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Role of Cu,Zn-SOD in the synthesis of endogenous vasodilator hydrogen peroxide during reactive hyperemia in mouse mesenteric microcirculation in vivo

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Yada T, Shimokawa H, Morikawa K, Takaki A, Shinozaki Y, Mori H, Goto M, Ogasawara Y, Kajiya F. Role of Cu,Zn-SOD in the synthesis of endogenous vasodilator hydrogen peroxide during reactive hyperemia in mouse mesenteric microcirculation in vivo. *Am J Physiol Heart Circ Physiol* 294: H441–H448, 2008. First published November 16, 2007; doi:10.1152/ajpheart.01021.2007.—We have recently demonstrated that endothelium-derived hydrogen peroxide (H_2O_2) is an endothelium-derived hyperpolarizing factor and that endothelial Cu/Zn-superoxide dismutase (SOD) plays an important role in the synthesis of endogenous H_2O_2 in both animals and humans. We examined whether SOD plays a role in the synthesis of endogenous H_2O_2 during in vivo reactive hyperemia (RH), an important regulatory mechanism. Mesenteric arterioles from wild-type and Cu,Zn-SOD$^{-/-}$ mice were continuously observed by a pencil-type charge-coupled device (CCD) intravital microscope during RH (reperfusion after 20 and 60 s of mesenteric artery occlusion) in the cyclooxygenase blockade under the following four conditions: control, catalase alone, N^G-monomethyl-L-arginine (L-NMMA) alone, and L-NMMA + catalase. Vasodilatation during RH was significantly decreased by catalase or L-NMMA alone and was almost completely inhibited by L-NMMA + catalase in wild-type mice, whereas it was inhibited by L-NMMA and L-NMMA + catalase in the Cu,Zn-SOD$^{-/-}$ mice. RH-induced increase in blood flow after L-NMMA was significantly increased in the wild-type mice, whereas it was significantly reduced in the Cu,Zn-SOD$^{-/-}$ mice. In mesenteric arterioles of the Cu,Zn-SOD$^{-/-}$ mice, Tempol, an SOD mimetic, significantly increased the ACh-induced vasodilatation, and the enhancing effect of Tempol was decreased by catalase. Vascular H_2O_2 production by fluorescent microscopy in mesenteric arterioles after RH was significantly increased in response to ACh in wild-type mice but markedly impaired in Cu,Zn-SOD$^{-/-}$ mice. Endothelial Cu,Zn-SOD plays an important role in the synthesis of endogenous H_2O_2 that contributes to RH in mouse mesenteric smaller arterioles.

nitric oxide; endothelium-derived hyperpolarizing factor; arteriole; vasodilatation

THE ENDOTHELIUM SYNTHESIZES and releases endothelium-derived relaxing factors (EDRFs), including vasodilator prostaglandins, nitric oxide (NO), and as yet unidentified endothelium-derived hyperpolarizing factor (EDHF). Since the first reports on the existence of EDHFs (4, 8), several candidates for EDHF have been proposed (9), including cytochrome *P*-450 metabolites (2, 3), endothelium-derived K^+ channel (7), and electrical communications through gap junctions between endothelial cells and vascular smooth muscle cells (34). Matoba et al. (19a, 19b, 20) previously identified that endothelium-derived hydrogen peroxide (H_2O_2) is a primary EDHF in mesenteric arteries of mice, pigs, and humans. Morikawa et al. (24a, 25) subsequently confirmed that endothelial Cu/Zn-superoxide dismutase (SOD) plays an important role in synthesizing EDHF/H_2O_2 in mice and humans. Recently, our laboratory (41a, 42) confirmed that endogenous H_2O_2 plays an important role for autoregulation and protection against reperfusion injury in canine coronary microcirculation.

Reactive hyperemia (RH) is an important regulatory mechanism of the cardiovascular system in response to a temporal reduction in blood flow for which both mechanosensitive (e.g., myogenic and shear mediated) and metabolic regulatory processes may be involved (6, 14a, 28). For the RH response of canine coronary microcirculation, NO, ATP-sensitive K^+ channels, and adenosine may all be involved (11, 41). Shear stress plays a crucial role in modulating vascular tone by stimulating the release of EDRFs (8, 32), and all three EDRFs (PGI_2, NO, and EDHF) are involved in flow-induced vasodilatation (15, 18, 33, 44).

However, it remains to be examined whether endogenous H_2O_2 is involved in the vasodilator mechanism of RH and, if so, whether endothelial Cu,Zn-SOD plays a role in the synthesis of endogenous H_2O_2 during RH. The present study was thus designed to address these important issues in mice. Our laboratory (42, 44) previously reported that the contribution of EDHF to the vasodilatory mechanisms increases as the diameter of the vessel decreases. Thus, by employing a pencil-type charge-coupled device (CCD) intravital microscope with a high resolution, we focused on the arterioles with a diameter of <50 μm in vivo.

