Increased superoxide leads to decreased flow-induced dilation in resistance arteries of Mn-SOD-deficient mice

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AMPLE EVIDENCE suggests that endothelium-dependent, nitric oxide (NO)-mediated arteriolar dilation is impaired in many pathological conditions because of either decreased production or increased inactivation of NO or because of the synergistic effects of these two factors. Superoxide dismutases (SOD), the major antioxidant enzymes, importantly contribute to the regulation of the steady-state levels of superoxide (O$_2^•−$) and other reactive oxygen species. There are three isoforms of SOD: cytosolic SOD or copper zinc SOD (CuZn-SOD or SOD-1), mitochondrial SOD or manganese SOD (Mn-SOD or SOD-2), and extracellular CuZn-SOD (EC-SOD or SOD-3). The functional importance of each individual isoform in vascular protection against oxidative stress is still not resolved.

Previous studies using dietary restriction of copper and nonselective pharmacological inhibition of copper-containing SODs (6, 21, 29), as well as more recent studies using genetic models of deficiency of CuZn-SOD (7) and EC-SOD (10), demonstrated that the endogenous activity of copper-containing SODs is essential for endothelial dilator functions. Clinical studies found that reduced EC-SOD activity is closely associated with increased vascular oxidative stress and is responsible for the impaired endothelium-dependent vasodilatation in patients with coronary artery disease (14) and chronic heart failure (15). However, few studies have examined the specific role of Mn-SOD in the regulation of vascular endothelial function, especially in microvessels.

The physiological importance of Mn-SOD has been confirmed because SOD-2 gene knockout mice die of a cardiomyopathy within 2 wk after birth (9, 17, 24). Interestingly, overexpression of CuZn-SOD does not prevent neonatal death in mice that lack Mn-SOD (5), indicating an unique function of Mn-SOD in vivo. Mn-SOD heterozygous (Mn-SOD$^{+/−}$) and wild-type (WT) mice. Superoxide levels in the arteries of Mn-SOD$^{−/−}$ mice. Acetylcholine- and sodium nitroprusside-induced, but not adenosine-induced, dilations were also decreased in arteries of Mn-SOD$^{−/−}$ mice. Tempol or tiron (superoxide scavengers) increased flow-induced dilation in vessels of Mn-SOD$^{−/−}$ mice. A 41% reduction in endothelial nitric oxide synthase (eNOS) protein and a 37% reduction in eNOS activity were also found in the vessels of Mn-SOD$^{−/−}$ mice. Whereas there was no difference in eNOS protein in kidney homogenates of WT and Mn-SOD$^{−/−}$ mice, a significant reduction of nitric oxide synthase activity was found in Mn-SOD$^{−/−}$ mice, which could be restored by the administration of tiron. We conclude that an increased concentration of superoxide due to reduced activity of Mn-SOD, which inactivates nitric oxide and inhibits eNOS activity, contributes to the impaired vasodilator function of isolated mesenteric arteries of Mn-SOD$^{−/−}$ mice. These results suggest that Mn-SOD contributes significantly to the regulation of vascular function.

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sponsible for shear stress-induced dilation, is not fully understood. Thus this study was designed to compare endothelium-dependent NO-mediated vasodilator responses to shear stress and vasoactive agents in isolated arteries of Mn-SOD+/− and WT mice and to clarify the specific effect of Mn-SOD deficiency in the regulation of microvascular function.

METHODS

Animals. Male Mn-SOD heterozygous (n = 24) and WT (n = 26) mice, 20–23 wk old and ~30 g in weight, were used. Mice were interbred and genotyped at our facility (18). All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the current guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals.

