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Mechanisms of hydrogen peroxide-induced relaxation in rabbit mesenteric small artery

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Abstract

The effects of hydrogen peroxide were studied on isolated rabbit mesenteric small artery; rabbit superior mesenteric artery and mouse aorta were also studied as reference tissues. For mesenteric small artery, hydrogen peroxide (1 to 100 μM) relaxed a norepinephrine-stimulated artery in a concentration-dependent manner. The relaxation was not significantly affected by removal of the endothelium and was less pronounced in arteries contracted with high-KCl solution plus norepinephrine than in those contracted with norepinephrine alone. The relaxation response to hydrogen peroxide was increased by isobutylmethylxanthine and zaprinast, inhibited by diclofenac, methylene blue and dithiothreitol and unaffected by atropine, tetraethylammonium, superoxide dismutase, deferoxamine, dimethyl sulfoxide or the Rp stereoisomer of adenosine cyclic monophosphothioate. Hydrogen peroxide shifted concentration–contractile response curves for norepinephrine to the right and downwards. Norepinephrine and caffeine elicited a transient, phasic contraction of the mesenteric small artery exposed for 0.5, 1 and 2 min to a Ca^{2+}-free solution. Hydrogen peroxide inhibited the norepinephrine-induced contraction, and to a lesser extent the caffeine-induced contraction, and verapamil did not alter the contraction to norepinephrine. These pharmacological properties of hydrogen peroxide were similar to those of 8-bromo cGMP; 8-bromo cGMP inhibited more potently the norepinephrine-induced than the KCl-induced contraction and the contraction elicited by norepinephrine in Ca^{2+}-free solution. The present results suggest that hydrogen peroxide induces endothelium-independent relaxation of the rabbit mesenteric small artery precontracted with norepinephrine. The effects of hydrogen peroxide may be at least in part mediated by cGMP and cyclooxygenase products in the vascular smooth muscles now used. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: cGMP; Diclofenac; Hydrogen peroxide; Mesenteric small artery; Superoxide dismutase

1. Introduction

Hydrogen peroxide, an important byproduct of oxidative metabolism, can be produced and released from vascular endothelial cells and other cells, including activated phagocytic cells (Root and Metcalf, 1977; Pizzinat et al., 1999), contributing to acute tissue injury or inflammatory disorders, i.e. ischemia-reperfusion injury (Downey, 1990; Ueda and Shah, 1992; Zweier et al., 1994). Therefore, effects of exogenous hydrogen peroxide and exogenously generated hydrogen peroxide have been determined on contraction and relaxation of vascular preparations. It has been reported that hydrogen peroxide relaxes or contracts vascular smooth muscle, depending on the species, tissues and experimental protocols. Briefly, under resting tension, the drug has been reported to cause smooth muscle contraction (or vasospasm) in human umbilical artery (Okatani et al., 1997), rat pulmonary artery and aorta (Rhoades et al., 1990; Jin and Rhoades, 1997; Rodríguez-Martínez et al., 1998; Yang et al., 1998b), rabbit intrapulmonary artery (Sheehan et al., 1993) and canine basilar artery (Yang et al., 1999b). The contractile response is in part or totally due to a direct action of hydrogen peroxide on vascular smooth muscle and is likely to be mediated by several cellular transduction mechanisms including tyrosine kinase (Jin and Rhoades, 1997; Yang et al., 1999b), protein kinase C (Yang et al., 1999b), phospholipase A_2/arachidonate metabolism (Barlow and White, 1998; Yang et al., 1998b), phospholipase C (Sheehan et al., 1993), mitogen-activated protein kinase (Yang et al., 1999b), phos-

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phatidylinositol 3-kinase (Yang et al., 1999b) and voltage-gated Ca^{2+} channels (Yang et al., 1999b).

However, hydrogen peroxide elicits relaxation in rabbit and rat aorta (Zembowicz et al., 1993; Iesaki et al., 1994, 1996; Yang et al., 1999a), bovine pulmonary artery (Burke and Wolin, 1987; Burke-Wolin et al., 1991; Iesaki et al., 1999), porcine and canine coronary arteries (Rubanyi and Vanhoutte, 1986; Bény and Von der Weid, 1991; Barlow and White, 1998) and cat and canine cerebral arteries (Fraile et al., 1994; Yang et al., 1998a), when precontracted with certain agonists. The relaxations are either endothelium-independent (Burke and Wolin, 1987; Burke-Wolin et al., 1991; Barlow and White, 1998; Iesaki et al., 1999) or -dependent (Yang et al., 1999a). In canine coronary artery and rabbit aorta, hydrogen peroxide-induced relaxation has both endothelium-dependent and -independent components (Rubanyi and Vanhoutte, 1986; Zembowicz et al., 1993). The endothelium-dependent relaxation in response to hydrogen peroxide seems to be mediated by an enhanced production of cyclic (c) GMP through release of endothelial nitric oxide (Zembowicz et al., 1993; Cosentino and Katusi'c, 1995; Yang et al., 1999a), which may be related to an increase in Ca^{2+} concentrations in endothelial cells (Kimura et al., 1992; Doan et al., 1994; Yang et al., 1999a). Yang et al. (1999a) have suggested that the relaxing effects of hydrogen peroxide in rat aorta are mediated by some products of cytochrome *P*450 enzyme actions. On the other hand, there is evidence suggesting that the endothelium-independent relaxation is also mediated by cGMP (Burke-Wolin et al., 1991; Hayabuchi et al., 1998; Iesaki et al., 1999) and arachidonate metabolites (Barlow and White, 1998). In addition, it has been reported that, in canine cerebral artery, hydrogen peroxide-induced relaxation is acetylcholine-dependent (Yang et al., 1998a). It has been found that hydrogen peroxide fails to relax arteries precontracted by increases in extracellular K^+ (Barlow and White, 1998), hyperpolarizes directly pig coronary and rat carotid artery smooth muscle cells (Bény and Von der Weid, 1991; Krippeit-Drews et al., 1995) and increases intracellular Ca^{2+} concentrations in smooth muscle of rabbit and rat aorta, rat carotid and cerebral arteries (Roveri et al., 1992; Krippeit-Drews et al., 1995; Iesaki et al., 1996; Yang et al., 1998b, 1999b). In addition, it has been suggested that large conductance Ca^{2+}-activated K^+ channels are directly stimulated by hydrogen peroxide or indirectly stimulated via enhanced production of cGMP (Barlow and White, 1998; Hayabuchi et al., 1998). Thus, it seems likely that the relaxation response to hydrogen peroxide is at least in part due to the activation of Ca^{2+}-dependent K^+ channels, resulting from the increased Ca^{2+} concentrations in vascular smooth muscle. Furthermore, hydrogen peroxide has been suggested to suppress the agonist-induced increase in Ca^{2+}-sensitivity of contractile proteins (Iesaki et al., 1996).