METHODS

The present study was approved by the Animal Care and Use Committee of Kawasaki Medical School and conformed to the guidelines on animal experiments of Kawasaki Medical School and *the*

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Animal preparation. Male Cu,Zn-SOD$^{-/-}$ and control mice (10–16 wk of age) derived from breeding pairs of heterozygous (Cu,Zn-SOD$^{+/-}$) mice (Jackson Laboratory, Bar Harbor, ME) were used (25). They were placed on a heating blanket to maintain body temperature at 37°C throughout the experiment. The animals were anesthetized with 1% inhalational anesthesia of isoflurane. After tracheal intubation, they were ventilated with a mixture of room air and oxygen by a ventilator. The abdomen was opened, and a 24-Fr catheter was inserted into the abdominal aorta to measure aortic pressure. Mesenteric arterioles were continuously observed by a pencil-type intravital microscope (Nihon Kohden, Tokyo, Japan) (13).

Measurements of diameter by pencil-type intravital microscope. Mesenteric arterioles were visualized using a pencil-type intravital microscope (13). The system was modified for the visualization of microcirculation from our previous needle-probe CCD videomicroscope system (40). The microscopic images were monitored and recorded on a digital videocassette recorder (Sony, Tokyo, Japan) every 33 ms (30 frames/s). The spatial resolution of a static image of this system is 0.5 μm for ×600 magnification. The field of view is 367 × 248 μm, and the focal depth is 50 μm.

Measurements of regional blood flow in mesenteric arteries. Regional blood flow in mesenteric arteries was measured by the nonradioactive microsphere (15 μm; Sekisui Plastic, Tokyo, Japan) technique at the end of the experiments, as previously described (24). Briefly, a bolus (50 μl) of the microspheres suspension (5×10^5 spheres; Ce and Ba) were injected into the abdominal aorta at baseline and 5 s after the reperfusion of the mesenteric artery with confirming changes of the blood flow of the mesenteric artery by a CCD intravital microscope and without inducing hemodynamic changes (14). Mice were euthanized, and the mesenterium was extracted. The X-ray fluorescence of the stable heavy elements was measured by a wavelength-dispersive spectrometer (model PW 1480; Phillips, Eindhoven, the Netherlands). The relative increase in blood flow of mesenterium [microsphere count/tissue weight (g)] during RH from baseline was calculated.

Detection of H_2O_2 and NO production in mesenteric microvessels. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes, Eugene, OR) and diaminorhodamine-4M AM (DAR; Daiichi Pure Chemicals, Tokyo, Japan) were used to detect H_2O_2 and NO production in mesenteric microvessels, respectively, as previously described (41a). Briefly, fresh and unfixed mesenteric tissue was cut into several blocks and immediately frozen in an optimal cutting temperature compound (Tissue-Tek; Sakura Fine Chemical, Tokyo, Japan). After washout of the mesenteric tissue with phosphate-buffered solution under a normal temperature, fluorescent images of the microvessels were obtained 3 min after application of acetylcholine (ACh) by using a fluorescence microscope (Olympus BX51) (41a). We defined the baseline fluorescent intensity as the response in the vascular endothelium just after the injection of NO or H_2O_2 fluorescent dye. The fluorescence data at baseline (both DCF-DA and DAR) were obtained after the RH.

Experimental protocol. We performed four protocols. First, mesenteric arterioles in wild-type and Cu,Zn-SOD$^{-/-}$ mice were continuously observed by a pencil-type intravital microscope during RH (reperfusion after 20 and 60 s of mesenteric artery occlusion) with cyclooxygenase blockade [indomethacin, 5×10^{-5} mol/l topical administration (ta)] with the following four conditions: control, catalase alone [1,500 U·min^{-1}·100 g body wt^{-1} intra-arterial administration (ia) polyethylene glycol-catalase, a specific decomposer of H_2O_2], NO synthase inhibitor alone (10^{-4} mol/l ta L-NMMA), and L-NMMA + catalase (17). In the presence of indomethacin and L-NMMA, microspheres were administered at baseline and 5 s after the reperfusion into the abdominal aorta by bolus injection because RH peaked within 20–60 s after release from 20- and 60-s occlusion (29). Maximal vascular diameter was measured within 20 and 60 s after the reperfusion. Second, ACh (10^{-7} to 10^{-5} mol/l ta)-induced endothelium-dependent vasodilatation was examined under the control conditions and in the presence of Tempol, a SOD mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (50 μg·min^{-1}·100 g body wt^{-1} ia) (17), and Tempol + catalase. In the combined infusion protocol (Tempol or Tempol + catalase) in the presence of cyclooxygenase blockade + L-NMMA, the combined infusion was performed simultaneously for 20 min, ACh was infused for 10 min, and the vascular diameter was measured. Third, sodium nitroprusside (SNP; 10^{-7} to 10^{-5} mol/l ta, each 10 min)-induced endothelium-independent vasodilatation was examined in wild-type and Cu,Zn-SOD$^{-/-}$ mice. Fourth, fresh and unfixed mesenteric tissue was then cut into several blocks and immediately frozen in optimal cutting temperature compound.

