Vasomotor responses in MnSOD-deficient mice

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Vasomotor responses in MnSOD-deficient mice. Am J Physiol Heart Circ Physiol 287: H1141–H1148, 2004; 10.1152/ajpheart.01215.2003.—MnSOD is the only mammalian isoform of SOD that is necessary for life. MnSOD+/− mice die soon after birth, and MnSOD+/+ mice are more susceptible to oxidative stress than wild-type (WT) mice. In this study, we examined vasomotor function responses in aortas of MnSOD+/− mice under normal conditions and during oxidative stress. Under normal conditions, contractions to serotonin (5-HT) and prostaglandin F2α (PGF2α), relaxation to ACh, and superoxide levels were similar in aortas of WT and MnSOD+/− mice. The mitochondrial inhibitor antimycin A reduced contraction to PGF2α and impaired relaxation to ACh to a similar extent in aortas of WT and MnSOD+/− mice. The Cu/ZnSOD and extracellular SOD inhibitor diethyldithiocarbamate (DDC) paradoxically enhanced contraction to 5-HT and superoxide more in aortas of WT mice than in MnSOD+/− mice. DDC impaired relaxation to ACh and reduced total SOD activity similarly in aortas of both genotypes. Tiron, a scavenger of superoxide, normalized contraction to 5-HT, relaxation to ACh, and superoxide levels in DDC-treated aortas of WT and MnSOD+/− mice. Hypoxia, which reportedly increases superoxide, reduced contractions to 5-HT and PGF2α similarly in aortas of WT and MnSOD+/− mice. The vasomotor response to acute hypoxia was similar in both genotypes. In summary, under normal conditions and during acute oxidative stress, vasomotor function is similar in WT and MnSOD+/− mice. We speculate that decreased mitochondrial superoxide production may preserve nitric oxide bioavailability during oxidative stress.

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Vascular function. Thoracic aortas were carefully removed and placed in cold, oxygenated Krebs solution (containing (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 12 glucose). Aortas were then gently cleaned of excess fat and connective tissue, with care taken not to remove the adventitia.

Aortic segments 3–4 mm in length were suspended on wire hooks in individual water-jacketed organ baths containing Krebs solution with 95% O₂-5% N₂ and kept at 37°C. Resting tension was increased from 0 to 0.5 g over 1 h, and, after this equilibration period, the vessels were twice contracted with 80 mM KCl for 5 min. Vessels from 0 to 0.5 g over 1 h, and, after this equilibration period, the vessels were rinsed before and after each concentration-response curves. Mild hypoxia was generated by bubbling the organ bath with 9% O₂-86% N₂-5% CO₂, and severe hypoxia was generated with 95% N₂-5% CO₂. In some studies, vessels were twice contracted with 80 mM KCl for 5 min. Vessels from 0 to 0.5 g over 1 h, and, after this equilibration period, the vessels were rinsed before and after each concentration-response curves.

Vessels were treated with 0.02 μg/ml antimycin A (Sigma) for 15 min before responses were examined to ACh and SNP. Separate vessels were incubated with 1 mM DDC for 1 h before the concentration-response curves were performed. Mild hypoxia was generated by bubbling the organ bath with 9% O₂-86% N₂-5% CO₂, and severe hypoxia was generated with 95% N₂-5% CO₂. In some studies, vessels were pretreated with 1 mM dihydroxy-1,3-benzenedisulfonic acid (tiron, Sigma) for 10 min before other interventions. In some of the studies of severe hypoxia, vessels were incubated with 1 μM glybenclamide (Sigma) for 10 min or 100 μM N⁴-nitro-L-arginine (l-NAME, Sigma) for 30 min before determination of vasoconstrictor responses.

Superoxide levels. Lucigenin-enhanced chemiluminescence was used to detect superoxide as previously described (33, 37). Aortas were prepared as above, and the rings were kept in ice-cold Krebs solution until use. Bis-N⁴-methylacridinium nitrate (lucigenin, 5 μM, Sigma) was prepared fresh daily in PBS at least 30 min before use and kept in the dark at room temperature.