Flow-induced dilation. Flow-induced dilation (FID) was assessed in second-order mesenteric arteries [{−160 μm in maximal diameter (passive diameter, PD) at 80 mmHg of intravascular pressure] of Mn-SOD+/− and WT mice. Vessels, −1 mm in length, were isolated and cannulated in a water-jacketed (37°C) perfusion device, which consisted of two parallel perfusion chambers (1 ml in volume). Two vessels, one from each group, were perfused (1 ml/min) with 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered physiological salt solution containing (in mM) 142.0 NaCl, 5.0 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 10.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS at pH 7.4. Intravascular pressure was maintained at 80 mmHg by a pressure-servocontroller (Living Systems, Burlington, VT) connected to the outflow side of the chamber. Intraluminal flow was established by a syringe pump (model 100, KD Scientific), coupled with an inflow pressure transducer, which was connected at the inflow side of the chamber. During intraluminal flow, intravascular pressure was maintained by lowering outflow pressure to an amount equal to the increase in inflow pressure. Perfusion flow was increased from 0 to 15 μl/min, in 5 μl/min steps. FID was measured in control and after the administration of N-nitro-L-arginine methyl ester (L-NAME, 3 × 10−4 M), Tempol (10−5 M) or Tempol plus L-NAME. In separate experiments, dilations to ACh (10−8 to 10−6 M), sodium nitroprusside (SNP, 10−8 to 10−6 M), and adenosine (ADO, 10−7 to 10−5 M) were assessed in the arteries of Mn-SOD+/− and WT mice.

Because of the insufficient amount of protein that can be isolated from second-order mesenteric arteries of each mouse, in the following experimental protocols, isolated first-order or homogenates of pooled first- and second-order mesenteric arteries were used.

Chemiluminescent detection of O2·− concentration. Rate of release of O2·− in isolated first-order mesenteric arteries from mice of both genotypes was assayed by the lucigenin chemiluminescence method (26). Vessels were equilibrated in PSS at 37°C for 30 min and then transferred into 2 ml of Krebs-HEPES buffer containing lucigenin (5 μM). Chemiluminescence was measured continuously with a scintillation counter (LS 700, Beckman Instruments) for 10 min after dark adaptation was allowed. After that, every vessel was completely digested with 50 μl of 1 N NaOH and total protein was assayed. O2·− level was normalized to protein content of each vessel and expressed as counts per minute per microgram of protein.

Protein expression with Western blotting. First- and second-order mesenteric arteries of Mn-SOD+/− and WT mice were isolated and pooled. Homogenates containing 10 μg of protein were separated on SDS-PAGE gels (10% acrylamide), transferred to a polyvinyl diffu-roide membrane, and probed with primary antibodies of endothelial NO synthase (NOS) (eNOS), Mn-SOD (BD Transduction Laboratories), and CuZn-SOD (Calbiochem). Secondary antibodies were conjugated to horseradish peroxidase according to the Amersham ECL-Plus protocol. The exposed film was developed in a Kodak X-Omat developer. Image acquisition and density of specific bands on the film were obtained by an image system (Alpha Innotech).

NOS activity. NOS activity of pooled first- and second-order mesenteric arteries (10 μg/sample) and the kidney (100 μg/sample) of Mn-SOD+/− and WT mice was assayed by measuring the rate of conversion of radiolabeled L-[3H]citrulline from L-[3H]arginine (Amersham) by using an assay kit (Cat. 482700, Calbiochem) and a scintillation counter (Beckman LS6500). The reaction was carried out at 37°C for 60 min. The values of samples treated with L-NAME (3 × 10−4 M) were used as background controls, and the average of background readings from each batch of assays was extracted from the value of each sample to reflect actual NOS activity. Final production of L-[3H]citrulline was expressed as picomoles per hour per milligram protein. In kidney samples, NOS activities were assayed in control and in the presence of pyrogallol (0.2–0.8 mM) or 0.4 mM of pyrogallol plus 10 mM tiron.

SOD activity. SOD activity of mesenteric arteries was assessed by measuring the inhibition of pyrogallol autoxidation (22). First- and second-order mesenteric arteries were isolated. Forty micrograms of protein (pooled from 2 to 3 mice) were used in each measurement, and the reaction was monitored spectrophotometrically at room temperature for 3 min. The activity was calculated against a standard curve of SOD (0–1.6 U/ml, S-2515, Sigma). To assess Mn-SOD activity, KCN was added to vessel homogenates at a final concentration of 0.4 mM to inhibit copper-containing SOD activity.

Statistical analysis. Data are expressed as means ± SE; n refers the number of mice in each group. Statistical analysis was performed using repeated-measures of ANOVA followed by the Tukey-Kramer post hoc test and Student’s t-test. Statistical significance was accepted at a level of P < 0.05.