Although hydrogen peroxide at a concentration of 0.4 μM has been reported to be potent as a vasorelaxant in canine cerebral artery and rat aorta (Yang et al., 1998a, 1999a), many of the investigators mentioned above studied the vascular effects of hydrogen peroxide at concentrations ranging from 10 μM to 1 mM in various types of blood vessels. Since we found that hydrogen peroxide at relatively low concentrations (1–30 μM) was a vasorelaxant in rabbit mesenteric small artery, we attempted to explore the mechanisms underlying the response of the artery to hydrogen peroxide. The rabbit superior mesenteric artery and mouse aorta were also studied as reference tissues, and relaxation in response to enzymatically generated hydrogen peroxide was compared to the response to exogenously applied hydrogen peroxide.

2. Materials and methods

2.1. Vascular preparations and tension recording

Male Japan white rabbits (1.9–2.3 kg) and mice (20–25 g) were anesthetized with pentobarbitone sodium (Nembutal, 40 mg/kg, i.v.) and ether, respectively. The animals were killed by rapid exsanguination. The entire mesenterium (rabbit) and thoracic aorta (mouse) were removed and placed in cold Krebs–Henseleit bicarbonate (KHB) solution of the following composition (mM): NaCl 114, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and dextrose 10. The superior mesenteric artery, its second-order branches (diameter of approximately 90–130 μm) and aorta were cut into 2-mm rings without side-branches, and suspended between two pins under resting tensions of 1 g, 300 mg and 500 mg, respectively, in 5 ml of warmed (37°C) and oxygenated (95% O_2 and 5% CO_2) KHB solution for isometric recordings. The KHB solution contained 2 μM propranolol to avoid β-adrenoceptor-mediated responses (Fujimoto and Itoh, 1997). The endothelium was removed from the preparation by rubbing the intimal surface with a metal wire. After an equilibration period of 60–90 min, the preparations were contracted three times with 1–10 μM norepinephrine (mesenteric) and 1–3 μM 5-hydroxytryptamine (aorta) for 5 min at 40-min intervals until the responses were reproducible. The presence or absence of the endothelium was confirmed by the response to acetylcholine (1 μM) during one of the contractions elicited by the agonists. The experiments were carried out with endothelium-denuded preparations, unless otherwise stated in the text.

2.2. Relaxation responses to hydrogen peroxide, 8-bromo cyclic GMP and acetylcholine

The endothelium-intact and -denuded superior mesenteric arteries, mesenteric small arteries from rabbits and mouse aortas were contracted with norepinephrine (mesenteric) or with 5-hydroxytryptamine (aorta) to 75–85% (EC_{75}) of the maximum agonist response. As de-

scribed previously (Fujimoto and Itoh, 1997), the concentrations of norepinephrine were as follows (in μM): endothelium-intact vs. -denuded: mesenteric artery 9 vs. 1 and mesenteric small artery 2 vs. 1. In mouse aorta, the concentrations of 5-hydroxytryptamine were 1 μM for both endothelium-intact and -denuded preparations. After the contraction had reached steady state, cumulative concentration–response curves for hydrogen peroxide were obtained, with only one curve per preparation. In one of the paired preparations, the curve for hydrogen peroxide was made 15–50 min after, and during treatment with various drugs and enzymes. Another untreated or vehicle-treated preparation was used as control. In some experiments, the mesenteric small artery was contracted with 35.9 and 65.9 mM KCl in the presence and absence of 1–4 μM verapamil and then further contracted with norepinephrine to obtain a contraction similar to that elicited with norepinephrine (1 μM) alone; the concentration of norepinephrine used was varied to obtain a comparable increase in tone in each preparation. At the end of the experiment, papaverine (100 μM) was added to obtain the maximum relaxation. Since papaverine relaxed the preparations back to almost baseline tensions, relaxation responses to the vasorelaxant were expressed as percentages of the papaverine-induced relaxation (100%). Cumulative concentration–response curves for 8-bromo (Br) cGMP were also made with mesenteric small artery contracted with 65.9 mM KCl and norepinephrine. In some experiments with endothelium-intact mesenteric small artery, cumulative concentration–response curves for acetylcholine were made in the presence of both nitro-L-arginine (100 μM) and diclofenac (3 μM). Potencies of the drugs are expressed as EC_{50} values, where EC_{50} is the molar concentration producing 50% of the maximum drug response in a given concentration–response curve.

2.3. Contractile responses to norepinephrine

In endothelium-denuded mesenteric small artery, two cumulative concentration–contraction response curves for norepinephrine were made with an interval of 60 min between each determination. One of the paired preparations was treated with hydrogen peroxide (3, 10 and 30 μM) for 20 min before and during determination of the second curve; another untreated preparation was used to determine if any change in tissue sensitivity to norepinephrine occurred in the course of the experiments (Fujimoto et al., 1988). Results are expressed as percentages of the maximum norepinephrine response in the first concentration–response curve.

2.4. Norepinephrine- and caffeine-induced contractions in Ca^{2+}-free solution containing EGTA

The endothelium-denuded mesenteric small artery was contracted for 2 min with 10 μM norepinephrine three times at 50-min intervals in the normal KHB solution. Next, the artery was treated for 20 min with 10 μM hydrogen peroxide in the KHB solution and then contracted with 10 μM norepinephrine 0.5, 1 and 2 min after the preparation was exposed to a Ca^{2+}-free solution containing 0.6 mM EGTA with 10 μM hydrogen peroxide. In some experiments, the preparations were exposed for 20 min to the KHB solution containing hydrogen peroxide (3, 10 and 30 μM), verapamil (1 μM) or 8-Br cGMP (30–100 μM) and then contracted with 10 μM norepinephrine 2 min after the preparations were exposed to the Ca^{2+}-free solution containing 0.6 mM EGTA with hydrogen peroxide, verapamil or 8-Br cGMP (at the same concentrations). In another series of experiments, the preparations were contracted with 5 mM caffeine using the same protocol as that used for norepinephrine. The results are expressed as percentages of the last contractions elicited by each norepinephrine and caffeine in the normal KHB solution.