Statistical analysis. The results are expressed as means ± SE. Dose-response curves were analyzed by two-way ANOVA followed by the Scheffé's post hoc test for multiple comparisons. Vascular responses were analyzed by one-way ANOVA followed by the Scheffé's post hoc test for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

RESULTS

Hemodynamics and blood gases during RH. Throughout the experiments, mean aortic pressure and heart rate were constant and comparable (Tables 1 and 2), and P_{O_2}, P_{CO_2}, and pH were maintained within the physiological ranges (>70 mmHg P_{O_2}, 25–40 mmHg P_{CO_2}, and pH 7.35–7.45). Baseline mesenteric

Table 1. *Hemodynamics during RH*

		Control		Catalase		L-NMMA		L-NMMA + Catalase	
	n	B	RH	B	RH	B	RH	B	RH
MBP, RH 20, mmHg									
WT	10	83±7	85±9	81±7	82±7	82±8	81±6	83±8	82±6
Cu/Zn-SOD$^{-/-}$	10	85±12	87±10	83±8	82±8	82±8	82±8	82±8	80±9
MBP, RH 60, mmHg									
WT	5	86±8	88±7	87±7	88±7	88±8	87±7	88±7	87±7
Cu/Zn-SOD$^{-/-}$	5	88±8	86±8	87±6	89±9	88±7	88±8	89±6	90±11
HR, RH 20, beats/min									
WT	10	346±14	348±14	335±15	333±17	315±15	310±17	330±18	330±18
Cu/Zn-SOD$^{-/-}$	10	364±27	354±22	350±18	351±15	355±15	340±17	355±15	335±17
HR, RH 60, beats/min									
WT	5	351±31	361±9	353±21	356±13	358±10	364±37	354±22	355±15
Cu/Zn-SOD$^{-/-}$	5	346±18	356±25	356±15	361±19	351±31	361±9	358±10	364±27

Values are means ± SE; *n*, number of rats. RH, reactive hyperemia; L-NMMA, N^G-monomethyl-L-arginine; B, baseline; MBP, mean blood pressure; HR, heart rate; WT, wild-type.

Table 2. *Hemodynamics during administration of ACh and SNP*

	n	Control			Tempol		Tempol + Catalase			SNP		
		B	ACh 10^{-7}	ACh 10^{-6}	ACh 10^{-5}	ACh 10^{-7}	ACh 10^{-6}	ACh 10^{-5}	ACh 10^{-7}	ACh 10^{-6}	ACh 10^{-5}	B	SNP 10^{-7}	SNP 10^{-6}	SNP 10^{-5}
MBP, mmHg															
WT	10	90±7	88±7	87±7	89±6	91±10	90±11	84±11	91±8	86±8	86±8	78±13	77±13	76±13	76±13
Cu/Zn-SOD$^{-/-}$	10	93±11	92±10	94±10	97±10	98±16	97±15	99±15	91±8	91±8	88±8	81±8	77±10	75±11	74±11
HR, beats/min															
WT	10	361±9	340±17	340±17	340±17	342±24	336±25	336±25	320±19	330±27	330±27	370±57	364±37	351±31	351±31
Cu/Zn-SOD$^{-/-}$	10	386±11	381±24	371±21	396±13	386±11	377±15	377±15	362±22	356±25	356±25	372±21	369±6	358±10	346±18

Values are means ± SE; *n*, number of rats. SNP, sodium nitroprusside; ACh, acetylcholine.

arteriolar diameter was comparable in the absence and presence of inhibitors under the four different experimental conditions (Tables 3 and 4). Those different inhibitors (L-NMMA, catalase, and Tempol) did not affect basal diameter.

Mesenteric vasodilatation during RH. We were able to observe EDHF-sensitive smaller arterioles (18–66 μm) by using a newly developed pencil-type CCD intravital microscope with a higher resolution. In the mesenteric arterioles of wild-type mice, vasodilatation during RH to 20- and 60-s arterial occlusion was decreased by catalase or L-NMMA alone and was almost completely inhibited by L-NMMA + catalase (Fig. 1). In contrast, in mesenteric arterioles of Cu,Zn-SOD$^{-/-}$ mice, vasodilatation during RH to 20- and 60-s arterial occlusion was decreased by catalase alone and was almost completely inhibited by L-NMMA alone or L-NMMA + catalase (Fig. 1). Blood flow measurement by microsphere technique showed that in the presence of indomethacin and L-NMMA, RH-induced increase in blood flow was 232 ± 4% (20 s) and 331 ± 4% (60 s) of baseline in control and was sensitive to catalase (137 ± 4%, 20 s; and 147 ± 17%, 60 s) in the wild-type mice, whereas in the Cu,Zn-SOD$^{-/-}$ mice, the vasodilator response was significantly reduced to 125 ± 19% (20 s) and 145 ± 23% (60 s) in control and was insensitive to catalase (120 ± 24%, 20 s; and 139 ± 19%, 60 s) (Fig. 2). With the longer occlusion of the mesenteric artery, the shear stimulus for H_2O_2 release was significantly increased in the control condition and was significantly decreased by catalase.

Endothelium-dependent vasodilatation. In mesenteric arterioles of wild-type mice, endothelium-dependent vasodilatation to ACh (10^{-7} to 10^{-5} mol/l in the presence of indomethacin and L-NMMA) was unchanged with Tempol but significantly inhibited by the addition of catalase (Fig. 3). In contrast, in the mesenteric arterioles of the Cu,Zn-SOD$^{-/-}$ mice, the response to ACh was significantly enhanced with Tempol, a response that was sensitive to the addition of catalase (Fig. 3).