RESULTS

Flow-induced dilation. Isolated mesenteric arteries of Mn-SOD WT and Mn-SOD+/− mice developed active tone (51.7 ± 2.1 and 52.3 ± 1.1% PD, respectively) during a 1-h equilibration at 80 mmHg of intravascular pressure. In the presence of constant intravascular pressure, increases in perfusate flow from 0 to 15 μl/min elicited significant increases in arterial diameter in both groups of mice (Fig. 1). However, FID in the arteries of Mn-SOD+/− mice was significantly reduced compared with that in WT mice. For example, in response to 15 μl/min of flow, the diameter of vessels of Mn-SOD+/− mice

Fig. 1. Flow-induced dilation (total average of different experimental protocols) in mesenteric arteries of wild-type (WT, n = 14) and Mn-SOD+/− (HZ, n = 24) mice. *P < 0.05 between WT and HZ mice (ANOVA).
was 64.6 ± 2.3% PD compared with 74.5 ± 2.6% PD (P < 0.05) in vessels of WT mice. Inhibition of NO synthesis with L-NAME significantly decreased FID in vessels of WT mice (Fig. 2A) but had no effect on FID in vessels of Mn-SOD+/− mice (Fig. 2B). These results indicate that FID in the mesenteric arteries of Mn-SOD-deficient mice is impaired, and the mechanism involved is related to an impaired NO-mediated dilation.

To investigate whether inactivation of NO by O2•− radicals is responsible for the decreased FID in vessels of Mn-SOD+/− mice, FID was assessed in control and after incubation of vessels, intra- and extraluminally, with the O2•− scavenger Tempol (10−3 M) for 30 min. Tempol did not affect the basal diameter of arteries in either group of mice nor FID in vessels of WT mice but significantly increased FID in the vessels of Mn-SOD+/− mice (Fig. 3) to a level comparable to that in the vessels of WT mice. Tiron (an intracellular O2•− scavenger, 3 × 10−4 M) also significantly increased FID in the vessels of Mn-SOD+/− mice (data not shown). In the presence of Tempol, L-NAME inhibited FID not only in WT mice but also in the arteries of Mn-SOD+/− mice.

**Agonist-induced dilation.** ACh (10−8, 10−7, and 10−6 M), SNP (10−8, 10−7, and 10−6 M), and ADO (10−7, 10−6, and 10−5 M)-induced dilations were compared in the mesenteric arteries of Mn-SOD+/− and WT mice (Fig. 4). Dilation to ACh was greatly reduced in the vessels of Mn-SOD+/− mice. For example, dilation to 10−7 M of ACh decreased by 56% compared with that of vessels of WT mice. Dilation to SNP was also reduced in the vessels of Mn-SOD+/− mice, but the magnitude of reduction was less. For example, dilation to 10−7 M SNP was decreased by 26% compared with that in vessels of WT mice. In contrast, dilations to ADO were comparable in vessels of the two groups of mice.

**O2•− level and SOD activity.** Basal release of O2•− in the mesenteric arteries of Mn-SOD+/− and WT mice was assessed by lucigenin-derived chemiluminescence. Figure 5 shows that the level of O2•− in the vessels of Mn-SOD+/− mice was significantly higher than that in vessels of WT mice.

Protein levels of CuZn-SOD and Mn-SOD in mesenteric arteries of Mn-SOD+/− and WT mice were assessed by Western analysis. A decreased density of Mn-SOD bands was seen in the vessels of Mn-SOD+/− mice (Fig. 6A). The summary data of densitometric ratios, shown in Fig. 6B, further indicate that expression of Mn-SOD was significantly decreased (by 42%) in Mn-SOD+/− mice compared with that of WT mice. Expression of CuZn-SOD was not different between the two groups. In correspondence with the decreased Mn-SOD expression, total SOD activity in the vessels of Mn-SOD+/− mice decreased by 44% (Fig. 6C). To determine Mn-SOD activity, KCN (0.4 mM) was added to inhibit the activity of copper-containing SODs. In the presence of KCN, SOD activity was 47% lower in vessels of Mn-SOD+/− than in vessels of WT mice (Fig. 6C).
eNOS activity. To determine whether the impairment of eNOS function was an additional cause of the decreased FID in the vessels of Mn-SOD+/− mice, we assessed eNOS expression and NOS activity in the mesenteric arteries of Mn-SOD+/− mice compared with those of WT mice (Fig. 7). Contrary to our expectation, expression of eNOS protein was reduced in vessels of Mn-SOD+/− mice compared with those of WT mice (Fig. 7A). Figure 7B shows that the densitometric ratio of eNOS in the vessels of Mn-SOD+/− mice significantly decreased (by 41%). Basal production of L-[3H]citrulline in the homogenates of pooled mesenteric arteries of Mn-SOD+/− mice was also significantly decreased (by 37%) (Fig. 7C).

