Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization

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The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasodilating factors, including prostacyclin, NO, and endothelium-derived hyperpolarizing factor (EDHF). We have recently identified that endothelium-derived H_2O_2 is an EDHF in mesenteric arteries of mice and humans and in porcine coronary microvessels. However, the mechanism for the endothelial production of H_2O_2 as an EDHF remains to be elucidated. In this study, we tested our hypothesis that Cu,Zn-superoxide dismutase (Cu,Zn-SOD) plays a pivotal role in endothelium-dependent hyperpolarization, using control and *Cu,Zn-SOD*$^{-/-}$ mice. In mesenteric arteries, EDHF-mediated relaxations and hyperpolarizations were significantly reduced in *Cu,Zn-SOD*$^{-/-}$ mice with no inhibitory effect of catalase, while endothelium-independent relaxations and hyperpolarizations were preserved. Endothelial H_2O_2 production also was significantly reduced in *Cu,Zn-SOD*$^{-/-}$ mice. In Langendorff isolated heart, bradykinin-induced increase in coronary flow was significantly reduced in *Cu,Zn-SOD*$^{-/-}$ mice, again with no inhibitory effect of catalase. The exogenous SOD mimetic tempol significantly improved EDHF-mediated relaxations and hyperpolarizations and coronary flow response in *Cu,Zn-SOD*$^{-/-}$ mice. These results prove the novel concept that endothelial Cu,Zn-SOD plays an important role as an "EDHF synthase" in mice, in addition to its classical role to scavenge superoxide anions.

Introduction

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasodilators, including prostacyclin, NO, and endothelium-derived hyperpolarizing factor (EDHF) (1, 2). EDHF plays an important role in modulating vascular tone, especially in microvessels (1–3). Since the first reports on the existence of EDHF (4, 5), several candidates for EDHF have been proposed, including cytochrome P450 metabolites (6, 7), potassium ions (8, 9), and gap junctional electrical communication (10, 11). We have recently identified that endothelium-derived H_2O_2 is an EDHF in mesenteric arteries of mice

(3) and humans (12) and in porcine coronary microvessels (13). Subsequently other investigators have confirmed the importance of H_2O_2 as an endogenous EDHF in coronary microvessels (14, 15) and piglet pial arterioles (16). However, the mechanism for the endothelial production of H_2O_2 as an endogenous EDHF remains to be elucidated. In endothelial cells, H_2O_2 is mainly produced by superoxide dismutase (SOD) from superoxide anions.

There are three isoforms of SOD that dismutate superoxide anions to H_2O_2. Copper,zinc-SOD (Cu,Zn-SOD, or SOD1) is located in the cytosol and nucleus and to a lesser extent in the mitochondria, and is expressed in almost all cell types (17). Cu,Zn-SOD is reported to dismutate superoxide anions derived from eNOS during NO synthesis (18) and thereby prolongs the half-life of NO (19, 20). Manganese SOD (MnSOD, or SOD2) is located in the mitochondrial matrix and is the primary SOD isoform that dismutates superoxide anions generated by the respiratory chain (21). Extracellular SOD (ecSOD, or SOD3) is secreted extracellularly and is bound to sulfated polysaccharides such as heparan sulfate on cell surfaces (21). Cu,Zn-SOD is a major SOD in blood vessels, accounting for approximately 50–80% of total SOD activity (22, 23). Since we have found that endothelial production of H_2O_2/EDHF is reduced in eNOS-knockout (*eNOS*$^{-/-}$) mice (3), we hypothesized that H_2O_2 is produced by

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Nonstandard abbreviations used: endothelium-derived hyperpolarizing factor (EDHF); superoxide dismutase (SOD); copper,zinc-SOD (Cu,Zn-SOD); manganese SOD (MnSOD); extracellular SOD (ecSOD); prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); acetylcholine (ACh); calcium-activated potassium channels (KCa channels); N$^{\omega}$-nitro-L-arginine (L-NNA); 2′,7′-dichlorodihydrofluorescein diacetate (DCF).

endothelial Cu,Zn-SOD from superoxide anions (mainly derived from eNOS) and acts as an endogenous EDHF. In this study, we examined our hypothesis that endothelial Cu,Zn-SOD plays a pivotal role as an EDHF synthase using Cu,Zn-SOD knockout (*Cu,Zn-SOD*$^{-/-}$) mice.

Methods

Animals and tissue preparation. Male *Cu,Zn-SOD*$^{-/-}$ and control mice (10–16 weeks of age) derived from breeding pairs of heterozygous (*Cu,Zn-SOD*$^{+/-}$) mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used. Systolic blood pressure was measured by the tail-cuff method under conscious conditions before the animals were sacrificed (3). The animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and small mesenteric arteries, heart, and liver were excised to be used for the experiments. This study was approved by the Research Committee of the Kyushu University Graduate School of Medical Sciences.

Western blot analysis for SOD isoforms and eNOS. Western blot analysis was performed using an antibody that specifically recognizes proteins including Cu,Zn-SOD, MnSOD, ecSOD, and eNOS. Mesenteric arteries were homogenized and proteins were extracted. The same amount of extracted protein (10–20 μg for each experiment) was loaded for SDS-PAGE/immunoblot analysis. The regions containing SOD isoform proteins and eNOS proteins were visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, United Kingdom).

Cu,Zn-SOD activity. Cu,Zn-SOD activity in the mesenteric arteries was examined using a nitroblue tetrazolium method as described previously (24). Briefly, 20 μg of tissue protein was separated on a nondenaturing polyacrylamide gel. The SOD activity was visualized by initially soaking the gel in 2.43 mM nitroblue tetrazolium for 30 minutes, followed by incubation in a solution of 0.036 M potassium phosphate buffer containing 0.028 mM riboflavin and 280 mM tetramethyl ethylenediamine (24).