Endothelium-independent vasodilatation. Endothelium-independent vasodilatation to SNP (10^{-7} to 10^{-5} mol/l in the presence of L-NMMA + catalase) was comparable between the two strains (Table 4).

Detection of H_2O_2 and NO production in the mesenteric artery. Fluorescent microscopy with DCF-DA showed that vascular H_2O_2 production in mesenteric arterioles was significantly increased in response to ACh in wild-type mice compared with baseline but markedly impaired in Cu,Zn-SOD$^{-/-}$ mice (Fig. 4). In contrast, vascular NO production in mesenteric arterioles, as assessed by DAR fluorescent intensity, was significantly increased in response to ACh in wild-type mice compared with baseline and was unaltered in Cu,Zn-SOD$^{-/-}$ mice (Fig. 5).

DISCUSSION

The novel finding of the present study with a newly developed pencil-type CCD intravital microscope in vivo is that Cu,Zn-SOD plays an important role in the synthesis of endogenous H_2O_2, which is substantially involved in the mechanisms of RH-induced vasodilatation in mouse mesenteric circulation.

Impaired EDHF-mediated vasodilatation in Cu,Zn-SOD$^{-/-}$ mice in vivo. Matoba et al. (19a, 20) have previously identified that endothelium-derived H_2O_2 is an EDHF in mouse and

Table 3. *Diameter change during RH*

	Control		Catalase		L-NMMA		L-NMMA + Catalase	
	B	RH	B	RH	B	RH	B	RH
RH 20, μm								
WT	36±4	49±4†	36±4	44±4†	36±3	42±3*	36±4	38±3
Cu/Zn-SOD$^{-/-}$	36±4	48±5†	36±4	42±5†	36±4	38±4	36±3	38±4
RH 60, μm								
WT	40±4	55±4†	40±4	51±4†	40±5	47±4*	39±4	41±4
Cu/Zn-SOD$^{-/-}$	40±3	56±4†	40±3	51±4†	39±4	42±3	40±5	42±3

Values are means ± SE; *n*, number of arterioles per animal. *$P < 0.01$ vs. B.

human mesenteric microvessels. Subsequently, our laboratory (42) and others (23) have confirmed that endogenous H_2O_2 exerts important vasodilator effects in canine coronary microcirculation in vivo and in isolated human coronary microvessels, respectively. H_2O_2 can be formed from superoxide anions derived from several sources in endothelial cells, including endothelial NO synthase (eNOS), cyclooxygenase, lipoxygenase, cytochrome *P*-450 enzymes, and reduced NADP [NAD(P)H] oxidases. Gupte et al. (10) demonstrated that cytosolic NADH redox and Cu,Zn-SOD activity have important roles in controlling the inhibitory effects of superoxide anions derived from NADH oxidase. Morikawa et al. (24a, 25) have also demonstrated that endothelial Cu,Zn-SOD plays an important role in the synthesis of H_2O_2 in mouse and human mesenteric arteries in vitro.

In the present study, catalase or L-NMMA alone significantly, but not completely, inhibited the RH-induced vasodilatation of mesenteric arterioles in wild-type mice in vivo, whereas L-NMMA + catalase markedly attenuated the remaining vasodilatation. In contrast, in Cu,Zn-SOD$^{-/-}$ mice, L-NMMA alone significantly decreased the vasodilatation and blood flow in response to 20- and 60-s arterial occlusion (Figs. 1 and 2). These results obtained using a pencil-type CCD intravital microscope indicate that H_2O_2 exerts important vasodilator effects on mesenteric smaller arterioles during RH and that Cu,Zn-SOD plays an important role in the synthesis of endogenous H_2O_2 during RH in vivo. Koller and Bagi (14a) showed that RH in rat isolated coronary arterioles was sensitive to pressure/stretch and flow/shear stress. Miura et al. (23) also showed the important role of endogenous H_2O_2 in flow-induced vasodilatation of human coronary arterioles. Koller and Bagi (14a) also suggested that H_2O_2 contributes to the development of the early peak phase of RH but not the duration of reactive vasodilatation, whereas NO prolongs the later phase of RH in rat isolated coronary arterioles, suggesting that H_2O_2 released endogenously within the vascular wall changes hemodynamic forces. In the present study, peak blood flow was significantly decreased after catalase (Fig. 2), suggesting that flow-induced vasodilatation during the early phase of RH is indeed mediated by H_2O_2 in mouse mesenteric arterioles in vivo.

Compensatory vasodilator mechanism between H_2O_2 and NO. It is well known that coronary vascular tone is regulated by the interactions among hemodynamic forces and several endogenous vasodilators, including NO, H_2O_2, and adenosine (41a, 42). Koller and Bagi (14a) demonstrated that mechanosensitive mechanisms were activated by changes in pressure and flow/shear stress during RH in isolated coronary arterioles. A superoxide anion is dismutated to H_2O_2 by manganese SOD (Mn-SOD, mitochondrial matrix) and Cu,Zn-SOD. H_2O_2 diffuses across the mitochondrial membrane to act on vascular smooth muscle (45). Tsunoda et al. (35) demonstrated that Mn-SOD augmented RH during 60-s canine coronary ischemia and reperfusion. H_2O_2 generated in the arteriolar smooth muscle could cause the response of activation of cGMP in rat skeletal muscle arterioles (38). Kitakaze et al. (12) indicated that the augmentation of reactive hyperemic flow caused by SOD is attributed to the enhanced release of adenosine in canine coronary circulation. These endogenous vasodilators may play an important role in causing the compensatory vasodilatation of coronary microvessels during myocardial ischemia.

