Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 459 (2003) 65–73
www.elsevier.com/locate/ejphar

Mechanisms underlying the hydrogen peroxide-induced, endothelium-independent relaxation of the norepinephrine-contraction in guinea-pig aorta

Seigo Fujimoto*, Mayumi Mori, Hiromi Tsushima

Department of Cellular and Molecular Pharmacology, Nagoya City University Graduate School of Medical Sciences, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

Received 1 August 2002; received in revised form 15 November 2002; accepted 22 November 2002

Abstract

The mechanisms underlying the hydrogen peroxide-induced relaxation of the norepinephrine-contraction were studied by measuring isometric force, myosin light chain (MLC_{20}) phosphorylation and cyclic GMP in endothelium-denuded muscle from the guinea-pig aorta. Norepinephrine (5.2 ± 1.3 μM) produced a phasic, followed by a tonic contraction. Hydrogen peroxide (10 and 100 μM), glyceryl trinitrate (30 and 300 nM) and 8-bromo cyclic GMP (30 and 100 μM) did not change the basal tone, but reduced the norepinephrine-induced contraction. Phosphorylation of MLC_{20} (percentage of phosphorylated to total MLC_{20}) was increased 1 min ($5.9 \pm 1.0\%$ vs. $35.9 \pm 4.9\%$) and, to a lesser extent, 20 min ($3.7 \pm 1.7\%$ vs. $13.9 \pm 1.6\%$) after the addition of norepinephrine. Hydrogen peroxide (100 μM) did not modify basal MLC_{20} phosphorylation, but reduced the increase in MLC_{20} phosphorylation induced by 1-min exposure to norepinephrine ($20.9 \pm 4.1\%$). Its effect was abolished by catalase. When the tissue was incubated for 20 min with norepinephrine in the presence of hydrogen peroxide, norepinephrine-induced MLC_{20} phosphorylation was not changed ($13.6 \pm 1.5\%$), as compared to that in the absence of hydrogen peroxide. Hydrogen peroxide relaxed norepinephrine-stimulated aortas in a concentration-dependent fashion with EC_{50} values of 5.9 ± 0.2 μM. The relaxation was inhibited by soluble guanylate cyclase inhibitors and increased by an inhibitor of cyclic GMP-selective phosphodiesterase. In aorta precontracted with norepinephrine, hydrogen peroxide (100 μM) relaxed the tissue by $89 \pm 11\%$ and almost doubled tissue concentrations of cyclic GMP, whereas sodium nitroprusside (1 μM) relaxed the tissue by 100% and increased cyclic GMP concentrations 30-fold. It is suggested that the inhibitory effects of hydrogen peroxide on the norepinephrine-induced phasic and sustained contractions are explained by a decrease in MLC_{20} phosphorylation and by an alteration in MLC_{20} phosphorylation-independent mechanisms, respectively. The effects of hydrogen peroxide were in part mediated by cyclic GMP.

Keywords: Aorta; cGMP; Hydrogen peroxide; Myosin light chain; Norepinephrine

1. Introduction

Smooth muscle contraction is normally thought to be regulated by Ca^{2+} and involves reversible phosphorylation and dephosphorylation of the 20-kDa regulatory light chain subunit of myosin (MLC_{20}), by the Ca^{2+}/calmodulin-dependent myosin light chain kinase and myosin light chain phosphatase, respectively, and MLC_{20} phosphorylation allows activation of myosin adenosine triphosphatase by actin, resulting in contraction (Kitazawa et al., 1991; Somlyo and Somlyo, 1994). On the other hand, vascular contraction in response to some agonists is maintained despite a fall in MLC_{20} phosphorylation and intracellular Ca^{2+}, suggesting that the agonist induces an alteration in Ca^{2+}-sensitivity of contractile elements and MLC_{20} phosphorylation-independent contraction in vascular smooth muscle (Himpens et al., 1990). The agonist-induced increase in Ca^{2+} sensitivity may be mediated through a small G-protein Rho A and protein kinase C (Jiang and Morgan, 1987; Throckmorton et al., 1998). Rho A activates Rho kinase, which phosphorylates myosin light chain phosphatase (and inactivates phosphatase) (Kimura et al., 1996). Activation of protein kinase C results in

* Corresponding author. Tel.: +81-52-853-8150; fax: +81-52-842-0863.
E-mail address: fujimoto@med.nagoya-cu.ac.jp (S. Fujimoto).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)02825-X

phosphorylation of calponin and caldesmon (thus contraction) via mitogen-activated protein kinase activation (Walsh et al., 1994).