Glutathione peroxidase activity. Glutathione peroxidase activity of the liver was examined by the oxidation of NADPH in the presence of glutathione reductase, which catalyzes the reduction of oxidized glutathione formed by glutathione peroxidase (25). Liver was homogenized, proteins were extracted, and the sample protein and the reagent (containing 0.1 M Tris-HCl, 0.2 mM NADPH, 0.5 mM EDTA, 2 mM glutathione, and 1 unit of glutathione reductase) were incubated in a total volume of 1 ml for 2 minutes at 37°C. Then *t*-butyl hydroperoxide (0.07 mM) was added to the reagent well and absorbance was measured at 340 nm every 10 seconds for 2 minutes.

Catalase activity. Catalase activity of the liver was determined as previously described (25). Briefly, 2.25 ml phosphate buffer (1.0 M), 9 ml methanol (12 M), 1.8 ml H_2O_2 (44 mM), and 6.75 ml distilled water were mixed. Then 50 μl of the mixed solution, 0.5 μl sample protein,

and 49.5 μl phosphate buffer were incubated for 20 minutes, and 75 μl Purpald (Sigma-Aldrich, St. Louis, Missouri, USA) (25 mM in 2 N potassium hydroxide) was added to the reagent and this was incubated for 20 minutes. Finally, 25 μl of potassium periodate (65.2 mM) was added and the absorbance of the purple formaldehyde adduct was measured at 550 nm (25).

Organ chamber experiments. Experiments were performed in 37°C Krebs solution bubbled with 95% O_2 and 5% CO_2. Small mesenteric arteries (200–250 μm) were cut into rings and isometric tension was recorded in isolated arterial rings contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (3–10 μM) or KCl (40–60 mM) (3, 12). The extent of contraction in response to $PGF_{2\alpha}$ was adjusted to 50–70% of that induced by 60 mM KCl. We examined endothelium-dependent relaxations in response to acetylcholine (ACh) as well as endothelium-independent relaxations in response to sodium nitroprusside; NS-1619, an opener of the large conductance calcium-activated potassium (KCa) channels; and exogenous H_2O_2. The contribution of vasodilator prostaglandins, NO, and EDHF to ACh-induced endothelium-dependent relaxation was estimated by determining the inhibitory effects of indomethacin (10^{-5} M), N$^\omega$-nitro-L-arginine (L-NNA, 10^{-4} M), and KCl (40–60 mM), respectively (3, 12). We have confirmed that the addition of carboxy–2-(4-carboxyphenyl)-4, 4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO, a scavenger of NO) (26) to these concentrations of indomethacin and L-NNA does not further inhibit the endothelium-dependent relaxations to ACh, indicating that the blockade of eNOS with L-NNA may be complete (data not shown). In addition, we examined the inhibitory effect of 100 nM charybdotoxin (an inhibitor of large and intermediate-conductance KCa channels) and 1 μM apamin (an inhibitor of small-conductance KCa channels). The combination of the two KCa channel blockers has been widely used to evaluate EDHF-mediated relaxations (1). Indomethacin, L-NNA, charybdotoxin, apamin, and the SOD mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol, 1 mM) (27) was applied to organ chambers 30 minutes before precontraction with $PGF_{2\alpha}$, and catalase (6,250 U/ml) was applied 1 hour before the precontraction (3, 12, 13). When we used endothelium-denuded arteries, we removed endothelial cells mechanically (3, 12, 13).

Electrophysiological experiments. Electrophysiological experiments were performed in isolated small mesenteric arteries. The rings of small mesenteric arteries from both strains were placed in experimental chambers perfused with 37°C Krebs solution containing indomethacin (10^{-5} M) and L-NNA (10^{-4} M) (3). A fine glass capillary microelectrode was stuck into the smooth muscle cell from the adventitial side (3). Changes in membrane potentials produced by ACh were continuously recorded (3). The SOD mimetic tempol (1 mM) (27) was applied 30 minutes before membrane potential recordings, and catalase (6,250 U/ml) was applied 60 minutes before the experiment.

Detection of ACh-induced H$_2$O$_2$ production by endothelial cells. Detection of H$_2$O$_2$ production by endothelial cells with a peroxide-sensitive fluorescent dye was performed as previously described (3). Briefly, small mesenteric arteries of control and *Cu,Zn-SOD$^{-/-}$* mice were cut into rings and then opened longitudinally. The vascular strip was incubated with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF) for 15 minutes, then placed on a glass slide and observed using a laser confocal microscope at an excitation wavelength of 488 nm and an emission wavelength of 520 nm at 25°C. Fluorescence images of the endothelium were obtained before and 3 minutes after application of ACh, which was achieved by dropping HEPES buffer containing 10 μM ACh onto the glass slide. Relative fluorescence intensity was calculated using images obtained under basal conditions without ACh.

Langendorff isolated heart preparation. A Langendorff experiment was performed as previously described (28, 29). Briefly, hearts were quickly excised and placed in heparinized ice-cold buffer to arrest cardiac contraction. After all extracardiac tissues were removed, the aorta was carefully tied to an aortic cannula made from a 20-gauge blunted needle. Hearts were perfused retrogradely at a constant pressure of 80 mmHg with warm Krebs-Henseleit buffer in standard Langendorff fashion. Once they were cannulated and the perfusion had started, the hearts were surrounded by a water-jacketed organ bath for maintenance of constant temperature. They were paced electrically at a constant rate throughout the experiment. Coronary flow was recorded with a flow meter using an ultrasonic flow probe connected in-line with the aortic perfusate. Coronary flow increase in response to bradykinin, and the effects of catalase (1,250 U/ml) and tempol (1 mM) were examined.

Drugs and solutions. The ionic composition (mM) of Krebs solution was Na$^+$ 144, K$^+$ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, H$_2$PO$_4^-$ 1.2, HCO$_3^-$ 24, Cl$^-$ 129.7, glucose 5.5; that of HEPES buffer was Na$^+$ 143, K$^+$ 5.9, Mg^{2+} 1.2, Ca^{2+} 1.6, H$_2$PO$_4^-$ 1.2, Cl$^-$ 150.9, glucose 11, HEPES 5; and that of Krebs-Henseleit buffer was Na$^+$ 144, K$^+$ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, H$_2$PO$_4^-$ 1.2, HCO$_3^-$ 25, Cl$^-$ 127.7, glucose 11, EDTA 0.5, and pyruvate 2.0. Antibodies to Cu,Zn-SOD and MnSOD were obtained from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada) and eNOS antibody was obtained from Transduction Laboratories (Franklin Lakes, New Jersey, USA). Levcromakalim was obtained from Tocris Cookson Inc. (Ellisville, Missouri, USA) and sodium nitroprusside was obtained from Maruishi Seiyaku Co. (Osaka, Japan). DCF was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). All other agents were obtained from Sigma-Aldrich.

