57.9\(\pm\)6.1\% \(n=5\)) and SMemb/MHC-B-stained (87.5\(\pm\)3.2\% \(n=5\)) lesions compared with WT mice (25.5\(\pm\)1.9\% \(n=5\), \(p<0.01\)) (Fig. 6B, b). WT mice showed no evidence of SMemb/MHC-B (Fig. 6B, c). These data demonstrate that CuZnSOD modulates the regional content of \(\alpha\)-SMA and SMemb/MHC-B after injury.

Discussion

Post-injury vascular remodeling is characterized by VSMC activation, migration, and proliferation, which are modulated by redox-sensitive pathways. CuZnSOD, which is catalyzed by the generation of hydrogen peroxide from superoxide, was shown to modulate these pathways in vitro, but pathophysiological roles in vascular injury in vivo remains poorly understood. The present study demonstrated inflam-
matory cell infiltration during the early post-injury stage, accompanied by increased levels of vascular \( \mathrm{O}_2^{-} \) in CuZnSOD \(^{-/-}\) mice. The area expressing TNF-\( \alpha \) and the expression of adhesion molecules also increased, as did adhesion molecule (ICAM-1, VCAM-1) expression, in the medial region of arteries from CuZnSOD \(^{-/-}\) mice during the early post-injury stage, suggesting the activation of inflammation \(^{20}\). At 14 days post-injury, we observed degeneration of the media as well as increased numbers of \( \alpha \)-SMA- and SMemb/MHC-B-stained lesions in arteries from CuZnSOD \(^{-/-}\) mice, suggesting that CuZnSOD participates importantly in the suppression of early-stage vascular inflammation, resulting in the modulation of vascular remodeling following injury \textit{in vivo}.

VSMCs are major components of the vascular wall, and their functions alter as phenotypes change. Mechanical stress dedifferentiates VSMCs from the contractile state to the synthetic state. \( \alpha \)-SMA is a specific marker of smooth muscle \(^{15}\), and SMemb/MHC-B is a nonmuscle-type myosin heavy chain expressed specifically in embryonic and dedifferentiated VSMCs \(^{21}\). Therefore, SMemb is a useful molecular marker for embryonic-type SMCs. We took advantage of the unique feature of this MHC isoform for analysis of the spatial and chronological correlation of the proliferation and phenotypic modulation of SMCs in injured arteries. In our study, proliferating SMCs showed an embryonic phenotype, as indicated by the expression of SMemb, and such embryonic-type SMCs were detected in injured arteries of both WT and CuZnSOD \(^{-/-}\) mice at 7 days after injury (data not shown). On day 14, the SMCs in the injured arteries of WT mice had returned to the adult phenotype, as indicated by the loss of SMemb (Fig. 6). In

**Fig. 4.** RNA expression of iNOS and NOX2 in cuff injury. RNA expression of iNOS and NOX2 was assessed with real-time PCR. NOX2 tended to increase after cuff injury in both WT and CuZnSOD \(^{-/-}\) mice. Error bars = SEM. NS indicates not significant.

**Fig. 5.** Dihydroethidium immunolabeling of femoral arteries. Femoral artery staining showed no significant difference among groups (a, b). Seven days after cuff injury, arteries of CuZnSOD \(^{-/-}\) mice (d) revealed a markedly higher red-fluorescent signal that localized primarily in medial smooth muscle compared with WT mice (c). Adv, adventitia; SM, smooth muscle layer. Scale bars: 30 \( \mu \)m.
contrast, the intima and media in CuZnSOD−/− mice were positive for α-SMA and had expressed SMemb/MHC-B on day 14 (Fig. 6), indicating that lack of CuZnSOD induced prolonged SMC phenotypic changes after injury. Several previous reports have reported the phenotypic modulation of VSMCs by ROS. Shi et al.10 demonstrated decreased VSMC proliferation in transgenic mice that overexpress human CuZnSOD and catalase. Didion et al.12 reported that CuZnSOD deficiency impaired endothelial function in mice. These reports support our hypothesis that CuZnSOD may negatively regulate VSMC activation, migration, and proliferation through a direct or endothelium-mediated mechanism that minimizes vascular remodeling in vivo.