2.5. Drugs and solutions

The following drugs and enzymes were dissolved in distilled water and diluted with the KHB solution or Ca^{2+}-free solution: acetylcholine chloride (Sigma, St. Louis, MO, USA), Rp stereoisomer of adenosine 3′,5′-cyclic monophosphothioate triethylamine (Rp-cAMPS, Sigma), atropine sulfate (Sigma), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br cGMP, Sigma), caffeine anhydrous (Sigma), catalase (from bovine liver, Wako, Tokyo), deferoxamine mesylate (Sigma), diclofenac sodium (Sigma), DL-dithiothreitol (Sigma), glucose oxidase (from *Aspergillus niger*, Sigma), hydrogen peroxide (30%, Mitsubishi Gas Chem., Tokyo), 5-hydroxytryptamine creatinine sulfate (Merck, Germany), 3-isobutyl-1-methylxanthine (IBMX, Sigma), methylene blue (Katayama Chem., Tokyo), N^G-nitro-L-arginine (Peptide Institute, Minoh, Japan), (−)-norepinephrine bitartrate (Sigma), papaverine HCl (Wako), DL-propranolol HCl (Sigma), superoxide dismutase (Wako), tetraethylammoniun chloride (Wako) and verapamil HCl (Eisai, Tokyo). Glibenclamide (Sigma) and zaprinast (Sigma) were dissolved in dimethyl sulfoxide (Sigma). The Ca^{2+}-free solution was prepared by removing $CaCl_2$ from the normal KHB solution and adding ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Sigma) at a final concentration of 0.6 mM.

2.6. Statistical analysis

Results are reported as mean values ± S.E. of the number (n) of observations. All the data were analyzed with Student's *t*-test for paired or non-paired data. Statistical significance was assumed when the P value was less than 0.05.

3. Results

3.1. Relaxation responses to hydrogen peroxide and 8-bromo cGMP

Hydrogen peroxide did not significantly change basal levels of vascular smooth muscle tone in rabbit and mouse. The drug elicited concentration-dependent relaxation in endothelium-intact preparations of the superior mesenteric and mesenteric small arteries, which had been contracted with 9 and 2 μM norepinephrine, respectively (Fig. 1A and B). There was no apparent difference in potency or maximum response to hydrogen peroxide between the superior mesenteric and mesenteric small arteries (EC_{50} value in 10^{-5} M; 1.26 ± 0.40, $n = 7$ and 1.35 ± 0.29, $n = 12$, respectively). However, unlike the superior mesenteric artery, the mesenteric small artery responded significantly to hydrogen peroxide, 1 μM, by relaxation. Removal of the endothelium from the superior mesenteric and mesenteric small arteries did not alter the response to hydrogen peroxide when the tissues were pre-contracted with 1 μM norepinephrine. In Fig. 1, hydrogen peroxide-induced relaxation is expressed as a percentage of the response to 100 μM papaverine. The absolute values for the relaxation to papaverine were as follows (in mg); endothelium-intact vs.-denuded, 768 ± 33 vs. 668 ± 40 ($n = 7$, superior mesenteric) and 360 ± 30 vs. 319 ± 26 ($n = 8$–12, small mesenteric). Diclofenac (3 μM) inhibited the response of the endothelium-denuded arteries to hydrogen peroxide. Catalase (700 U/ml) almost completely abolished the response in the mesenteric small artery. Catalase boiled for 3 min failed to reduce the response to hydrogen peroxide (3–30 μM). Atropine (0.5 μM) did not inhibit the response to hydrogen peroxide in the mesenteric small artery (data not shown).

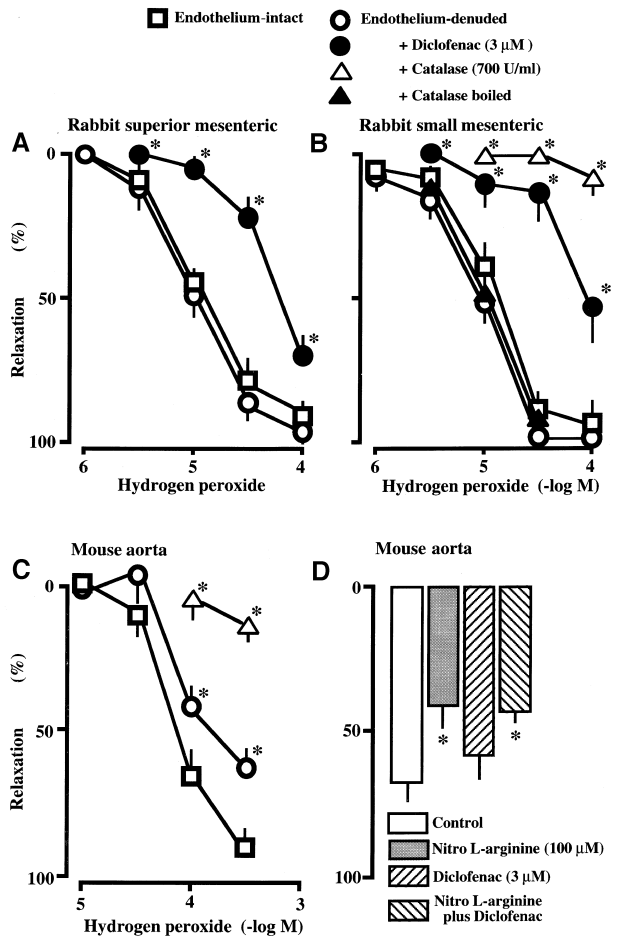

Fig. 1. Hydrogen peroxide-induced relaxation in rabbit superior mesenteric (A), mesenteric small arteries (B) and mouse aorta (C, D). To observe relaxation, rabbit and mouse blood vessels were contracted with norepinephrine and 5-hydroxytryptamine, respectively. See Materials and methods for concentrations of the contractile drugs used. The preparations used were endothelium-intact (□) and -denuded (○). The endothelium-denuded preparations were treated with diclofenac (3 μM, ●) for 30 min and catalase (700 U/ml, open triangle) for 20 min. In (B), the preparation was treated with catalase inactivated by boiling for 3 min (solid triangle). In (D), the endothelium-intact aorta was relaxed by 100 μM hydrogen peroxide in the absence and presence of nitro-L-arginine (100 μM), diclofenac (3 μM) or a combination of these drugs. Ordinate, papaverine (100 μM)-induced relaxations are expressed as 100%. Vertical bars represent S.E. of means ($n = 6$–12). * Significant difference from appropriate controls ($P < 0.05$).