In the present study, endothelium-dependent vasodilatation during RH (in the presence of L-NMMA) was almost completely inhibited by catalase in wild-type mice. In the Cu,Zn-SOD$^{-/-}$ mice, vasodilatation during RH remained under the control condition but was almost completely inhibited by L-NMMA (Fig. 1). The RH-induced increase in blood flow (in the presence of indomethacin and L-NMMA) was significantly inhibited by catalase in the wild-type mice but not in Cu,Zn-SOD$^{-/-}$ mice (Fig. 2). RH-induced increase in blood flow (in the presence of indomethacin and L-NMMA) remained in Cu,Zn-SOD$^{-/-}$ mice (Fig. 2). H_2O_2 may compensate for the loss of action of NO. H_2O_2 produced by SOD other than Cu,Zn-SOD may compensate for the loss of action of Cu,Zn-SOD-derived H_2O_2.

Table 4. *Diameter change during administration of ACh and SNP*

	ACh				SNP			
	B	ACh 10^{-7}	ACh 10^{-6}	ACh 10^{-5}	B	SNP 10^{-7}	SNP 10^{-6}	SNP 10^{-5}
WT, μm	36±3	41±3*	45±3†	49±4†	35±4	39±4*	43±5†	46±5†
Cu/Zn-SOD$^{-/-}$, μm	36±4	38±4	41±4*	44±4†	34±4	38±4*	41±4†	44±4†

Values are means ± SE; *n*, number of arterioles per animal. *$P < 0.05$; †$P < 0.01$ vs. B.

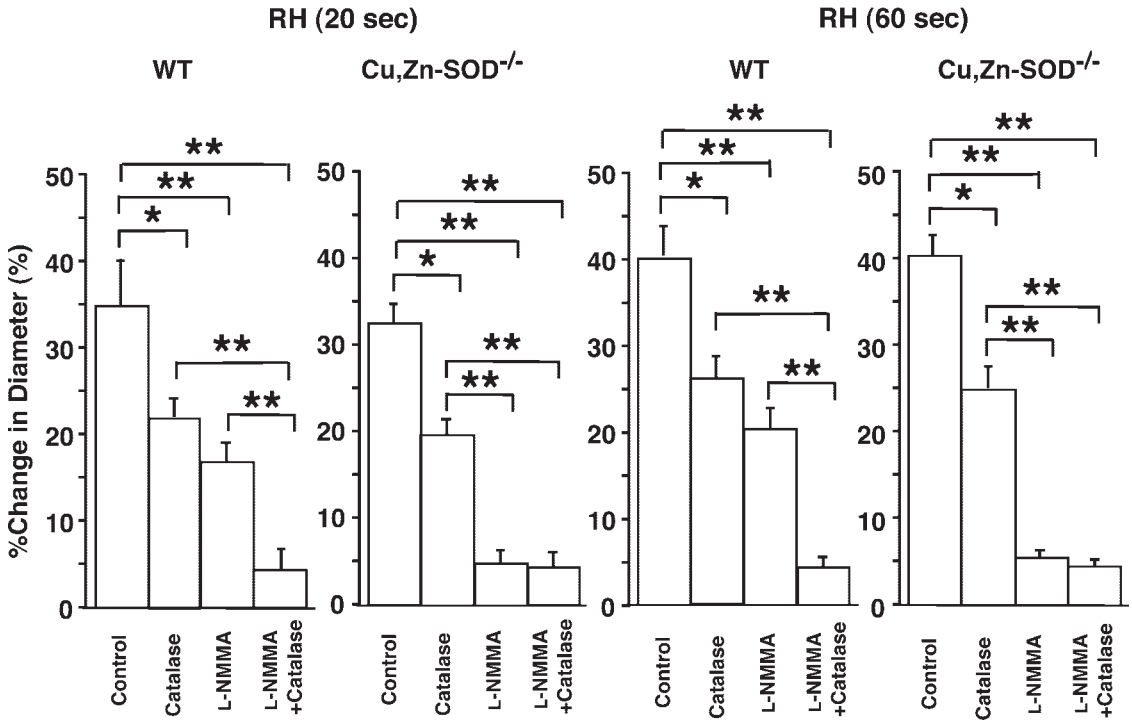

Fig. 1. Mesenteric vasodilatation during reactive hyperemia (RH). In the wild-type (WT) mice, vasodilatation during RH was inhibited by catalase or N^G-monomethyl-L-arginine (L-NMMA) and further inhibited by L-NMMA + catalase. In the Cu,Zn-SOD$^{-/-}$ mice (Cu,Zn-SOD$^{-/-}$), vasodilatation during RH was inhibited by catalase and markedly inhibited by L-NMMA, and the remaining response was not inhibited by catalase. The number of arterioles per animals used was 10/5 for each group. *$P < 0.05$; **$P < 0.01$.