Vessel rings were studied under control conditions and after treatment with antimycin A (0.02 μg/ml) for 15 min. In other experiments, DDC (1 mM) or an equivalent volume of PBS was applied, and the rings were incubated for 1 h at 37°C before measurements of superoxide were made. In some experiments, vascular rings were incubated with 100 μM N⁴-nitro-L-arginine methyl ester (l-NAME, Sigma) for 30 min, with or without DDC, before superoxide determination. After superoxide measurement, vessels were cut open longitudinally and dried flat on plastic plates. The surface area of the vessels was measured using digital photos of the vessels and the supplied tools in Scion Image (NIH, v4.0.2). Data are expressed as relative light units per second per millimeter squared.

Immunoblotting. Aortas were removed and cleaned as described above. Aortas were frozen in liquid N₂ and pulverized with a pestle, followed by homogenization in 100 μl of room temperature homogenization buffer (1% SDS, 10 mM EDTA, and protease inhibitor cocktail, Roche; Basel, Switzerland). Samples were then boiled for 15 min and centrifuged at 15,000 g for 15 min. The protein concentration of the supernatant was determined using the Lowry method (Bio-Rad; Hercules, CA).

Samples were diluted 1:1 with 2× Laemmli buffer and boiled for 15 min before being loaded (5–10 μg) into wells of 4–20% SDS gels (Ready Gel, Bio-Rad). After electrophoresis at a constant 150 V for 1.5 h, proteins were transferred to nitrocellulose membranes using the semidyed cell transfer method (Bio-Rad) at a constant 40 mA/gel for 45 min.
min. After transfer, equal loading was assured by staining the nitrocelulose blots for 5 min using 0.1% Ponceau S (Sigma). The blots were then blocked in cold blocking solution (5% nonfat milk and 1% BSA) for 1 h. Blots were then incubated overnight at 4°C with the MnSOD primary rabbit antibody, which has activity toward both human and mouse MnSOD (65) (1:1,000, a gift from Dr. Larry Oberley, University of Iowa, and now available from Upstate Biotechnology; Lake Placid, NY).

Blots were then rinsed in PBS, blocked for 1 h at room temperature, and then exposed to the horseradish peroxidase-conjugated secondary antibody (1:10,000 anti-rabbit) for 1 h at room temperature. After blots were rinsed in PBS, bands were detected by chemiluminescence (Supersignal West Femto Maximum Sensitivity; Pierce; Rockford, IL). Densitometric analysis was performed using a Bio-Rad Flour-S Technology; Lake Placid, NY).

Immunostaining. Aortas were removed and cleaned as described above. Vessels were then frozen and cut in a cryostat at 8°C. Sections were then blocked with 8% BSA and 2% normal goat serum for 1 h before the primary antibody (anti-MnSOD made in rabbit, 1:1,000) was applied and incubated overnight at 4°C.

The sections were then rinsed with PBS for 15 min, incubated in 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity, and washed in distilled H2O for 5 min. Sections were then rinsed with cold 15 min in PBS and blocked in 8% BSA for 1 h. MnSOD was then visualized using a Vectastain Elite ABC kit (PK-6101, Vector Labs; Burlingame, CA) following the manufacturer’s directions. Sections were then counterstained with hematoxylin and cosin, dehydrated, cleared, and coverslipped. Specificity of the antibodies was demonstrated by a lack of staining in conditions where no primary antibody was applied (data not shown).

SOD activity. Total SOD activity in aortic homogenates was measured using the nitroblue tetrazolium (NBT; Sigma) assay (47). The blots were then blocked in cold blocking solution (5% nonfat milk and 1% BSA) for 1 h. Blots were then incubated overnight at 4°C with the MnSOD primary rabbit antibody, which has activity toward both human and mouse MnSOD (65) (1:1,000, a gift from Dr. Larry Oberley, University of Iowa, and now available from Upstate Biotechnology; Lake Placid, NY).