Statistics. Results are expressed as mean ± SEM. Dose-response curves were analyzed by two-way ANOVA followed by the Scheffé post-hoc test for multiple comparisons. Other values were analyzed by paired and unpaired Student's *t* test or one-way ANOVA. *P* < 0.05 was considered to be statistically significant.

Results

Characterization of Cu,Zn-SOD$^{-/-}$ mice. There was no significant difference in systolic blood pressure (mmHg) between the *Cu,Zn-SOD$^{-/-}$* mice (117 ± 3) and the control mice (120 ± 4) (*n* = 17 each). Western blot analysis showed that expression of Cu,Zn-SOD was absent in mesenteric arteries from the *Cu,Zn-SOD$^{-/-}$* mice, whereas the expression of MnSOD or ecSOD was unchanged (Figure 1a). The Cu,Zn-SOD activity in mesenteric arteries, which was examined by a nitroblue tetrazolium method (24), was totally absent in the *Cu,Zn-SOD$^{-/-}$* mice (Figure 1b). By contrast, the activities of glutathione peroxidase and catalase in the liver, both of which dismutate H$_2$O$_2$, were unchanged in the knockout mice (Figure 1, c and d, respectively). Western blot analysis for eNOS showed that the extent of eNOS expression was comparable between the two strains (Figure 1e).

Endothelium-dependent relaxations. In mesenteric arteries of control mice, endothelium-dependent relaxations in response to ACh were resistant to indomethacin (10^{-5} M) or L-NNA (10^{-4} M) but were significantly inhibited by KCl (40–60 mM) (Figure 2a). In mesenteric arteries of *Cu,Zn-SOD$^{-/-}$* mice, endothelium-dependent relaxations in response to ACh were slightly attenuated compared with those of control mice, and L-NNA markedly inhibited the relaxations (Figure 2b). Figure 2c shows
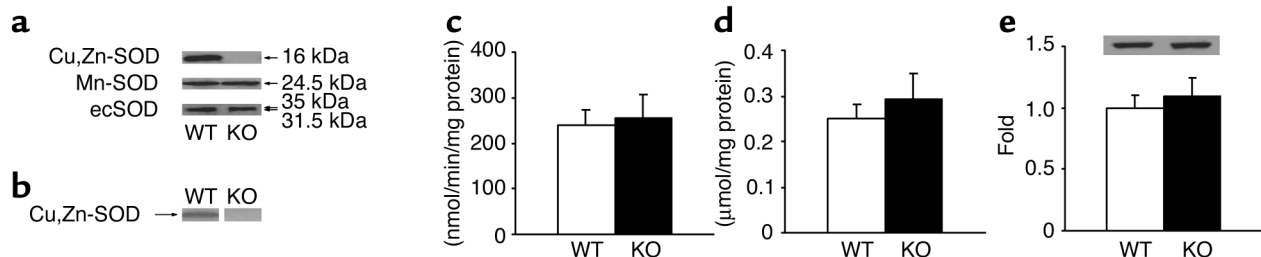

Figure 1
Characterization of *Cu,Zn-SOD$^{-/-}$* mice. (**a**) Western blot analysis for SOD isoforms in mesenteric arteries from control (WT) and *Cu,Zn-SOD$^{-/-}$* (KO) mice. Cu,Zn-SOD expression was absent in the *Cu,Zn-SOD$^{-/-}$* mice, whereas the expression of MnSOD and ecSOD was unaltered. (**b**) Cu,Zn-SOD activity of mesenteric arteries from the two strains. Cu,Zn-SOD activity was absent in the *Cu,Zn-SOD$^{-/-}$* mice. Comparable activities of glutathione peroxidase (**c**) and catalase (**d**) of the liver in the two strains (*n* = 5 each). (**e**) Western blot analysis for eNOS expression in mesenteric arteries (*n* = 5 each). eNOS protein expression was comparable between the two strains.

the relative contributions of prostacyclin, NO, and EDHF to the endothelium-dependent relaxations of mesenteric arteries from both strains when using KCl as an inhibitor of EDHF-mediated responses. In mesenteric arteries, EDHF-mediated relaxations were significantly reduced, while NO-mediated relaxations were significantly increased in $Cu,Zn-SOD^{-/-}$ mice compared with control mice. The combination of charybdotoxin and apamin also significantly inhibited EDHF-mediated relaxations in both strains (Figure 2, d and e). The EDHF-mediated relaxations, as evaluated by the inhibitory effect of the combination, were also significantly reduced in $Cu,Zn-SOD^{-/-}$ mice (Figure 2f).

Endothelium-independent relaxations. Endothelium-independent relaxations in response to sodium nitroprusside (Figure 3a) and to NS-1619 (Figure 3b) were comparable between the two strains. Furthermore, relaxations of endothelium-denuded mesenteric arteries in response to exogenous H_2O_2 were comparable between the two strains (Figure 3c).

Endothelium-dependent and -independent hyperpolarizations. Electrophysiological recordings of membrane potentials with the microelectrode technique in mesenteric arteries demonstrated that endothelium-dependent hyperpolarizations induced by ACh (10^{-5} M) were significantly reduced in the $Cu,Zn-SOD^{-/-}$ mice compared with control mice (Figure 4a). By contrast, endothelium-independent hyperpolarizations induced by levcromakalim were comparable between the two strains (Figure 4b).