Improvement of ACh-induced vasodilatation by Tempol in Cu,Zn-SOD$^{-/-}$ mice. It was previously reported that Tempol, a cell membrane-permeable SOD mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl, decreased oxidative stress in the spontaneously hypertensive rat (31). In the present study, Tempol significantly improved the ACh-induced vasodilatation in Cu,Zn-SOD$^{-/-}$ mice, whereas catalase abolished the beneficial effect of Tempol (Fig. 3), indicating that the effect of Tempol was mediated by endogenous H_2O_2 in vivo. In contrast, Tempol had no enhancing effect on the ACh-induced vasodilatation in control mice (Fig. 3), suggesting that a sufficient amount of SOD is present in this strain. In Cu,Zn-SOD$^{-/-}$ mice, L-NMMA did not abolish the ACh-induced vasodilation, and the DCF-DA stain showed remaining fluorescent intensity (Fig. 4). Thus the residual vasodilatation could be caused by the following possible mechanisms. First, NO may also be synthesized in a nonenzymatic manner (27). Nonenzymatic synthesis of NO could occur in the presence of NADPH, glutathione, and L-cysteine, etc., opposing the effects of NOS inhibition (27). Second, the effects of L-NMMA may be limited since it is known that L-NMMA does not abolish NO production (1). H_2O_2 produced from vascular smooth muscle cells and other tissues may also contribute to the residual vasodilation (5, 30). Third, the contribution of other proposed

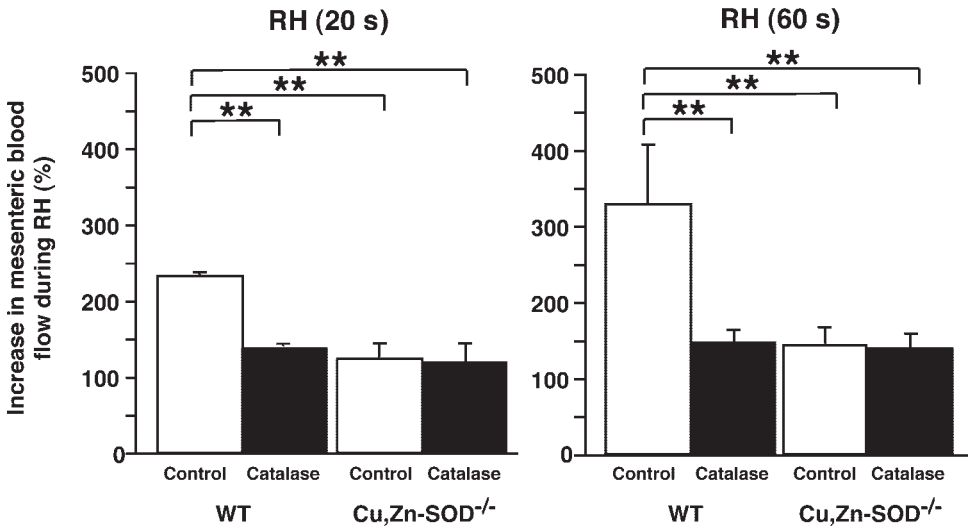

Fig. 2. The increase in mesenteric blood flow during RH. In the presence of indomethacin and L-NMMA, RH-induced increase in blood flow was sensitive to catalase in the WT mice, whereas in the Cu,Zn-SOD$^{-/-}$, the vasodilation was significantly reduced in control and was insensitive to catalase. The number of animals used was 5 for each group. **$P < 0.01$.

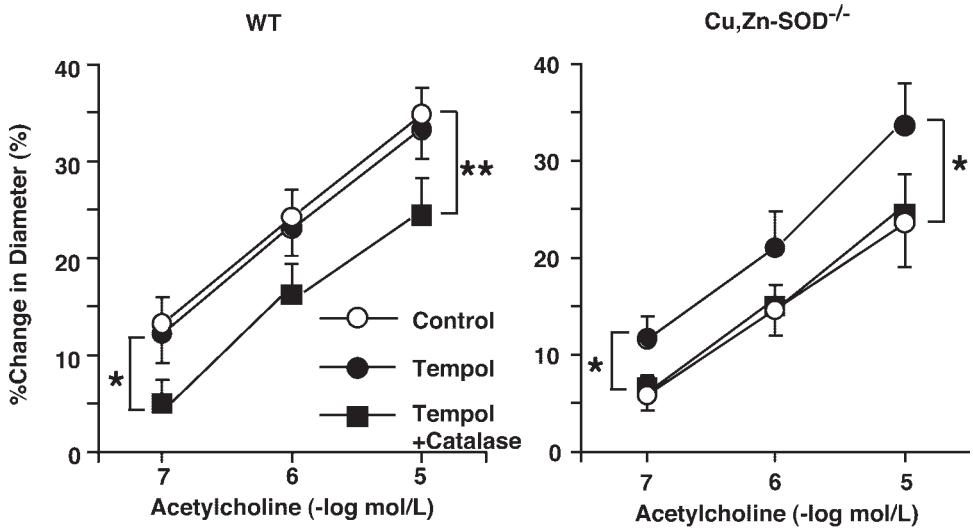

Fig. 3. Endothelium-dependent relaxations to ACh. In the WT mice, endothelium-dependent vasodilatation to ACh (in the presence of indomethacin and L-NMMA) was unchanged with Tempol but significantly inhibited by the addition of catalase. In the Cu,Zn-SOD$^{-/-}$, the vasodilation was significantly enhanced with Tempol, where the response was sensitive to the addition of catalase. The number of arterioles per animals used was 10/5 for each group. *$P <$ 0.05; **$P < 0.01$.