Hydrogen peroxide, an important byproduct of normal cellular oxidative metabolism (Raimondi et al., 2000), which is found in human plasma at micromolar concentrations (Lacy et al., 1998; Liu and Zweier, 2001), has been implicated in the pathophysiology of a variety of cardiovascular disorders including hypertension, myocardial ischemia and atherosclerosis (Hearse et al., 1993; Ross, 1993; Lacy et al., 1998). However, the mechanisms by which hydrogen peroxide brings about these disorders still remain unclear. Hydrogen peroxide causes a contraction or a relaxation in vascular smooth muscle, depending on the species, tissues and baseline tone. Under resting tension, hydrogen peroxide contracts rat, porcine and rabbit pulmonary arteries (Rhoades et al., 1990; Sheehan et al., 1993; Jin and Rhoades, 1997; Pelaez et al., 2000a,b), rat aorta (Rodríguez-Martînez et al., 1998; Yang et al., 1998b), dog cerebral artery (Yang et al., 1999b) and human umbilical artery (Okatani et al., 1997). It has been suggested that the hydrogen peroxide-induced contraction is either Ca^{2+}-dependent in dog basilar artery (Yang et al., 1999b) or Ca^{2+}-independent in rat, porcine and rabbit pulmonary arteries (Jin et al., 1991; Sheehan et al., 1993; Pelaez et al., 2000a,b), and is mediated by tyrosine kinase (Jin and Rhoades, 1997; Yang et al., 1998b, 1999b), phospholipase A_2/arachidonate metabolism (Barlow and White, 1998; Yang et al., 1998b), phospholipase C (Sheehan et al., 1993), phosphatidylinositol 3-kinase (Yang et al., 1999b) or voltage-gated Ca^{2+} channels (Yang et al., 1999b). In addition, there are controversial results suggesting that hydrogen peroxide-induced contraction may or may not be mediated by protein kinase C and mitogen-activated protein kinase (Bass and Berk, 1995; Rao et al., 1995; Guyton et al., 1996; Jin and Rhoades, 1997; Yang et al., 1999b; Pelaez et al., 2000b). MLC_{20} phosphorylation seems not to be required for hydrogen peroxide-induced contraction in rat pulmonary and systemic arterial and venous smooth muscle (Pelaez et al., 2000a).

In agonist-stimulated artery, hydrogen peroxide causes endothelium-dependent or -independent relaxation. Briefly, hydrogen peroxide-induced relaxation is mediated by the stimulated release of nitric oxide from the endothelium in rat aorta, canine and cat cerebral arteries and guinea-pig nasal mucosa vasculature (Fraile et al., 1994; Yang et al., 1998a, 1999a; Hirai et al., 2000). On the other hand, hydrogen peroxide relaxes directly smooth muscles of rabbit and mouse mesenteric arteries (Matoba et al., 2000; Fujimoto et al., 2001), bovine and rabbit pulmonary arteries (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; Hayabuchi et al., 1998; Barlow et al., 2000). It seems likely that hydrogen peroxide is an endothelium-derived relaxing factor which acts on vascular smooth muscle (Vanhoutte, 2001). Hydrogen peroxide causes hyperpolarization as a result of activation of ATP-sensitive or Ca^{2+}- and voltage-activated K^+ channels in the vascular smooth muscles (Wei et al., 1996; Sobey et al., 1997; Barlow and White, 1998; Hayabuchi et al., 1998; Barlow et al., 2000; Matoba et al., 2000). However, we have found that the relaxation response of rabbit mesenteric artery to hydrogen peroxide is not inhibited by glibenclamide (an ATP-sensitive K^+ channel blocker) and tetraethylammonium (a non-selective Ca^{2+}-sensitive K^+ channel blocker) (Fujimoto et al., 2001). In addition, it has been suggested that the hydrogen peroxide-induced relaxation is mediated by an enhanced formation of phospholipase A_2 and lipoxygenase metabolites (Barlow et al., 2000) and of cyclic GMP via soluble guanylate cyclase activation in smooth muscle (Burke and Wolin, 1987; Burke-Wolin et al., 1991; Zembowicz et al., 1993; Hayabuchi et al., 1998; Iesaki et al., 1999; Fujimoto et al., 2001). On the other hand, the relaxation in response to hydrogen peroxide seems not to be mediated by cyclic GMP in dog and cat cerebral arteries (Wei et al., 1996; Iida and Katusic, 2000) or in dog trachea (Lorenz et al., 1999).