Inhibitory effect of catalase on EDHF-mediated responses. In the presence of indomethacin (10^{-5} M) and L-NNA (10^{-4} M), catalase significantly inhibited EDHF-mediated relaxations of mesenteric arteries from control mice (Figure 5a). By contrast, catalase had no inhibitory effect on the EDHF-mediated relaxations of mesenteric arteries from $Cu,Zn-SOD^{-/-}$ mice (Figure 5b). Catalase significantly inhibited the hyperpolarization response in control mice (Figure 5c) but not in $Cu,Zn-SOD^{-/-}$ mice (Figure 5d).

ACh-induced H_2O_2 production by endothelial cells. In the control mice, ACh significantly increased the fluorescence intensity in endothelial cells (Figure 6, a and b), while in the $Cu,Zn-SOD^{-/-}$ mice, ACh-induced fluorescence intensity was significantly reduced compared with the control mice (Figure 6c). Figure 6d
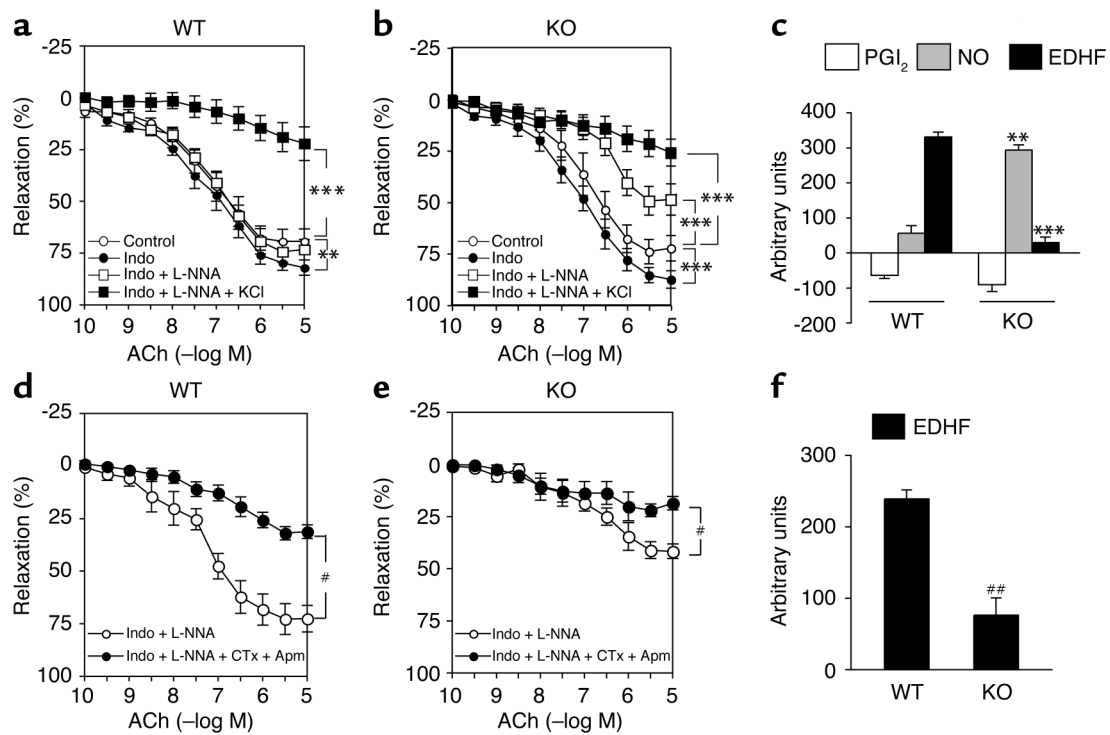

Figure 2

Reduced EDHF-mediated relaxations in $Cu,Zn-SOD^{-/-}$ mice. Endothelium-dependent relaxations of mesenteric arteries from control WT mice (**a**) and from $Cu,Zn-SOD^{-/-}$ mice (**b**) ($n = 7$ each) are shown. Indo, indomethacin (10^{-5} M); L-NNA, 10^{-4} M; KCl, 40–60 mM. In mesenteric arteries of control mice (**a**), endothelium-dependent relaxations were resistant to indomethacin or L-NNA, whereas in those of $Cu,Zn-SOD^{-/-}$ mice (**b**), indomethacin and L-NNA markedly inhibited the relaxations. $**P < 0.01$, $***P < 0.0001$. (**c**) Relative contribution of prostacyclin (PGI_2), NO, and EDHF to the endothelium-dependent relaxations of mesenteric arteries in response to ACh. In mesenteric arteries from $Cu,Zn-SOD^{-/-}$ mice, EDHF-mediated relaxations were significantly attenuated and NO-mediated relaxations were significantly enhanced. $**P < 0.01$, $***P < 0.0001$ vs. WT. (**d** and **e**) Inhibitory effect of the combination of charybdotoxin (CTx) and apamin (Apm) on EDHF-mediated relaxations. The combination significantly inhibited EDHF-mediated relaxations in both strains. $^{#}P \leq 0.0001$. (**f**) Relative contribution of EDHF to the endothelium-dependent relaxations of mesenteric arteries as evaluated by the inhibitory effects of charybdotoxin and apamin. EDHF-mediated relaxations were also significantly reduced in $Cu,Zn-SOD^{-/-}$ mice. $^{##}P \leq 0.01$.
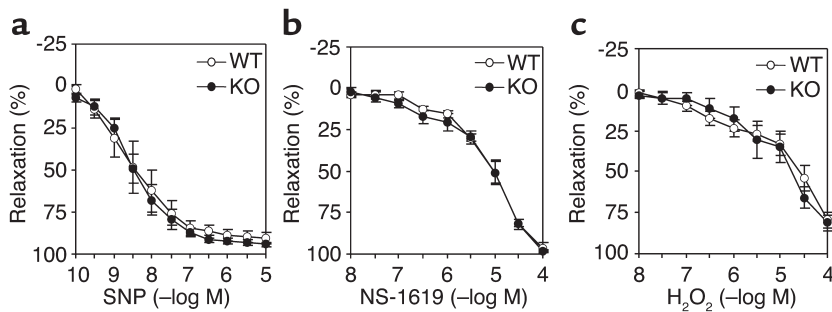
Figure 3

Preserved vascular smooth muscle cell responses in *Cu,Zn-SOD*$^{-/-}$ mice. (**a**) Endothelium-independent relaxations in response to sodium nitroprusside (SNP) of mesenteric arteries from control mice and *Cu,Zn-SOD*$^{-/-}$ mice (*n* = 6–8). (**b**) Endothelium-independent relaxations in response to NS-1619 of mesenteric arteries from control mice and *Cu,Zn-SOD*$^{-/-}$ mice (*n* = 6–8). Those endothelium-independent relaxations were comparable between the two strains. (**c**) Exogenous H_2O_2-induced relaxations of mesenteric arteries without endothelium (*n* = 6 each). H_2O_2 caused a comparable extent of endothelium-independent relaxations in the two strains.

summarizes the relative fluorescence intensity in response to ACh in both strains.