EDHF candidates, such as *P*-450 metabolites (2, 3) and potassium ion (7), may contribute to the residual vasodilatation. Although RH and ACh have different mechanisms of vasodilator effects, they also share the same flow-induced vasodilator mechanism.

Endothelium-independent vasodilatation in Cu,Zn-SOD$^{-/-}$ mice. Microvascular dysfunction in hypercholesterolemic rats was confined to the endothelium because the dilator response to SNP and adenosine was unchanged (37). In the present study, endothelium-independent vasodilatation in response to SNP was comparable between the two genotypes, suggesting that vasodilatation properties of vascular smooth muscle cells were preserved in the Cu,Zn-SOD$^{-/-}$ mice in vivo.

Detection of vascular H_2O_2 and NO production. Our laboratory (41a) has recently demonstrated that vascular production of H_2O_2 and NO after ischemia-reperfusion is enhanced in small coronary arteries and arterioles in vivo, respectively. It was previously shown that a ACh-induced increase in fluorescence intensity in endothelial cells of the mesenteric artery is significantly reduced in Cu,Zn-SOD$^{-/-}$ mice (25). In the present study, vascular H_2O_2 production, as assessed by DCF-DA fluorescent intensity in mesenteric arterioles, was markedly impaired

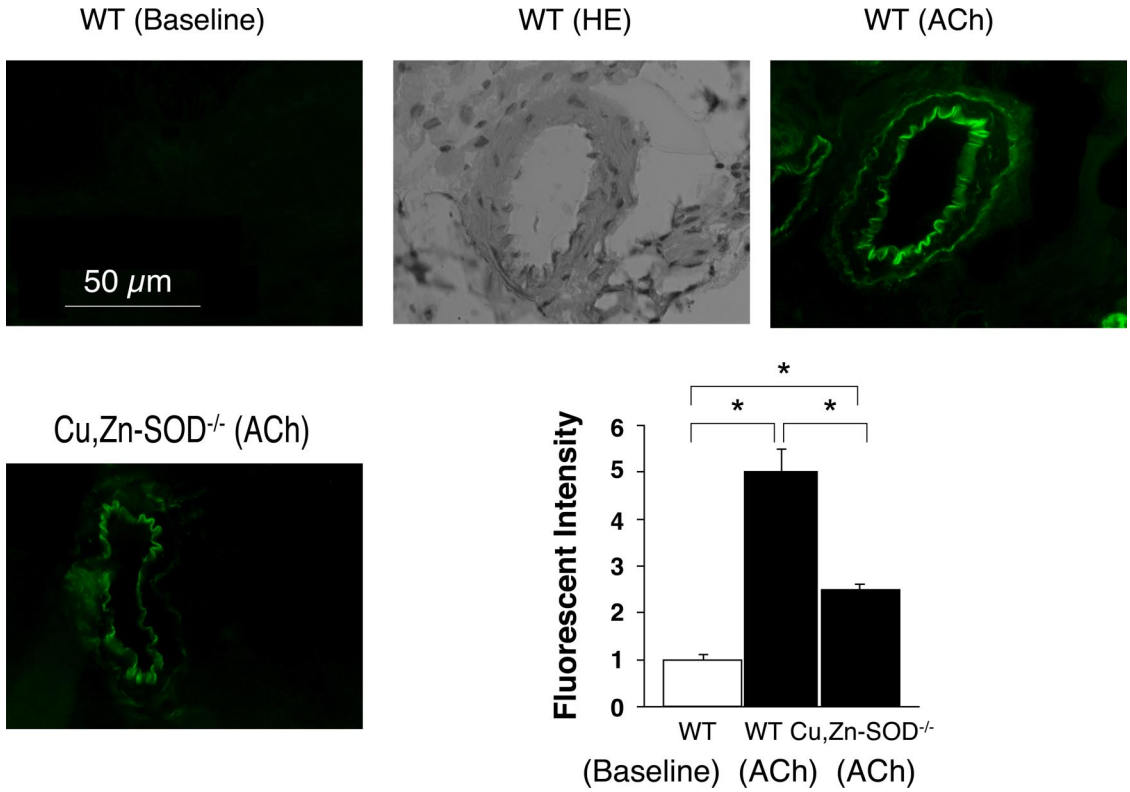

Fig. 4. Detection of vascular H_2O_2 production. Vascular H_2O_2 production in mesenteric arterioles was significantly increased in response to ACh in WT mice but markedly impaired in Cu,Zn-SOD$^{-/-}$. The number of arterioles per animals used was 10/5 for each group. *$P < 0.05$. HE, Hematoxylin eosin.