Hydrogen peroxide inhibits norepinephrine-induced transient contraction in the absence of extracellular Ca^{2+} (Fujimoto et al., 2001) and agonist-induced increase in Ca^{2+} sensitivity of vascular and tracheal smooth muscles (Iesaki et al., 1996; Lorenz et al., 1999). There is evidence that cyclic GMP inhibits the agonist-induced Ca^{2+} sensitization of contractile elements in vascular smooth muscle (Rembold et al., 2000; Sauzeau et al., 2000; Nauli et al., 2001). The present study made use of guinea-pig aorta, since hydrogen peroxide-induced relaxation in this tissue has received less attention and blood vessels are highly heterogeneous. Thus, the present study was designed to determine the effects of hydrogen peroxide on force development, cyclic GMP concentrations and MLC_{20} phosphorylation in aorta without endothelium.

2. Materials and methods

2.1. Tissue preparation and measurement of isometric tension

The animal care and research protocols were in accordance with the guidelines of our university. Male Hartley guinea-pigs (250–350 g) were anesthetized with pentobarbitone sodium (Nembutal, 40 mg/kg, i.v.) and killed by rapid exsanguination. The thoracic aortas were isolated 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 aorta was cut into rings (1.7–2 mm in diameter and 1.5–2 mm in length), and the endothelium of the preparations was removed by gently rubbing the intimal surface with a metal wire. Each aorta ring was stretched to

the resting tension of 1.6 g in 4 ml of warmed (37 °C) KHB solution, oxygenated with a 95% O_2 and 5% CO_2 gas mixture for isometric recording. The solution contained 3 μM propranolol to avoid β-adrenoceptor-mediated responses (Fujimoto and Itoh, 1997). After an equilibration period of 60–90 min, conditioning contractions were induced 3 times with 1–10 μM norepinephrine for 5–10 min at 40-min intervals. During one of the contractions, the presence or absence of endothelium was confirmed by the response to acetylcholine (1 μM).

2.2. Contractile responses to norepinephrine

Aorta rings were contracted with 1–100 μM norepinephrine to determine EC_{85} values (75–85% of the maximum contraction) for norepinephrine-induced contraction. Thereafter, the preparations were contracted twice with norepinephrine at the EC_{85} value for 25 min at 90-min intervals. The second contraction was made 15–30 min after and during treatment with hydrogen peroxide (10 and 100 μM), glyceryl trinitrate (30 and 300 μM) or 8-bromo cyclic GMP (30 and 100 nM). Isometric tension was recorded as a function of time on a strip chart recorder (Nihon Kohden Kogyo, Tokyo). The time course of force development during norepinephrine-induced contractions in the presence of these drugs was expressed as a percentage of the maximum norepinephrine response in the first contraction.