To further investigate the influence of O2•− on NOS activity, we assessed eNOS expression and NOS activity from kidney homogenates of both groups of mice. Protein expression of eNOS was not different in the two groups (data not shown). However, L-[3H]citrulline production was significantly decreased (by 46%) in the Mn-SOD+/− mice compared with that of the WT mice (Fig. 8A). In WT mice, pyrogallol, a O2•− donor, dose dependently (0.2–0.8 mM) decreased NOS activity. In the presence of pyrogallol (0.4 mM), tiron (10 mM) fully restored NOS activity (Fig. 8B). Furthermore, in kidney homogenates of Mn-SOD+/− mice, tiron (10 mM) significantly increased NOS activity that became comparable to that of WT mice (Fig. 8C). These data indicate that an increased level of O2•− in Mn-SOD+/− mice inhibits the activity of NOS.

**DISCUSSION**

The present study is the first to provide evidence for the role of Mn-SOD in the maintenance of endothelium-dependent, flow- or shear stress-induced dilation by use of Mn-SOD+/− mice and demonstrates that the impaired NO-mediated FID in the arteries of Mn-SOD+/− mice is most likely due to an increased O2•− level and a decreased activity of eNOS.
It has been reported that in small mesenteric arteries of mice, the ACh-induced relaxation, in the absence of eNOS activity, is mainly mediated by H₂O₂-induced hyperpolarization of vascular smooth muscle cells (23). The mechanism involved in the ACh-induced synthesis of H₂O₂ is dependent on the function of CuZn-SOD (27). In the present study, ACh-induced dilation in arteries of Mn-SOD⁺⁻ mice is reduced (Fig. 4). Whether the reduced response is due to inactivation of NO by increased level of O₂⁻ or to decreased H₂O₂-mediated dilation (eNOS activity and SOD activity are both reduced in these vessels) is not clear, and more extensive studies would be needed to address these questions. Nevertheless, the results showing that SNP-induced dilations are reduced in the mesenteric arteries of Mn-SOD⁺⁻ mice (Fig. 4) (indicating an increased interaction between NO and O₂⁻ within the vascular wall) and that dilation to ADO, which serves as a control for smooth muscle-dependent dilation, as well as the basal tone of arteries are not different in Mn-SOD⁺⁻ and WT mice, suggest that increased mitochondria-derived O₂⁻ impairs endothelium-dependent agonist-induced dilations and that impaired NO-mediated responses are associated with Mn-SOD deficiency.

Several mechanisms may contribute to the impaired NO-mediated FID in the arteries of Mn-SOD⁺⁻ mice. One of them could be that oxidative stress significantly contributes to endothelial dysfunction via inactivation of NO by an increased formation of O₂⁻ (3, 19), as has been shown in variety of pathological states (4). In the present study, we found that basal release of O₂⁻ in the mesenteric arteries of Mn-SOD⁺⁻ mice was increased twofold over control (Fig. 5). Although, it is not clear whether application of shear stress would elicit an additional increase in the release of O₂⁻ in the vessels of Mn-SOD⁺⁻ mice, the results showing that administration of Tempol by scavenging O₂⁻ restores FID, reveal an imbalance among the level of O₂⁻, the activity of SOD, and the bioavailability of NO in response to shear stress in vessels of Mn-SOD⁺⁻ mice (Fig. 3). It can be debated whether O₂⁻ as a charged molecule can cross mitochondrial membranes. Recent studies suggest that O₂⁻ is released from the mitochondria into...
when released into the cytosol would inactivate NO, resulting in reduced endothelium-dependent and NO-mediated dilations. In addition, O$_2^\cdot$ produced in smooth muscle and perhaps other cells in the vascular wall could also inactivate NO generated in endothelial as well as smooth muscle cells. This concept is supported by the observed O$_2^\cdot$-dependent reduction in dilation of vessels of Mn-SOD$^{+/-}$ mice to SNP.