To observe relaxation, both endothelium-intact and -denuded mouse aortas were contracted with 1 μM 5-hydroxytryptamine to 203 ± 17 mg ($n = 6$) and 229 ± 21 mg ($n = 6$), respectively. The relaxation response to hydrogen peroxide was reduced by the removal of endothelium and catalase (700 U/ml) (Fig. 1C). Hydrogen peroxide was less potent in the mouse aorta than in the rabbit mesenteric arteries (EC_{50} value in 10^{-5} M; 6.31 ± 0.09, $n = 6$, $P < 0.05$ vs. rabbit arteries). The response of endothelium-intact aorta to hydrogen peroxide (100 μM) was partially reduced by nitro-L-arginine (100 μM) but not diclofenac (3 μM)(Fig. 1D). The inhibitory effect of nitro-L-arginine was not changed in the presence of diclofenac.

In the endothelium-denuded mesenteric small artery, the relaxation response to hydrogen peroxide was not significantly altered in the presence of dimethyl sulfoxide (7 mM), deferoxamine (1 mM) or superoxide dismutase (200 U/ml) (Fig. 2A and B). When the artery was treated with dithiothreitol (1 mM) for 15 min, the response to hydrogen peroxide was reduced as compared to that in the control tissue (Fig. 2A), and the hydrogen peroxide-induced relaxation was reversed by dithiothreitol (1 mM), which did not change papaverine-induced relaxation (Fig. 2C). Dimethyl sulfoxide, deferoxamine, superoxide dismutase and dithiothreitol at the concentrations used did not significantly change the basal levels of vascular smooth muscle tone or the norepinephrine-induced contraction.

The response to hydrogen peroxide was significantly increased by isobutylmethylxanthine (5 μM) and zaprinast (5 μM), reduced by methylene blue (10 μM) and unchanged by Rp-cAMPS (25 μM) (Fig. 3). Since methylene blue alone increased the basal vascular tone by 30–80 mg, the tension was re-adjusted.

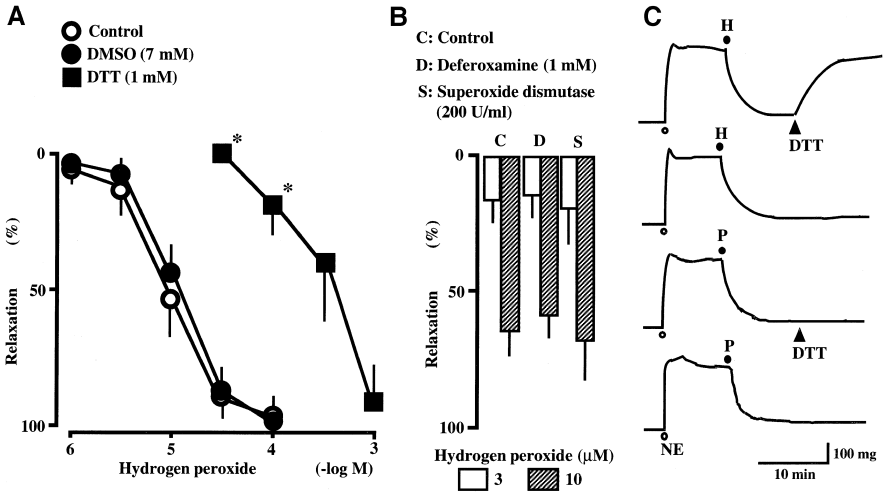

Fig. 2. (A) Concentration–response curves for hydrogen peroxide-induced relaxation in endothelium-denuded mesenteric small artery. The curves were made in the absence (○) and presence of 7 mM dimethyl sulfoxide (DMSO, ●) and 1 mM dithiothreitol (DTT, ■). Ordinate, papaverine-induced relaxations are expressed as 100%. Vertical bars represent S.E. of means ($n = 5$–10). * Significant difference from appropriate controls ($P < 0.05$). (B) Relaxation response of the mesenteric small artery to hydrogen peroxide at 3 μM (open column) and 10 μM (hatched column) in the absence (C) and presence of deferoxamine (D, 1 mM) and superoxide dismutase (S, 200 U/ml).Vertical bars represent S.E. of means ($n = 4$–8). (C) Effects of DTT on relaxations elicited by hydrogen peroxide (H, 100 μM) and papaverine (P, 100 μM). The arteries which had been contracted with norepinephrine (NE, 1 μM) were relaxed by hydrogen peroxide or papaverine to almost pre-contraction levels. For hydrogen peroxide, the decrease of relaxation to original contraction levels was obtained by adding DTT (1 mM) into the bath solution. For papaverine, however, the relaxant effect was not affected by DTT.

The relaxation response to hydrogen peroxide was reduced in the artery precontracted with norepinephrine (0.31 ± 0.09 μM) in 35.9 mM KCl solution and, to a greater extent, in the artery precontracted with norepinephrine (0.23 ± 0.09 μM) in 65.9 mM KCl solution as compared to that in the artery precontracted with 1 μM norepinephrine in the normal KHB solution (Fig. 4A). In Fig. 4A, results are expressed as percentages of papaverine-induced relaxation (absolute values; norepinephrine alone, 281 ± 21 mg, $n = 7$, norepinephrine in 35.9 mM KCl, 223 ± 37 mg, $n = 6$ and norepinephrine in 65.9 mM KCl, 282 ± 31 mg, $n = 6$). In another series of experiments, arteries were contracted with 65.9 mM KCl and the contraction was completely reversed by 1–4 μM verapamil, an L-type Ca^{2+} channel blocker. The artery was again contracted to 213 ± 22 mg ($n = 12$) with norepinephrine (10 μM) after the tension reached the basal level in the presence of verapamil. In these arteries, hydrogen peroxide (10 and 30 μM) elicited greater relaxations than in the control tissue contracted with a combination of 65.9 mM KCl and norepinephrine (Fig. 4B). 8-Br cGMP (10–100 μM) was also more potent as a vasorelaxant in the norepinephrine-stimulated artery than in the artery contracted with the combination of 65.9 mM KCl and norepinephrine (Fig. 5A). However, unlike hydrogen peroxide, 8-Br cGMP still elicited a marked relaxation in the depolarized artery. Tetraethylammonium at 10 mM did not alter the relaxation in response to hydrogen peroxide (Fig. 4C), although the drug markedly reduced the relaxation response of endothelium-intact artery to acetylcholine in the presence of diclofenac and nitro-L-arginine (Fig. 4D). Glibenclamide (10 μM) did not significantly alter the response to hydrogen peroxide (data not shown).