2.3. Relaxation responses to hydrogen peroxide

The tissue was contracted with norepinephrine at the EC_{85} value. After the contraction had reached steady state, cumulative concentration–relaxation response curves for hydrogen peroxide were obtained, with one curve per preparation. Similar experiments were done with preparations that had been treated with methylene blue (10 μM), 1H-[1,2,4]oxadiazolo[4,3,α]quinoxalin-1-one (ODQ, 10 μM) or zaprinast (5 μM) for 20–50 min. Methylene blue increased the basal tone of the preparation by 150–300 mg, the tension was re-adjusted. At the end of the experiment, papaverine (100 μM) was added to obtain the maximum relaxation. The relaxation response to hydrogen peroxide was expressed as a percentage of the papaverine-induced relaxation.

2.4. Radioimmunoassay of cyclic GMP

Radioimmunoassay techniques were used to determine the levels of cyclic GMP. Strips (4 mm width, 3–4 mm length) were immersed in control KHB solution containing isobutylmethylxanthine (0.3 mM) and propranolol (3 μM), and bubbled with a 95% O_2 and 5% CO_2 gas mixture and kept at 37 °C, pH 7.4. After 1 h, the strips were incubated for another 15 min in a KHB solution containing hydrogen peroxide (100 μM), isobutylmethylxanthine and propranolol. The strips were further incubated for 1 min after the addition of norepinephrine (10 μM). All strips were removed from the solution and quick-frozen in liquid nitrogen. In another series of experiments, aorta strips which had been suspended in the KHB buffer under the resting tension of 1.6*g* were contracted 4 times with 5 μM norepinephrine for 15–20 min at 60-min intervals. During the last contraction, hydrogen peroxide (100 μM) or sodium nitroprusside (1 μM) was further added to the KHB solution. Within 15 s after the tissue had been relaxed for 15–20 min with these drugs in the presence of isobutylmethylxanthine (30 μM), the tissue was frozen in liquid nitrogen.

The tissues were homogenized in 0.2 ml of ice-cold 6% trichloroacetic acid. The homogenate was centrifuged at 3000 rpm at 4 °C and the supernatant was extracted 3 times with 1 ml of water-saturated ethylether. The cyclic GMP level was determined with a radioimmunoassay kit (Yamasa Shoyu, Tokyo) and was expressed as pmol/mg protein. Protein assay was done with a *DC* Protein Assay Kit (Bio-Rad, California).

2.5. Measurement of MLC_{20} phosphorylation

MLC_{20} phosphorylation was measured using protocols similar to those used in our previous experiments with guinea-pig ileum (Mori and Tsushima, 2000). Briefly, strips of endothelium-denuded aorta (4 mm in width and 1 mm in length) were incubated for 1 and 20 min in the KHB solution containing norepinephrine (10 μM) with propranolol (3 μM). In some experiments, the strips were incubated for 1 min with norepinephrine 15 and 30 min after treatment with hydrogen peroxide (100 μM) and catalase (700 U/ml), respectively. The strips were then quick-frozen with 10% trichloroacetic acid in acetone-dry ice containing 10 mM dithiothreitol and allowed to reach room temperature. The strips were then washed with acetone for 5 min to remove residual trichloroacetic acid. Proteins, including MLC_{20}, were extracted in urea-glycerol-polyacrylamide gel electrophoresis (PAGE) sample buffer (8 M urea, 20 mM Tris, 23 mM glycine, 10 mM dithiothreitol, 0.004% bromophenol blue and saturated sucrose, pH 8.6) or sodium dodecyl sulfate-PAGE sample buffer for 60 min by sonication. Protein concentrations were determined using the Bradford-dye-binding assay (Bio-Rad). A total of 10 μM of protein was separated by urea-glycerol-PAGE or sodium dodecyl sulfate-PAGE followed by electrophoretic transfer of the protein to nitrocellulose membrane. The membrane was blocked with 5% skim milk in phosphate-buffered saline overnight at 4 °C, and incubated with anti MLC_{20} antibody or anti phosphorylated MLC_{20} antibody for 3 h at room temperature. After incubation with secondary antibody, blots of non-phosphorylated and phosphorylated MLC_{20} were detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Tokyo). The blots of enhanced chemiluminescence were quantitated with Lumi-Imager™ F1 (Roche

Diagnostics, Germany). The extent of MLC_{20} phosphorylation was expressed as a percentage of phosphorylated forms to total MLC_{20}.