H$_2$O$_2$ plays an important role in many vascular beds as an endogenous vasodilator. In human coronary resistance arteries, FID is mediated by H$_2$O$_2$ (25), and the mitochondria are believed to be the source of H$_2$O$_2$ generated in response to shear stress (20). With the application of these mechanisms to our findings, the reduced FID in vessels of Mn-SOD$^{+/-}$ mice may also result from impaired H$_2$O$_2$-mediated dilation, because insufficiency of Mn-SOD could potentially result in a reduced ability to convert O$_2^\cdot$ in mitochondria to H$_2$O$_2$. However, our results showed that the magnitude of the remaining FID after inhibition of NO synthesis with l-NAME in the vessels of WT and Mn-SOD$^{+/-}$ mice were comparable, suggesting that the impaired FID in arteries of Mn-SOD$^{+/-}$ mice is mainly due to reduced NO bioavailability caused by an increased level of mitochondrial O$_2^\cdot$.

With regard to the decreased eNOS protein expression in the arteries of Mn-SOD$^{+/-}$ mice (Fig. 7), we have no ready mechanistic explanation. The reduction seems to be tissue specific because eNOS protein was not found to be reduced in the kidney or myocardium (12). Nevertheless, the reduced basal production of NO could contribute to the impaired NO-mediated FID in the vessels of these mice. In addition, the increased O$_2^\cdot$ concentration in Mn-SOD$^{+/-}$ mice may inhibit eNOS activity via several mechanisms to reduce FID (see below).

Increased level of O$_2^\cdot$ and reduced activity of eNOS in Mn-SOD$^{+/-}$ mice. In the present study, we found that an acute increase in O$_2^\cdot$ level by administering an O$_2^\cdot$ donor pyrogallol decreased eNOS activity in the kidney homogenates of Mn-SOD WT mice (Fig. 8B). Conversely, in Mn-SOD$^{+/-}$ mice, an acute decrease in the O$_2^\cdot$ level by administering the O$_2^\cdot$ scavenger tiron increased eNOS activity (Fig. 8C). These results suggest that an increased level of O$_2^\cdot$ inhibits eNOS activity. In contrast, in the mesenteric vessels of Mn-SOD$^{+/-}$ mice, it seems likely that the reduction (41\%) in eNOS protein is responsible for the reduced basal production of NO (Fig. 7, B and C). However, we cannot rule out the possibility that under a stimulation of shear stress, the inhibitory role of O$_2^\cdot$ on the activation of eNOS may provide for additional mechanisms that could contribute to the reduced FID in vessels of Mn-SOD$^{+/-}$ mice. It has been demonstrated by many studies that the formation of peroxynitrite, as a result of the reaction of O$_2^\cdot$ with NO, inhibits in variety of vascular functions that are dependent on the activation of eNOS. One of the underlying mechanisms is the uncoupling of eNOS via the oxidation of BH$_4^\cdot$ (16). On the other hand, it has also been demonstrated that peroxynitrite may attack the zinc-thiolate center of eNOS directly to cause enzymatic uncoupling (34). In either case, eNOS uncoupling decreases NO synthesis and increases the O$_2^\cdot$ level.

Recently, the role of oxidative stress-induced formation of asymmetric dimethylarginine (ADMA), an endogenous eNOS inhibitor, in contributing to cardiovascular dysfunction has attracted considerable attention. It has been demonstrated that
oxidative stress increases ADMA concentrations via upregulation of ADMA synthesis or inhibition of the ADMA degrading enzyme dimethylaminohydrolase. In the presence of high levels of ADMA, eNOS can become a source of vascular O$_2^-$ production that further enhances vascular oxidative stress burden (31, 32). In patients with peripheral arterial disease, FID is impaired, accompanied by an enhanced plasma concentration of ADMA (30). Thus a variety of O$_2^-$-related mechanisms may contribute to the decreased eNOS activity in mesenteric vessels and kidney homogenates of Mn-SOD$^{-/-}$ mice.

In summary, this study elucidates the specific role of Mn-SOD in the regulation of shear stress-dependent mechanisms in microvessels. The results demonstrate that deletion of the gene for Mn-SOD blunts eNOS activity and causes impaired flow- and agonist-induced dilation, caused by increased O$_2^-$ levels.

REFERENCES