Several studies have reported that ROS might act as intracellular signaling molecules, and inflammatory cytokines increase adhesion molecules via redox-sensitive signaling3. Adhesion molecule expression on VSMC activates macrophage accumulation, leading to vascular growth and medial hypertrophy22. The present study also demonstrates increased expression of major adhesion molecules ICAM-1 and VCAM-1 in arteries from CuZnSOD−/− mice (Fig. 3). An in vitro study showed that TNF-α increases VCAM-1 expression in human umbilical vein endothelial cells (HUVECs)23. Adenovirus-mediated gene transfer of CuZnSOD but not catalase decreased VCAM-1 expression induced by TNF-α, suggesting that superoxide participates in adhesion molecule expression. Superoxide can also elevate transcription factors such as activator protein-1 (AP-1) or nuclear factor κB (NF-κB) activated by TNF-α24. Indeed, SOD catalyzes the generation of H₂O₂ from superoxide, and these two molecules are distinguishable25. In CuZnSOD−/− mice, DHE staining showed elevated levels of vascular superoxide; therefore, in addition to the induction of oxidative stress, ROS may influence phenotypic changes in vascular injury26.

CuZnSOD deficiency did not only weaken the removal of superoxide, but also increased the source of ROS/RNS. In RT-PCR, iNOS expression was markedly increased in CuZnSOD−/− mice with cuff injury. iNOS participates in the inflammatory process of vas-

Fig. 6. (A) Histological staining of cuffed femoral arteries (day 14) of WT (a, b, c) and CuZnSOD−/− (d, e, f) mice. Sections were stained with elastica van Gieson (a, d), α-SMA (b, e), or SMemb/MHC-B (c, f). Scale bars: 100 μm. (B) Quantitative analysis of percent medial area (a), percent α-SMA-positive area (b), and percent SMemb/MHC-B-positive area (c) 14 day after cuff injury. n=5 per group. Error bars=SEM.
culature, which is expressed in white blood cells, endothelial cells, smooth muscle cells, and adventitia fibroblast. A large amount of NO is produced from iNOS, which may also cause oxidative stress. Because NOX2 expression was also elevated in cuff injury, superoxide generation was supposed to be increased and peroxynitrite should be generated with a diffusion-limited reaction between NO and superoxide. Peroxynitrite plays a role in vascular inflammatory processes, such as the induction of apoptosis or activation of matrix metalloproteinases. iNOS and NOX2 are potential sources of ROS/RNS and peroxynitrite generation could contribute to the phenotype changes observed in this study.

Our study detected no significant differences in neointimal formation following arterial injury in CuZnSOD−/− mice and WT mice, with the result that may have been influenced by several factors. First, the antioxidant function of native CuZnSOD may be irrelevant in post-injury neointimal formation, which may relate not only to superoxide but also to other ROS, e.g. H₂O₂ or hydroxy-radicals. An anerogenic diet did not reduce fatty-streak lesions in CuZnSOD transgenic mice, suggesting that the prospective effects of CuZnSOD against vascular oxidative stress might be limited. Second, ecSOD may replace CuZnSOD in vascular injury. Leite et al. reported that CuZnSOD expression decreased following balloon injury in rabbits, whereas ecSOD expression increased consistently. Third, the expression of native CuZnSOD may not be enough to suppress ROS induced by vascular injury. Kuo et al. reported that adenovirus-mediated CuZnSOD overexpression reduced post-injury neointimal formation in rabbits, suggesting that reducing neointimal formation may require supra-physiological levels of SOD. SODs have peroxidase properties, and overexpression of SOD may suppress various kinds of ROS.

Conclusions

In conclusion, we show here that CuZnSOD participates importantly in adhesion molecule suppression and inflammatory cell infiltration by decreasing the amount of superoxide in the vessel during the early stage following injury, leading to post-injury modulation of medial remodeling, but not neointimal formation in vivo. Our observations suggest a distinguishable role of superoxide in medial inflammation following vascular injury.

Conflict of Interest

The authors have no conflicts to disclose.

Acknowledgments

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