Coronary flow response in isolated heart. In the presence of indomethacin (10^{-5} M) and L-NNA (10^{-4} M), bradykinin-induced increase in coronary flow was 201% ± 9% of baseline in the control mice, whereas in the *Cu,Zn-SOD*$^{-/-}$ mice, the response was significantly reduced at 151% ± 7% (Figure 7a). Catalase significantly inhibited the coronary flow response in the control mice (153% ± 7%) but not in the *Cu,Zn-SOD*$^{-/-}$ mice (Figure 7a). Thus, the inhibitory effect of catalase on the coronary flow response was markedly reduced in the *Cu,Zn-SOD*$^{-/-}$ mice compared with the control mice (Figure 7b).

Effect of SOD mimetic supplementation on EDHF-mediated relaxations in Cu,Zn-SOD$^{-/-}$ *mice.* Since SOD is a large molecule that penetrates the endothelium with difficulty, we used a cell-permeable SOD mimetic, tempol (27). In the presence of indomethacin (10^{-5} M) and L-NNA (10^{-4} M), tempol did not significantly improve EDHF-mediated relaxations of mesenteric arteries from control mice, and the relaxations were again significantly inhibited by catalase (Figure 8a). By contrast, in mesenteric arteries from *Cu,Zn-SOD*$^{-/-}$ mice, tempol significantly improved the EDHF-mediated relaxations, and the enhancing effect of tempol was abolished by catalase (Figure 8b). Membrane potential recordings demonstrated that in control mice, tempol did not enhance endothelium-dependent hyperpolarizations, whereas in *Cu,Zn-SOD*$^{-/-}$ mice, it significantly enhanced the responses (Figure 8c). Importantly, this enhancing effect of tempol was totally abolished by catalase. In isolated heart studies, tempol did not enhance the bradykinin-induced increase in coronary flow in the control mice, but significantly improved the response in the *Cu,Zn-SOD*$^{-/-}$ mice (Figure 8d). In a separate experiment, the enhancing effect of tempol was abolished by catalase (control, 144% ± 8% vs. tempol plus catalase, 147% ± 9%, *n* = 3).

Discussion

The novel finding of this study is that Cu,Zn-SOD plays a pivotal role in EDHF synthesis in both the mesenteric and the coronary microvessels in mice, further supporting our hypothesis that H_2O_2 is an EDHF. Although the role of Cu,Zn-SOD has been emphasized only for its effect of scavenging superoxide anions (19, 20), its biological role in maintaining vascular homeostasis remains to be fully elucidated. The present study demonstrates a novel role of Cu,Zn-SOD as an "EDHF synthase" in maintaining vascular homeostasis.

Characterization of Cu,Zn-SOD$^{-/-}$ *mice.* In *Cu,Zn-SOD*$^{-/-}$ mice, the expression of Cu,Zn-SOD protein was absent, while there was no upregulation of the other two SODs (MnSOD and ecSOD). Furthermore, the activities of glutathione peroxidase and catalase were comparable between the control and the *Cu,Zn-SOD*$^{-/-}$ mice, suggesting that these endogenous H_2O_2-scavenging enzymes are not involved in the reduced EDHF responses in *Cu,Zn-SOD*$^{-/-}$ mice. Regarding the other two isoforms of SOD, a role for MnSOD in synthesizing EDHF/H_2O_2 is less likely because MnSOD is located in mitochondria (21) and thus does not seem to be involved in EDHF synthesis. We have also ruled out the possible role of ecSOD, because EDHF-mediated responses were not altered by heparin (unpublished observations).

Reduced EDHF-mediated responses in Cu,Zn-SOD$^{-/-}$ *mice.* Upregulated NO compensates the reduced contribution of EDHF to endothelium-dependent relaxations in *Cu,Zn-SOD*$^{-/-}$ mice, confirming the importance of endothelial Cu,Zn-SOD as an EDHF synthase. EDHF-mediated relaxations were predominant in small
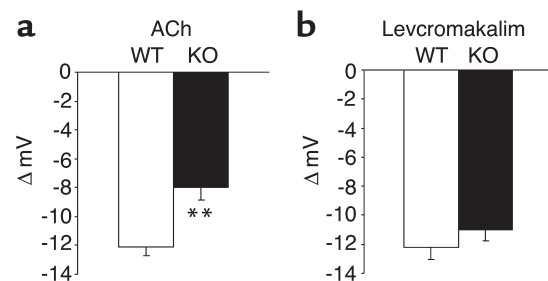