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**DISCUSSION**

This is the first study to examine vascular function in MnSOD-deficient mice. The major new findings of the study are as follows: 1) under normal conditions, vasomotor function and superoxide levels are similar in aortas of WT and MnSOD$^{+/−}$ mice; 2) the mitochondrial inhibitor antimycin A impairs endothelium-dependent relaxation to ACh to a similar degree in aortas of WT and MnSOD$^{+/−}$ mice without detectably increasing superoxide levels; 3) inhibition of Cu/ZnSOD and ECSOD with DCC enhances contraction to 5-HT in aortas of WT but not MnSOD$^{+/−}$ mice, whereas relaxation to ACh...
was similarly impaired by DDC in aortas of both genotypes; 4) after DDC treatment, aortic superoxide levels were greater in WT mice than in MnSOD \(^{+/−}\) mice; and 5) contraction to 5-HT and PGF\(_2\alpha\) was impaired during hypoxia similarly in both genotypes, and vasomotor responses to hypoxia were similar in aortas of WT and MnSOD \(^{+/−}\) mice. Overall, these finding suggest that aortas of MnSOD \(^{+/−}\) mice are not more susceptible to endothelial dysfunction caused by acute oxidative stress.

**MnSOD location and content.** In aortas of WT and MnSOD \(^{+/−}\) mice, MnSOD immunostaining was intense in the endothelium and was diffuse in the smooth muscle and adventitia. Consistent with a previous study (13), aortic MnSOD protein expression was reduced by 45% in MnSOD \(^{+/−}\) mice. Total SOD activity was similar in aortas of the two genotypes. Because MnSOD comprises 10% (or less) of total SOD activity in the mouse aorta (49), measurement of total SOD activity may not be sufficiently sensitive to detect a reduction in SOD activity in MnSOD \(^{+/−}\) mice. Likewise, it is difficult to determine whether the other isoforms of SOD have compensated for the reduction of MnSOD. When considered with previous studies (13, 21, 29), however, it is probable that Cu/ZnSOD and ECSOD are not upregulated in aortas of MnSOD \(^{+/−}\) mice. We cannot rule out the possibility that changes in other proteins may influence vascular function and/or superoxide levels in MnSOD-deficient mice.

**Vasomotor function and superoxide levels under normal conditions.** As we hypothesized, contraction, endothelium-dependent relaxation, and superoxide levels were similar in aortas of WT and MnSOD \(^{+/−}\) mice under normal conditions. Previous studies suggest that oxidative stress is confined to mitochondria of MnSOD \(^{+/−}\) mice and that subjecting MnSOD \(^{+/−}\) mice to oxidative stress is required to observe a more severe phenotype in MnSOD \(^{+/−}\) mice (2, 25, 29, 58, 64).

**Effects of antimycin A.** In isolated mitochondria, antimycin A stimulates superoxide production (43, 48), but, as with other electron transport chain inhibitors, this may not occur in intact tissues (32, 39). In the present study, antimycin A impaired contraction to PGF\(_2\alpha\), which is concordant with effects in other models (50, 63). Antimycin A alters Ca\(^{2+}\) handling (50) and decreases ATP levels (3, 8, 68), each of which could explain the reduction of contraction to PGF\(_2\alpha\) observed in the present study.

Antimycin A impairs relaxation to ACh in rabbit aortic strips (12), and there is an association between endothelial dysfunction and ATP depletion (51, 52). It is conceivable that ATP depletion and not superoxide production per se is the primary mechanism for endothelial dysfunction and reduced contraction produced by antimycin A seen in this study.

**Effects of DDC.** Contrary to our hypothesis, there was no difference in the response to ACh in aortas of WT and MnSOD \(^{+/−}\) mice treated with DDC. Previous studies have also shown that DDC impairs endothelium-dependent relaxation in the aorta (10, 14, 30, 31, 35, 40). DDC did, however, significantly enhance contraction to 5-HT in aortas of WT mice but not MnSOD \(^{+/−}\) mice. To our knowledge, the present data are the first to demonstrate that DDC enhances contraction to 5-HT. Interestingly, Cu/ZnSOD-deficient mice also display endothelial dysfunction typified by impaired relaxation to ACh and enhanced contraction to 5-HT (11).

DDC may act as a NO- spin trap (59), but our data are consistent with the concept that DDC produces endothelial dysfunction by increasing superoxide. The superoxide scavenger tiron restored relaxation to ACh and contraction to 5-HT to control levels in aortas of both WT and MnSOD \(^{+/−}\) mice treated with DDC. Others have also found that scavengers of superoxide protect vessels from DCC-induced endothelial dysfunction (9, 38, 40, 41, 61). Because reducing superoxide with tiron restores endothelial function in the presence of DDC, the effects of DDC appear to be mediated primarily by elevated superoxide levels and not direct interactions between NO- and DDC.