2.6. Drugs and solutions

The following were used: acetylcholine chloride (Sigma, St. Louis, MO), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo cyclic GMP, Sigma), catalase (from bovine liver, Wako, Tokyo), DL-dithiothreitol (Sigma), hydrogen peroxide (30%, Mitsubishi Gas Chem., Tokyo), 3-isobutyl-1-methyl-xanthine (Sigma), methylene blue (Katayama Chem., Tokyo), (−)-norepinephrine bitartrate (Sigma), papaverine hydrochloride (Wako), DL-propranolol hydrochloride (Sigma), sodium nitroprusside (Wako) and trichloroacetic acid (Wako). Anti MLC_{20} antibody and anti phosphorylated MLC_{20} were purchased from Sigma, and anti mouse immunoglobulin M peroxidase-labelled antibody (Biosource International, California) was used as a secondary antibody. Glyceryl trinitrate (Nippon Kayaku, Tokyo) was an injectable preparation for clinical use. 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, Sigma) and zaprinast (Sigma) were dissolved in dimethyl sulfoxide (Sigma).

2.7. Statistical analysis

The results are reported as mean values ± S.E. of the number (n) of observations. The one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test was used. Statistically significant differences were accepted when $P<0.05$ with Student's t-test for two mean values and with the one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test for multiple means.

3. Results

3.1. Inhibitory effects of hydrogen peroxide, glyceryl trinitrate and 8-bromo cyclic GMP on norepinephrine-induced contraction

The aorta strips were contracted by norepinephrine at concentrations (EC_{85}) ranging from 1.6 to 10 μM (mean value: 5.2 ± 1.3 μM, $n=11$). The agonist produced an initial phasic, followed by a secondary tonic, contraction (Fig. 1). The maximum contraction obtained 10 min after the first application of norepinephrine was 571 ± 53 mg ($n=11$). Hydrogen peroxide (10 and 100 μM) did not significantly alter the basal levels of vascular smooth muscle tone. When hydrogen peroxide was added to the KHB solution 15 min before, and during the contraction with norepinephrine, the phasic and tonic contractions were suppressed in a concentration-dependent manner (Fig. 1). Catalase (700 U/ml), when added to the KHB solution 30 min before addition of hydrogen peroxide, abolished the inhibitory effect of hydrogen peroxide (data not shown).

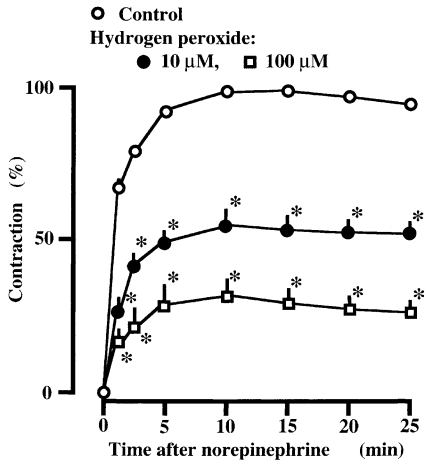

Fig. 1. Time course of changes in the contractile response to norepinephrine in the presence and absence of hydrogen peroxide. Norepinephrine (5.2 ± 1.3 μM) was added to the KHB solution 15 min after the tissue was exposed to hydrogen peroxide at 10 (●) and 100 μM (□). Control (○). Ordinate, norepinephrine-induced contraction is expressed as a percentage of the maximum norepinephrine response in the first contraction. Abscissa, time after addition of norepinephrine into the KHB buffer (min). Vertical bars represent S.E. of means ($n=5–6$). *Significantly different from control value at a given time ($P<0.05$).

Glyceryl trinitrate (30 and 300 nM) and 8-bromo cyclic GMP (30 and 100 μM) were added into the KHB buffer 30 min before, and during the contraction with norepinephrine. These drugs did not change the basal tone of aorta strips, but significantly reduced the ability of the preparation to contract in response to norepinephrine (Fig. 2).