Figure 4

Endothelium-dependent and -independent hyperpolarizations of mesenteric arteries. (**a**) Endothelium-dependent hyperpolarizations of mesenteric arteries in response to ACh (10^{-5} M) were significantly attenuated in the *Cu,Zn-SOD*$^{-/-}$ mice (*n* = 4–5). *******P* < 0.01 vs. WT. (**b**) Endothelium-independent hyperpolarizations of mesenteric arteries in response to levcromakalim (10^{-5} M) were comparable between the two strains (*n* = 4–5).
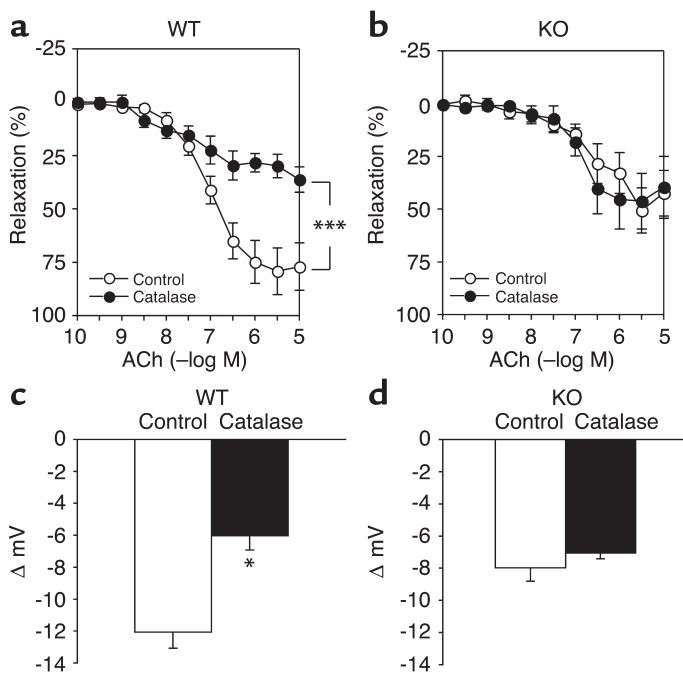
Figure 5

Inhibitory effect of catalase on EDHF-mediated relaxations and hyperpolarizations of mesenteric arteries. (**a**) In mesenteric arteries from control mice, catalase (6,250 U/ml) in the presence of indomethacin and L-NNA significantly inhibited EDHF-mediated relaxations ($n = 4$ each). ***$P < 0.0001$. (**b**) In mesenteric arteries from $Cu,Zn\text{-}SOD^{-/-}$ mice, catalase (6,250 U/ml) had no inhibitory effect on the remaining EDHF-mediated relaxations ($n = 4$ each). (**c**) Catalase significantly inhibited endothelium-dependent hyperpolarizations in response to ACh (10^{-5} M) of mesenteric arteries from control mice ($n = 3$). *$P \leq 0.05$. (**d**) Catalase had no effect on endothelium-dependent hyperpolarization of mesenteric arteries from $Cu,Zn\text{-}SOD^{-/-}$ mice ($n = 3$).

mesenteric arteries, a finding consistent with our previous studies (2, 3, 30). EDHF-mediated relaxations, as evaluated by the combination of charybdotoxin and apamin, also were reduced in $Cu,Zn\text{-}SOD^{-/-}$ mice, further confirming the reduced EDHF response in those mice. Both the expression and the activity of Cu,Zn-SOD are enhanced by fluid shear stress in endothelial cells but not in smooth muscle cells in the human aorta (31). This finding is consistent with our present study because shear stress stimulates the synthesis of EDHF by the endothelium (32).
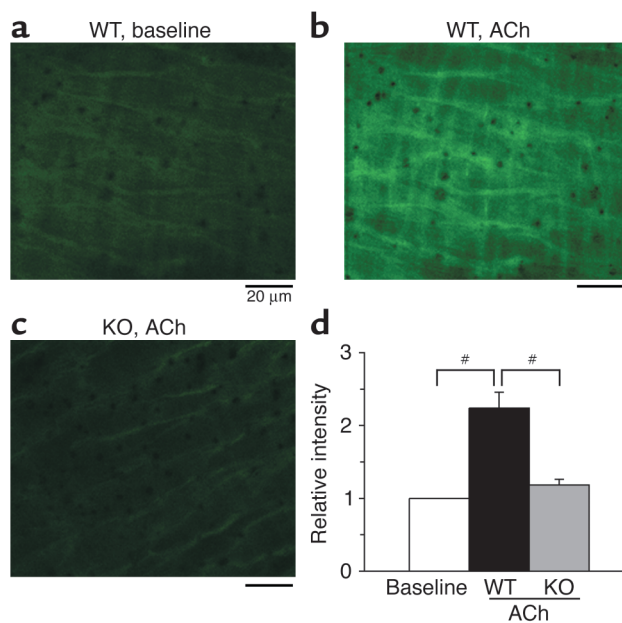
In the carotid artery of $Cu,Zn\text{-}SOD^{-/-}$ mice, NO-mediated relaxations were attenuated and contractile responses of vascular smooth muscle to serotonin and phenylephrine were enhanced (23). In the present study, however, NO-mediated relaxations were enhanced to partially compensate for the endothelium-dependent relaxations in mesenteric arteries. It is unlikely that upregulated eNOS is involved in this compensation because eNOS expression was unaltered in $Cu,Zn\text{-}SOD^{-/-}$ mice. Although the mechanism for the upregulation of NO-mediated responses remains to be elucidated, this could explain, at least in part, the absence of hypertension in those mice.

Several abnormalities have been reported in the cardiovascular system of $Cu,Zn\text{-}SOD^{-/-}$ mice. In those mice, myocardial ischemia-reperfusion injury was enhanced (33), as was cerebral injury after transient ischemia (34). It remains to be elucidated to what extent Cu,Zn-SOD contributes to those abnormalities as an EDHF synthase in $Cu,Zn\text{-}SOD^{-/-}$ mice.

H_2O_2 plays an important role in many blood vessels under physiological conditions as an endogenous relaxing and hyperpolarizing factor. Indeed, H_2O_2 causes relaxations and hyperpolarizations (3, 12, 13, 16, 35, 36). We were recently able to demonstrate the endothelial production of H_2O_2 as an endogenous EDHF in
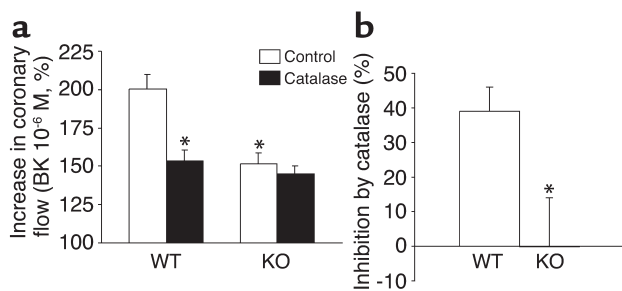

Figure 6

ACh-induced production of H_2O_2 by the endothelium. H_2O_2 production by the endothelium was detected using confocal microscopy with DCF. (**a**) Basal fluorescence intensity. (**b**) Application of ACh significantly increased the fluorescence intensity in endothelial cells in a control mouse. (**c**) ACh-induced increase in fluorescence intensity was significantly reduced in $Cu,Zn\text{-}SOD^{-/-}$ mice. (**d**) Summary of ACh-induced increase in fluorescence intensity in endothelial cells. ACh significantly increased the fluorescence intensity in control mice but not in $Cu,Zn\text{-}SOD^{-/-}$ mice ($n = 5$ each). #$P \leq 0.0001$.