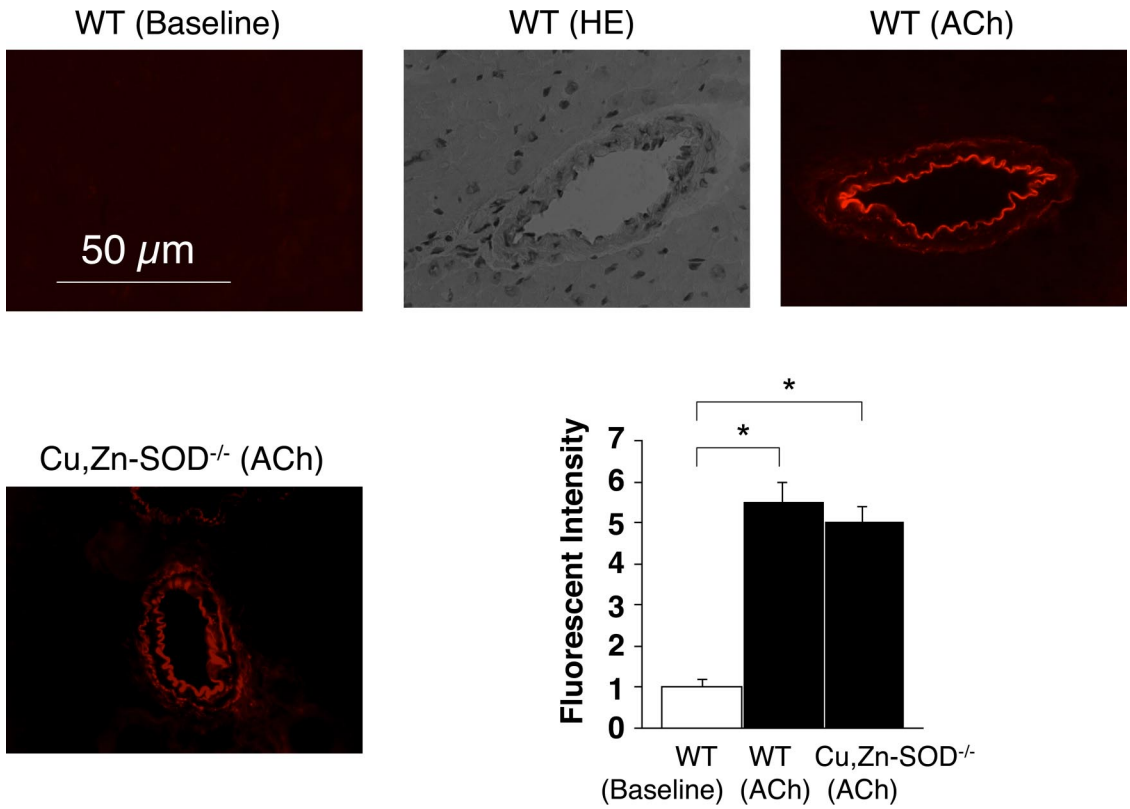

Fig. 5. Detection of vascular nitric oxide (NO) production. Vascular NO production in mesenteric arterioles was significantly increased in response to ACh in WT mice and unaltered in Cu,Zn-SOD$^{-/-}$. The number of arterioles per animals used was 10/5 for each group. *$P < 0.05$.

in Cu,Zn-SOD$^{-/-}$ mice (Fig. 4). These findings indicate that endothelial production of H_2O_2 is significantly impaired in Cu,Zn-SOD$^{-/-}$ mice, confirming the importance of the enzyme in endothelial synthesis of H_2O_2.

In the previous study by Morikawa et al. (25), eNOS protein expression was comparable between Cu,Zn-SOD$^{-/-}$ and wild-type mice. In the present study, vascular NO production in small mesenteric artery was unaltered in Cu,Zn-SOD$^{-/-}$ mice compared with wild-type mice (Fig. 5). NO could compensate for the loss of action of H_2O_2, although there are still many uncertainties about the local cellular dynamics of superoxide anions and NO.

Study limitations. Several limitations should be mentioned for the present study. First, we estimated blood flow in the mesenteric circulation using microspheres. We were unable to calculate the absolute values of local blood flow or shear stress because of the methodological limitations. However, since the flow measurement with microspheres was performed at the end of the experiments, it should not have influenced other results. Second, we used Cu,Zn-SOD$^{-/-}$ mice in the present study, where unknown compensatory mechanisms may be operative, and we were unable to elucidate the mechanism(s) for the remaining EDHF-mediated responses in those mice.

Clinical implications. RH is an important regulatory mechanism of the cardiovascular system, reflecting the flow reserve in response to a brief period of cessation of flow. An impaired flow reserve in resistance vessels is a hallmark of microvascular dysfunction with coronary risk factors. Hypertension is associated with structural alterations in the microcirculation and a reduced endothelium-dependent dilation in conduit arteries (19). It is well known that abnormality in Cu,Zn-SOD is noted in several diseases, including hypertension and diabetes mellitus (36, 39).

In conclusion, endogenous H_2O_2 exerts important vasodilator effects of mesenteric smaller arterioles during RH, especially at the low level of NO, and that Cu,Zn-SOD plays an important role in the synthesis of endogenous H_2O_2 during RH in vivo.

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