Similar to previous studies, we found that DDC reduced total aortic SOD activity and increased superoxide (19, 35, 38, 40, 61). Surprisingly, superoxide levels in DCC-treated aortas were greater in WT mice. This unexpected result may provide an explanation for the finding that contraction to 5-HT was greater in DCC-treated aortas of WT mice, because contraction to 5-HT is modulated by NO- levels (26) and is increased by oxidative stress (17, 18, 55). Nevertheless, it is surprising that WT mice have higher levels of superoxide than MnSOD \(^{+/−}\) mice. Two explanations were considered: increased production of NO- or reduced production of superoxide under basal conditions in MnSOD \(^{+/−}\) mice.

Consistent with the findings that relaxation to ACh and contraction to 5-HT are similar in control aortas of WT and MnSOD \(^{+/−}\) mice, inhibition of NO- synthase with L-NAME did not increase superoxide in vehicle- or DCC-treated aortas of either WT or MnSOD \(^{+/−}\) mice. Thus there is evidence showing that release of NO- is not greater in the aortas of MnSOD \(^{+/−}\) mice than in WT mice. The possibility that basal superoxide production is reduced in MnSOD \(^{+/−}\) mice is considered below.
Effect of hypoxia. Similar to previous studies in other models (23, 46), we found that severe hypoxia attenuated contractile responses in aortas of both WT and MnSOD+/− mice. Because impairment of contraction was not greater in MnSOD+/− mice than in WT mice, the primary mechanism for impairment may not be directly related to superoxide. It is possible that other events during hypoxia, such as increased K+ conductance (53, 54) or altered responses to Ca2+ in smooth muscle (46), underlie the reduced contraction during hypoxia observed in this study.

Hypoxia produces relaxation in systemic arteries, although specific mechanism(s) are not completely defined. Similar to previous studies (45, 53, 54), we demonstrated that responses of precontracted vessels differ depending on the severity of the hypoxia. Mild hypoxia simply relaxed aortic rings, whereas the response to severe hypoxia was triphasic.

As in previous studies (7, 67), we found that the first two phases of the response to severe hypoxia were dependent on NO-synthesis, but maximum relaxation was NO-independent. Consistent with some (23, 46) but not all studies (53, 54), we did not find that glybenclamide-sensitive KATP channels are involved in the sustained relaxation to hypoxia. Mechanisms that mediate the large, sustained relaxation, while almost certainly not NO- or KATP channel dependent, are not clear.

In some (6, 32) but not other (7, 42, 46, 66) models, generation of ROS from mitochondria contributes to the vascular response to hypoxia. Although levels of MnSOD are reduced, the aortas of WT and MnSOD+/− mice respond similarly to hypoxia, and the ROS scavenger tiron did not alter vasomotor responses to hypoxia in either genotype. These findings suggest that increased ROS levels may not be important for vasomotor response to hypoxia in the mouse aorta.

In summary, levels of MnSOD are reduced in aortas of MnSOD+/− mice, and, under normal conditions, vasomotor function and superoxide levels are similar in aortas of WT and MnSOD+/− mice. Interestingly, after DDC treatment, contraction to 5-HT and superoxide levels were greater in aortas of WT mice than in MnSOD+/− mice, suggesting that superoxide is reduced in aortas of MnSOD+/− mice under normal conditions.

Previous studies have shown that mitochondrial oxygen consumption is reduced in MnSOD+/− mice (20, 29). Mitochondrial oxygen consumption is directly related to superoxide production (22, 57). We speculate that decreased oxygen consumption by mitochondria could contribute to the finding that the aortas of MnSOD+/− mice have lower superoxide levels than those of WT mice. In support of this hypothesis, impairment of vasomotor function was not greater in aortas of MnSOD+/− mice than in WT mice, and less superoxide was observed in aortas of MnSOD+/− mice after DDC treatment than in WT mice. Therefore, it seems likely that under normal conditions, aortic mitochondria produce less superoxide in MnSOD+/− mice than in WT mice.

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