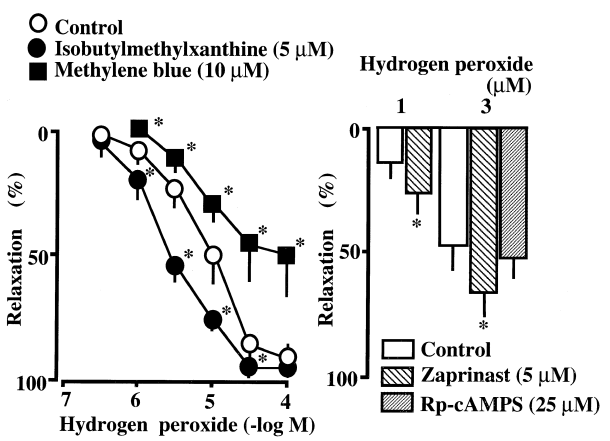

Fig. 3. Left panel: effects of isobutylmethylxanthine and methylene blue on hydrogen peroxide-induced relaxation. Concentration–response curves for hydrogen peroxide were determined 20–30 min after and during treatment with isobutylmethylxanthine (5 μM, ●) and methylene blue (10 μM, ■). Right panel: effects of zaprinast (5 μM) and Rp-cAMPS (25 μM) on the relaxation to hydrogen peroxide (1 and 3 μM). The artery was treated for 50 min with these drugs. Ordinate, papaverine (100 μM)-induced relaxations are expressed as 100%. Vertical bars represent S.E. of means ($n = 6$–15). * Significant difference from controls ($P < 0.05$).

3.2. Relaxation response to enzymatically generated hydrogen peroxide

When glucose oxidase (7.5 and 15 mU/ml) was applied to the norepinephrine-stimulated, endothelium-de-

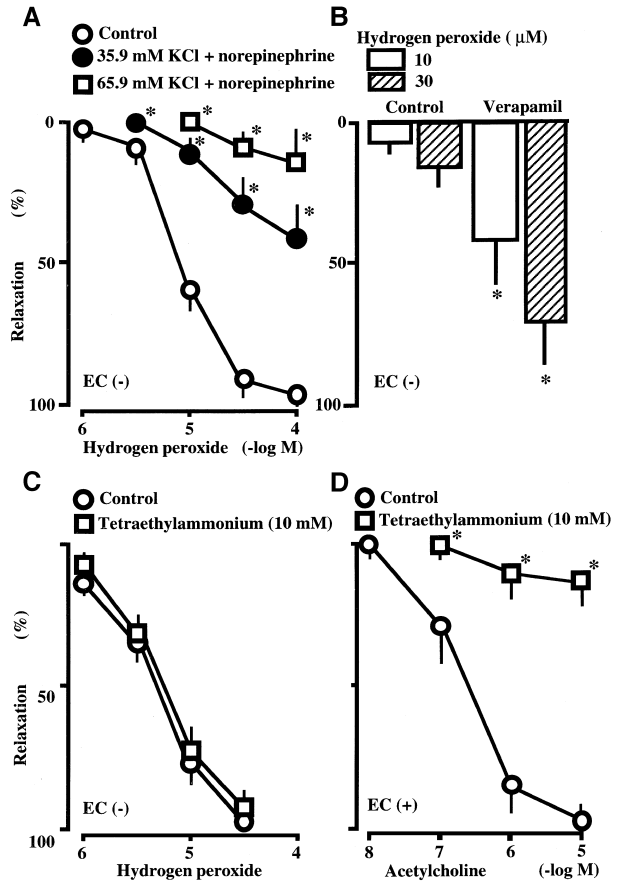

Fig. 4. Effects of high-KCl solution and tetraethylammoniun on hydrogen peroxide- or acetylcholine-induced relaxations. (A) The endothelium-denuded tissues were contracted with norepinephrine at 0.31 ± 0.09 μM (223 ± 37 mg, $n = 5$) and 0.23 ± 0.09 μM (282 ± 31 mg, $n = 5$) in the presence of KCl at concentrations of 35.9 mM (●) and 65.9 mM (□), respectively. The control preparations were contracted with norepinephrine (1 μM) alone (281 ± 21 mg, $n = 7$, ○). (B) The tissues which had been contracted with norepinephrine (10 μM) and KCl (65.9 mM) in the presence of verapamil (1–4 μM) (213 ± 22 mg, $n = 12$) were relaxed with hydrogen peroxide at 10 (open column) and 30 μM (solid column). The control artery was contracted with norepinephrine and KCl (65.9 mM). (C) Concentration–response curves for hydrogen peroxide were determined in the presence (□) and absence (○) of tetraethylammoniun (10 mM). $n = 6$. (D) Concentration–response curves for acetylcholine-induced relaxation in the presence (□) and absence (○) of tetraethylammoniun (10 mM) were made with endothelium-intact artery. The KHB solution contained diclofenac (3 μM) and nitro-L-arginine (100 μM) to inhibit cyclooxygenase and nitric oxide synthase, respectively. $n = 6$. Vertical bars represent S.E. of means. * Significant difference from controls ($P < 0.05$). EC (+) and EC (−) indicate endothelium-intact and -denuded preparations, respectively.

nuded mesenteric small artery, the tissue responded by relaxation (Fig. 6). The tissue was completely relaxed by glucose oxidase (45 mU/ml) to the levels produced by papaverine, and the response to glucose oxidase (45 mU/ml) was markedly reduced by either catalase (700 U/ml) or dithiothreitol (1 mM). The response to glucose oxidase (15 U/ml) was not significantly altered by deferoxamine (1 mM) and superoxide dismutase (200 U/ml) (data not shown).

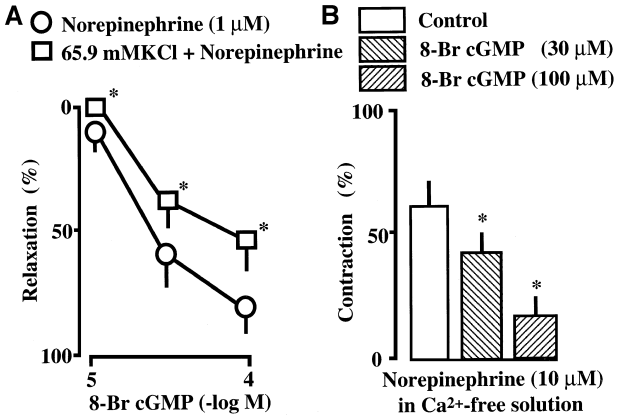

Fig. 5. Effect of high-KCl solution on relaxation response to 8-Br cGMP (A) and inhibitory effect of 8-Br cGMP (30 and 100 μM) on norepinephrine-induced contraction in a Ca^{2+}-free solution containing 0.6 mM EGTA (B). Same explanations as in Figs. 4 and 8 (or in Materials and methods).