Figure 7
Reduced coronary flow increase in *Cu,Zn-SOD*$^{-/-}$ mice. (**a**) Coronary flow increase in response to bradykinin (BK) (10^{-6} M) in isolated heart was significantly attenuated in the *Cu,Zn-SOD*$^{-/-}$ mice compared with the control mice (*n* = 6 each). Catalase significantly inhibited the coronary flow increase in control mice, but not in *Cu,Zn-SOD*$^{-/-}$ mice. *$P < 0.05$ vs. WT control. (**b**) The inhibitory effect of catalase on bradykinin-induced increase in coronary flow was absent in *Cu,Zn-SOD*$^{-/-}$ mice. *$P < 0.05$ vs. WT.

porcine coronary microvessels by an electron spin resonance method (13). It has recently been reported that endothelial production of superoxide anions from eNOS and the subsequent synthesis of H_2O_2 are increased in the aorta of deoxycorticosterone acetate–salt hypertensive rats and that this eNOS-derived H_2O_2 is involved in endothelium-dependent relaxations in response to calcium ionophore A23187 (37). The increase in endothelial H_2O_2 production was due to oxidation of tetrahydrobiopterin that resulted in eNOS uncoupling (37). However, endothelial production of superoxide anions from eNOS is noted even under physiological conditions without deficiencies of tetrahydrobiopterin (18). Thus, we consider that endothelial production of superoxide anions by eNOS

and subsequent synthesis of H_2O_2 by Cu,Zn-SOD are physiological phenomena and play a pivotal role in maintaining vascular homeostasis.

Preserved vascular smooth muscle responses in Cu,Zn-SOD$^{-/-}$ *mice.* Endothelium-independent relaxations in response to sodium nitroprusside, NS-1619, and exogenous H_2O_2 were comparable between the two strains and endothelium-independent hyperpolarizations induced by levcromakalim also were unchanged in *Cu,Zn-SOD*$^{-/-}$ mice, suggesting that relaxation properties of vascular smooth muscle cells were preserved in those mice.

Decreased inhibitory effect of catalase on EDHF-mediated relaxations and hyperpolarizations in Cu,Zn-SOD$^{-/-}$ *mice.* In mesenteric arteries from control mice, pretreatment with catalase significantly inhibited EDHF-mediated
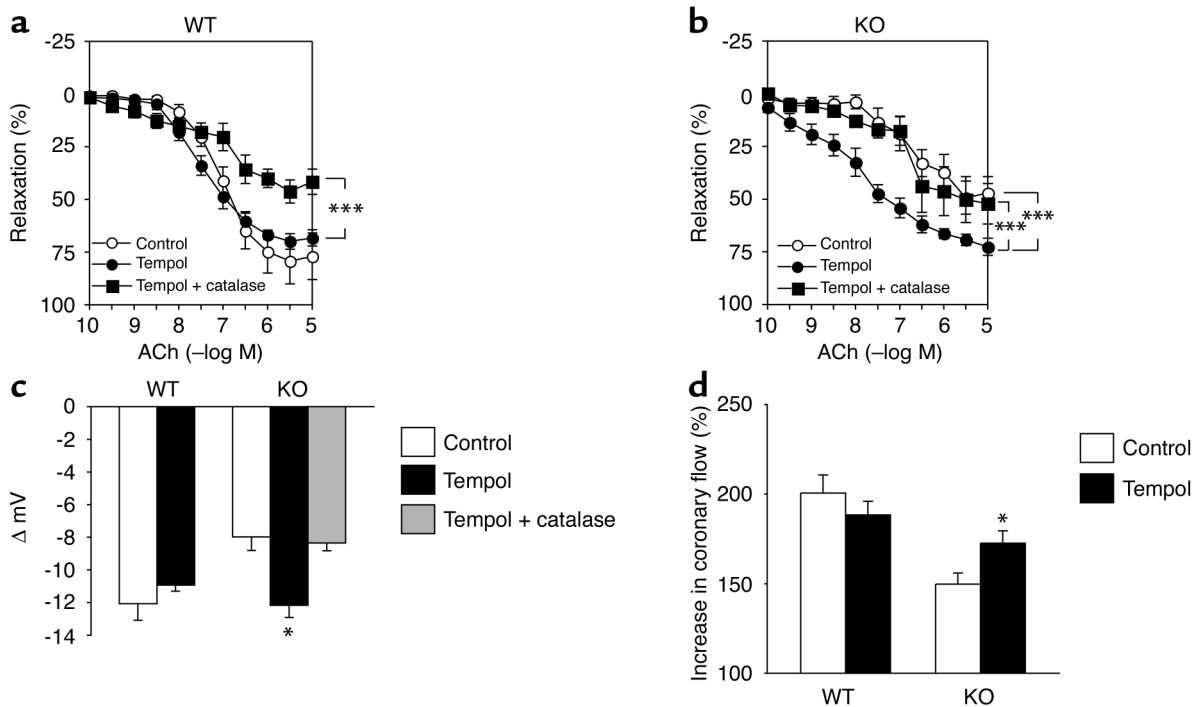