3.3. Effect of hydrogen peroxide on contractile response to norepinephrine

Norepinephrine (10 nM–100 μM) elicited concentration-dependent contractions in endothelium-denuded preparations of the mesenteric small artery (Fig. 7). The 20-min incubation with hydrogen peroxide (3–30 μM) shifted the concentration–response curves for norepinephrine to the right and downwards. The EC_{50} values for norepinephrine were as follows: control; $3.55 \pm 0.24 \times 10^{-7}$ M ($n = 15$), hydrogen peroxide, 3 μM; $4.47 \pm 0.38 \times 10^{-7}$ M ($n = 5$), 10 μM; $5.62 \pm 0.31 \times 10^{-7}$ M ($P < 0.05$ from control, $n = 5$), 10 μM; $1.78 \pm 0.09 \times 10^{-6}$ M ($P < 0.05$ from control, $n = 5$).

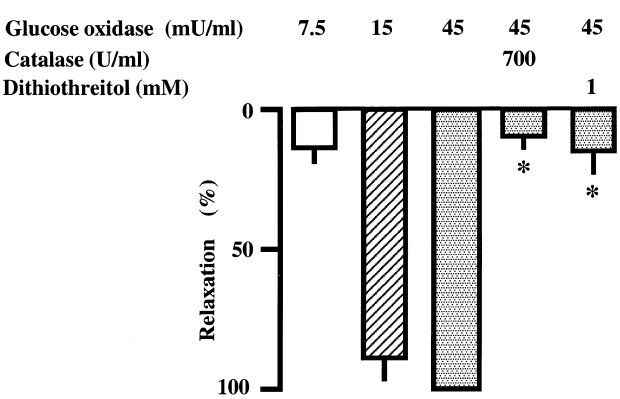

Fig. 6. Inhibitory effects of catalase and dithiothreitol on relaxation elicited by glucose oxidase. The artery responded to glucose oxidase (7.5–45 mU/ml) by relaxation. The preparations were incubated with catalase (700 U/ml) or dithiothreitol (1 mM) for 20–30 min before the addition of glucose oxidase (45 mU/ml). Results represent means ± S.E. ($n = 7$). * Significant difference from glucose oxidase alone ($P < 0.05$). Vertical bars represent S.E. of means.

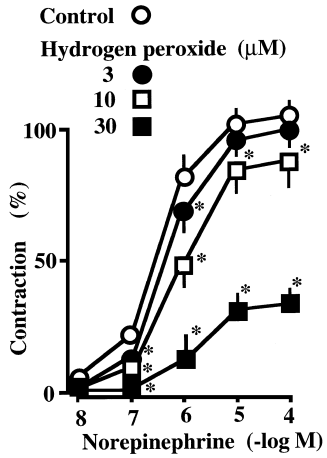

Fig. 7. Concentration–response curves for norepinephrine-induced contractions in the presence of hydrogen peroxide, 3 (●), 10 (□) and 30 μM (■). The medium contained 2 μM propranolol (a β-adrenoceptor antagonist). Control (○). Ordinate; the maximum norepinephrine response in the first concentration–response curve is expressed as 100%. Vertical bars represent S.E. of means ($n = 6–18$). * Significant difference from controls ($P < 0.05$).

3.4. Contractile responses to norepinephrine and caffeine in Ca^{2+}-free solution

Norepinephrine (10 μM) elicited a short-lived, phasic contraction in a Ca^{2+}-free solution containing 0.6 mM EGTA. In Fig. 8A, the peak contraction is expressed as a percentage of the maximum norepinephrine contraction obtained in the normal KHB buffer (100%; 607 ± 31 mg, $n = 16$). The contraction was decreased in relation to the time of exposure to the Ca^{2+}-free solution. When the mesenteric artery was suspended for 20 min in the KHB buffer containing 10 μM hydrogen peroxide and then contracted with 10 μM norepinephrine 0.5, 1 and 2 min after the artery was exposed to the Ca^{2+}-free solution containing 10 μM hydrogen peroxide, hydrogen peroxide reduced the contraction in the Ca^{2+}-free solution (Fig. 8A). In addition, the artery was also exposed for 20 min to 3, 10 and 30 μM hydrogen peroxide in the KHB buffer and then contracted with 10 μM norepinephrine 2 min after the artery was exposed to the Ca^{2+}-free solution with hydrogen peroxide at the same concentrations. Under these conditions, the contraction in response to norepinephrine was attenuated by hydrogen peroxide in a concentration-dependent manner (Fig. 8B). When the artery was treated with verapamil or 8-Br cGMP instead of hydrogen peroxide, verapamil (1 μM) did not significantly alter the contraction to norepinephrine (Fig. 8B), but 8-Br cGMP (30 and 100 μM) inhibited it (Fig. 5B).

The arteries were contracted with caffeine (5 mM) using the same protocol as that used for norepinephrine. The maximum contraction in response to caffeine was 290 ± 33 mg ($n = 16$) in the normal KHB buffer. Caffeine produced a phasic contraction of the artery in the Ca^{2+}-free solution. Hydrogen peroxide inhibited the caffeine-induced contraction but to a lesser extent than the norepinephrine-induced contraction. For instance, hydrogen peroxide (10 μM) failed to alter the response to caffeine after exposure for 0.5 min in the Ca^{2+}-free solution (Fig. 8C) and the drug at 3 μM did not inhibit the contraction to caffeine after exposure for 2 min in the Ca^{2+}-free solution (Fig. 8D).

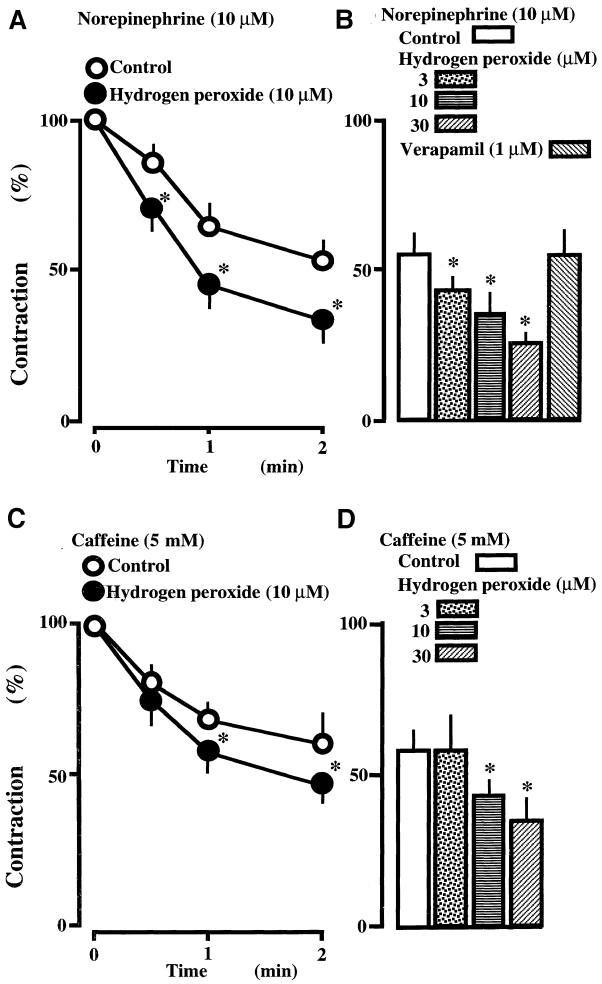

Fig. 8. Effects of hydrogen peroxide on norepinephrine (A, B)- and caffeine (C, D)-induced contractions in a Ca^{2+}-free solution containing 0.6 mM EGTA. In (A), the tissues were incubated with hydrogen peroxide (10 μM) for 20 min in the normal KHB solution and for another 0.5, 1 and 2 min in the Ca^{2+}-free solution containing hydrogen peroxide (10 μM). Then, the tissues were contracted with 10 μM norepinephrine. In (B), the preparations were incubated with hydrogen peroxide, 3, 10 and 30 μM or verapamil, 1 μM for 20 min in the KHB solution and for another 2 min in the Ca^{2+}-free solution containing EGTA (0.6 mM) and hydrogen peroxide or verapamil at the same concentrations. Then, the tissues were contracted with 10 μM norepinephrine. In (C) and (D), the arteries were contracted with caffeine (5 mM) using the same protocol as that used for norepinephrine in (A) and (B), respectively. Ordinate, norepinephrine- or caffeine-induced contractions in the Ca^{2+}-free solution as percentages of contractions obtained in the KHB solution (100%). Abscissa in (A) and (C), exposure time (min) in the Ca^{2+}-free solution. Vertical bars represent S.E. of means ($n = 5–9$). * Significant difference from appropriate controls ($P < 0.05$).

4. Discussion

Although hydrogen peroxide is known to relax some peripheral arteries (Zembowicz et al., 1993; Barlow and White, 1998; Yang et al., 1998a, 1999a; Iesaki et al., 1999), the effects of hydrogen peroxide on small arteries such as the second branches of mesenteric artery have received less attention. The present results indicated that hydrogen peroxide evoked relaxation of endothelium-intact mesenteric small artery of rabbits (precontracted with norepinephrine). In this artery, hydrogen peroxide was five times more potent as a vasorelaxant than in the mouse aorta but equipotent in the superior mesenteric artery of the same rabbit. However, unlike the superior mesenteric artery, the mesenteric small artery did respond to 1 μM hydrogen peroxide by relaxation. The mesenteric small artery was more responsive to hydrogen peroxide than bovine pulmonary artery and rabbit aorta (Burke-Wolin et al., 1991; Zembowicz et al., 1993), but less responsive than canine cerebral artery and rat aorta, tissues which responded to hydrogen peroxide at a concentration as low as 0.4 μM (Yang et al., 1998a, 1999a). The minimum concentrations (1–3 μM) of hydrogen peroxide required for relaxation in the mesenteric small artery were close to concentrations of hydrogen peroxide estimated in rat liver perfusate (see White et al., 1976; Barlow and White, 1998). In addition, it has been reported that isolated and stimulated human neutrophils and polymorphonuclear leucocytes can produce/release hydrogen peroxide, which reaches concentrations of 10 μM–1 mM/10^6 cells (Nathan, 1987; Shappell et al., 1990; Negri et al., 1991). Together with these findings, the present results suggest that hydrogen peroxide is physiologically or pathophysiologically important as an endogenous vasodilator.

Nitro-L-arginine (a nitric oxide synthase inhibitor) and removal of the endothelium attenuated but did not completely abolish the hydrogen peroxide-induced relaxation in mouse aorta, suggesting that hydrogen peroxide has a direct action on the smooth muscle and an indirect action via endothelium-derived nitric oxide, as suggested before for canine coronary artery and rabbit and rat aortas (Rubanyi and Vanhoutte, 1986; Zembowicz et al., 1993; Yang et al., 1999a). On the other hand, the ability of the mesenteric small and superior mesenteric arteries to relax in response to hydrogen peroxide was not affected by removal of the endothelium, suggesting that the relaxation is due to the direct action of hydrogen peroxide on smooth muscle, as reported previously for bovine and rabbit pulmonary arteries (Burke and Wolin, 1987; Burke-Wolin et al., 1991). Therefore, the following experiments were performed with endothelium-denuded mesenteric small artery, unless otherwise stated. The relaxation response to exogenous hydrogen peroxide and enzymatically generated hydrogen peroxide was abolished by catalase, an enzyme which hydrolyses hydrogen peroxide, but was not changed by dimethyl sulfoxide (a hydroxy radical scavenger), deferoxamine (a cell-membrane permeable iron chelator) and superoxide dismutase, suggesting that hydrogen peroxide is the major causative substance. In addition, dithiothreitol (a sulfhydryl group reductant) markedly inhibited the relaxation caused by hydrogen peroxide and enzymatically generated hydrogen peroxide and the force decline induced by hydrogen peroxide was reversed by subsequent exposure to dithiothreitol. Thus, it is very likely that the mechanisms underlying the relaxation in response to hydrogen peroxide include sulfhydryl oxidation. Unlike that of canine cerebral artery (Yang et al., 1998a), the response of rabbit mesenteric small artery to hydrogen peroxide was unaffected by atropine, suggesting that, in this artery, the relaxation is not mediated by acetylcholine.