Figure 8
Effect of supplementation of SOD mimetic on EDHF-mediated relaxations in *Cu,Zn-SOD*$^{-/-}$ mice. (**a**) In the presence of indomethacin and L-NNA, the SOD mimetic tempol (1 mM) did not improve the EDHF-mediated relaxations of mesenteric arteries from control mice, which were significantly inhibited by catalase (6,250 U/ml) (*n* = 6 each). (**b**) In mesenteric arteries from *Cu,Zn-SOD*$^{-/-}$ mice, tempol (1 mM) in the presence of indomethacin and L-NNA significantly enhanced EDHF-mediated relaxations, an effect that was abolished by catalase (6,250 U/ml) (*n* = 5 each). ***$P < 0.0001$. (**c**) Tempol did not improve hyperpolarizations in control mice but did so in *Cu,Zn-SOD*$^{-/-}$ mice, and that effect was abolished by simultaneous administration of catalase (6,250 U/ml). (**d**) In isolated heart, tempol did not improve the coronary flow responses in control mice, but did so in *Cu,Zn-SOD*$^{-/-}$ mice. *$P \leq 0.05$.

relaxations, suggesting that those relaxations were mediated by H_2O_2 in control mice, a finding consistent with our previous studies (3). By contrast, in mesenteric arteries from *Cu,Zn-SOD*$^{-/-}$ mice, the inhibitory effect of catalase was absent, suggesting that the contribution of H_2O_2 to EDHF-mediated relaxations is markedly reduced in those mice. Catalase also significantly inhibited endothelium-dependent hyperpolarization in control mice, while it had no inhibitory effect in *Cu,Zn-SOD*$^{-/-}$ mice. These results further support our hypothesis that endothelial Cu,Zn-SOD plays a pivotal role in the production of H_2O_2/EDHF.

Decreased formation of ACh-induced H_2O_2 in Cu,Zn-SOD$^{-/-}$ *mice*. Microscopy of DCF-stained vascular endothelium demonstrated that ACh-induced fluorescence intensity was significantly reduced in *Cu,Zn-SOD*$^{-/-}$ mice compared with control mice. This suggests that ACh-induced endothelial production of H_2O_2 was significantly decreased in *Cu,Zn-SOD*$^{-/-}$ mice, further confirming the importance of Cu,Zn-SOD in endothelial synthesis of H_2O_2/EDHF.

Reduced coronary flow response in Cu,Zn-SOD$^{-/-}$ *mice*. In *Cu,Zn-SOD*$^{-/-}$ mice, EDHF-mediated increase in coronary flow was also significantly reduced, and the remaining coronary flow was no longer sensitive to catalase. These results indicate that catalase-sensitive, H_2O_2-mediated relaxations of coronary microvessels were almost absent in *Cu,Zn-SOD*$^{-/-}$ mice, confirming the importance of Cu,Zn-SOD in the coronary circulation.

Improvement of EDHF-mediated responses by an SOD mimetic in Cu,Zn-SOD$^{-/-}$ *mice*. Since SOD is a large molecule and penetrates the endothelium only with difficulty, we used the cell-permeable SOD mimetic tempol. Tempol is a stable nitroxide and has been reported to act as an SOD mimetic and to dismutate superoxide into H_2O_2 (27, 38). In the present study, tempol significantly improved the EDHF-mediated relaxations and hyperpolarizations of mesenteric arteries and also significantly improved the coronary flow response to bradykinin in *Cu,Zn-SOD*$^{-/-}$ mice, further confirming the pivotal role of the enzyme in endothelium-dependent hyperpolarization. Catalase completely inhibited this effect of tempol, indicating that the effect of tempol was mediated by H_2O_2. By contrast, tempol had no augmenting effect on the EDHF-mediated relaxations and hyperpolarizations of mesenteric arteries and coronary flow responses in control mice, suggesting that a sufficient amount of SOD is present in those mice.

Limitations of the study. Several limitations of the present study should be mentioned. First, we did not demonstrate the endothelial source of superoxide anions that are dismutated into H_2O_2. Since EDHF-mediated relaxations are significantly attenuated in *eNOS*$^{-/-}$ mice, it is possible that eNOS is one of the important sources of superoxide anions (3). Other possible sources of superoxide anions and the details of the interaction between eNOS and other enzymes remain to be examined in future studies. Second, while we were able to demonstrate the reduced H_2O_2 pro-

duction by the endothelium in *Cu,Zn-SOD*$^{-/-}$ mice using confocal microscopy with DCF, we were unable to quantitatively measure endothelial H_2O_2 production because a sufficient amount of mesenteric microvessels was not available in mice. However, we have recently demonstrated with an electron spin resonance method that endothelial cells produce H_2O_2, at least in micromolar quantities, in porcine coronary microvessels under physiological conditions (13). Third, the mechanisms for the compensatory role of NO in *Cu,Zn-SOD*$^{-/-}$ mice remain to be elucidated. Fourth, we did not elucidate the mechanisms for the remaining EDHF-mediated responses in *Cu,Zn-SOD*$^{-/-}$ mice. Fifth, although we demonstrated the importance of Cu,Zn-SOD as an EDHF synthase in mesenteric arteries in vitro and in the coronary circulation ex vivo, the importance of the enzyme in EDHF-mediated responses in vivo remains to be examined.

Clinical implications. EDHF plays an important role in human arteries, especially in microvessels (39, 40). We have recently observed that SOD mimetics also improve EDHF-mediated relaxations and hyperpolarizations in human mesenteric arteries (unpublished observations). It has been reported that abnormality in Cu,Zn-SOD processing and mutation of genes encoding Cu,Zn-SOD is involved in the pathogenesis of several diseases, including diabetes mellitus, hypertension, amyotrophic lateral sclerosis, Down syndrome, and malignant breast diseases (41–44). It has also been reported that Cu,Zn-SOD is important for ovary function (45) and hearing function (46). EDHF-mediated relaxations are impaired in several disease states, including hypercholesterolemia and aging (39), which may promote the progression of vascular disease, especially at microvascular levels. It remains to be examined to what extent Cu,Zn-SOD contributes to the above-mentioned physiological and pathophysiological conditions as an EDHF synthase.

In summary, the present study provides the novel concept that endothelial Cu,Zn-SOD plays a pivotal role as an EDHF synthase.

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