We then studied the effects of diclofenac (a cyclooxygenase inhibitor), methylene blue (a soluble guanylate cyclase inhibitor), zaprinast (a cGMP-selective phosphodiesterase inhibitor) and isobutylmethylxanthine (a nonselective phosphodiesterase inhibitor) on the hydrogen peroxide-induced relaxation, since several lines of evidence have suggested that the endothelium-independent relaxation due to hydrogen peroxide is mediated by arachidonic acid metabolites and cGMP in vascular smooth muscle (Zembowicz et al., 1993; Hayabuchi et al., 1998; Iesaki et al., 1999). The present findings that diclofenac and methylene blue inhibited, and the phosphodiesterase inhibitors increased, the hydrogen peroxide-induced relaxation and that 8-Br cGMP was vasorelaxant suggest that the vasorelaxing effects of hydrogen peroxide are mediated by intracellular accumulation of cyclooxygenase products of arachidonic acid and cGMP. Further studies are needed to identify the cyclooxygenase products. In porcine coronary artery, hydrogen peroxide-induced, endothelium-independent relaxation was mediated by lipoxygenase metabolites of arachidonic acid (Barlow and White, 1998). On the contrary, the ability of rabbit aorta and cat cerebral arteriole to relax in response to hydrogen peroxide was not affected by inhibitors of cyclooxygenase and soluble guanylate cyclase (Iesaki et al., 1994; Wei et al., 1996). Furthermore, the endothelium-independent relaxation response of bovine pulmonary artery to hydrogen peroxide was reported to be independent of cyclooxygenase metabolites but mediated by cGMP (Burke and Wolin, 1987). It was also found that rat aorta relaxation was independent of cyclooxygenase but required formation of cGMP (Yang et al., 1999a). It seems likely that there are regional and species differences in the mechanisms underlying the hydrogen peroxide-induced relaxation. We confirmed the previous finding that the response to hydrogen peroxide was not altered by Rp-cAMPS, an inhibitor of cAMP-mediated responses (Hayabuchi et al., 1998).

We found that the vasorelaxations in response to hydrogen peroxide and 8-Br cGMP were more marked in norepinephrine-stimulated arteries than in arteries depolarized by high-KCl solution. These results are consistent with findings reported previously (Lincoln, 1983; Burke and

Wolin, 1987; Iesaki et al., 1996; Barlow and White, 1998). Also, verapamil (1–4 μM, an L-type Ca^{2+} channel blocker), which completely prevented KCl (65.9 mM)-induced contractions, increased the response to hydrogen peroxide in norepinephrine (10 μM)-contracted arteries in the presence of 65.9 mM KCl. These findings suggest that hydrogen peroxide is not an inhibitor of voltage-dependent Ca^{2+} influx and that membrane depolarization does not affect the response to hydrogen peroxide in the rabbit mesenteric small artery. We also found that tonic contraction in response to norepinephrine was non-competitively inhibited by hydrogen peroxide (Fig. 7). It seems likely that the agonist-induced contraction is more susceptible to the inhibitory effect of hydrogen peroxide than is that produced by high-KCl solution. The former is induced by Ca^{2+} influx from extracellular space and by Ca^{2+} release from internal stores with an increase in Ca^{2+} sensitivity of contractile proteins, while the latter is induced by increased concentrations of intracellular Ca^{2+} without a significant increase in Ca^{2+} sensitivity. Unlike the relaxation to hydrogen peroxide, which was almost completely abolished by 65.9 mM KCl, that to 8-Br cGMP was still apparent in the artery depolarized by 65.9 mM KCl. Thus, these pharmacological properties of hydrogen peroxide may be partly explained by those of cGMP.

Electrophysiological studies have shown that hydrogen peroxide hyperpolarizes the vascular smooth muscle cell membrane by opening of K^+ channels; the results are conflicting as far as subtype of K^+ channels involved. Hydrogen peroxide opens large-conductance Ca^{2+}-sensitive K^+ channels directly or indirectly via enhanced formation of cGMP in rat carotid, rat cerebral or porcine coronary arteries (Krippeit-Drews et al., 1995; Sobey et al., 1997; Barlow and White, 1998; Hayabuchi et al., 1998). On the contrary, Wei et al. (1996) found that hydrogen peroxide relaxed cat cerebral artery through the activation of ATP-sensitive K^+ channels by a mechanism not involving the stimulation of soluble guanylate cyclase. In our studies, however, tetraethylammonium (a non-selective Ca^{2+}-sensitive K^+ channel blocker) and glibenclamide (an ATP-sensitive K^+ channel blocker) failed to affect the hydrogen peroxide-induced, endothelium-independent relaxation of norepinephrine-induced contraction, although tetraethylammonium did inhibit acetylcholine-induced, endothelium-dependent relaxation resistant to inhibitors of cyclooxygenase and nitric oxide synthase, a relaxation which is accounted for by hyperpolarization in smooth muscle cells resulting from activation of K^+ channels by an enhanced release of an endothelium-derived hyperpolarizing factor (Fujimoto et al., 1999). We, therefore, ruled out a role for these K^+ channels in the relaxation response of the rabbit mesenteric small artery to hydrogen peroxide. This discrepancy may be explained partly by differences in species and vascular regions or preparations between our and other studies.

In spite of its relaxant effect on vascular smooth muscle, hydrogen peroxide at concentrations as high as 0.3–1 mM induced a biphasic increase in intracellular Ca^{2+} concentrations in vascular smooth muscle as the result of release of Ca^{2+} from internal stores (an initial and fast transient increase) and Ca^{2+} influx across the cell membrane (a sustained increase) (Roveri et al., 1992; Krippeit-Drews et al., 1995). In addition, Grover et al. (1992) have reported that hydrogen peroxide at concentrations (3–30 μM) similar to those used in our experiments inactivates Ca^{2+} pumps and increases membrane permeability to Ca^{2+} in plasma membrane and, more so, in endoplasmic reticulum of pig coronary artery. We found that hydrogen peroxide, and also 8-Br cGMP, reduced the short-lived, transient contraction induced by norepinephrine in the Ca^{2+}-free solution and the contraction was not altered by verapamil (1 μM), suggesting that the contraction is due to the stimulated release of Ca^{2+} from intracellular storage sites. Likewise, hydrogen peroxide attenuated the caffeine-induced contraction in the Ca^{2+}-free solution, although the mechanisms underlying the release of Ca^{2+} are different for norepinephrine and for caffeine (Itoh et al., 1992). Hydrogen peroxide more effectively inhibited the contraction elicited by norepinephrine than that induced by caffeine. In addition, there is evidence suggesting that hydrogen peroxide may suppress the agonist-induced increase in Ca^{2+} sensitivity of the contractile apparatus (Iesaki et al., 1996).

In conclusion, hydrogen peroxide at concentrations as low as 1–100 μM inhibits the norepinephrine-induced contraction of the rabbit mesenteric small artery via its direct action on smooth muscle. The contraction of the artery elicited by membrane depolarization with high-KCl solution is less affected by hydrogen peroxide than the agonist-induced contraction. Hydrogen peroxide is not an inhibitor of voltage-dependent Ca^{2+} influx in the tissues now studied. The hydrogen peroxide-induced relaxation is affected by the redox state of sulfhydryl group and is partly mediated through diclofenac-sensitive pathways and by an accumulation of cGMP. Enzymatically generated hydrogen peroxide also elicits relaxation in norepinephrine-stimulated mesenteric artery of the rabbit.

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