3.2. Effect of hydrogen peroxide on tissue cyclic GMP concentrations

In the first series of experiments in which the aorta strips were not under tension, when the strips were incubated with norepinephrine at 10 μM for 1 min, tissue concentrations of cyclic GMP remained unchanged (Fig. 3A), and treatment of the tissue for 15 min with hydrogen peroxide (100 μM) resulted in a slight but significant increase in tissue cyclic GMP concentrations. In strips which had been treated with hydrogen peroxide for 15 min, further treatment for 1 min with norepinephrine in the continuous presence of hydrogen peroxide resulted in a significant increase in arterial levels of cyclic GMP. In the second series of experiments, the aorta strips, which had been suspended under the resting tension, were contracted with norepinephrine. Hydrogen peroxide (100 μM) and sodium nitroprusside (1 μM) relaxed the precontracted aorta strips to 84 ± 11% ($n=8$) and 100% ($n=5$), respectively, of the amplitude of the contraction produced with norepinephrine,

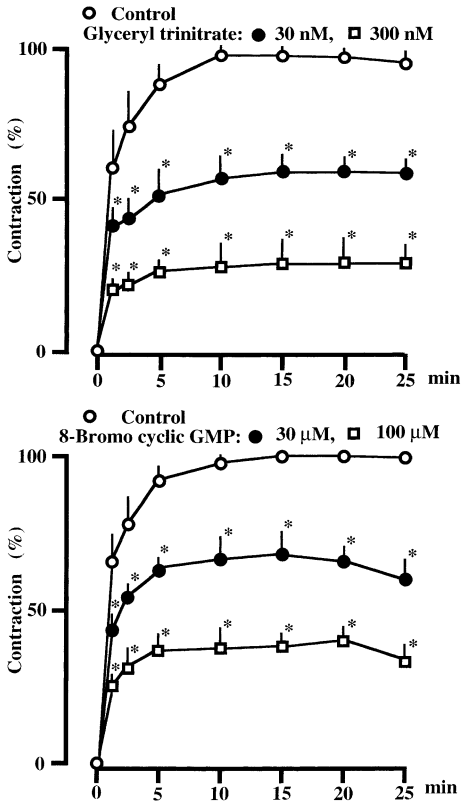

Fig. 2. Inhibition by glyceryl trinitrate and 8-bromo cyclic GMP of norepinephrine-induced contraction. Glyceryl trinitrate at 30 (●) and 300 nM (□), and 8-bromo cyclic GMP at 30 (●) and 100 μM (□) were added into the KHB buffer 30 min before, and during the contraction with norepinephrine. Control (○). Ordinate, responses to norepinephrine are expressed as percentages of the maximum norepinephrine response in the first contraction. Abscissa, time after norepinephrine (min). Vertical bars represent S.E. of means ($n=6$). *Significantly different from controls ($P<0.05$).

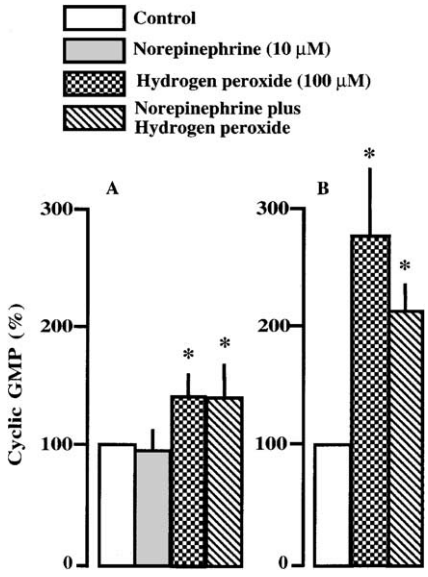

Fig. 3. Effects of hydrogen peroxide on aorta strip cyclic GMP concentrations in the presence and absence of norepinephrine. The preparations in panel B but not in panel A were stretched under the resting tension. Ordinate, cyclic GMP concentrations in control tissues are expressed as 100% (28.8 ± 5.8 pmol/mg protein, $n=4$ in panel A and 2.0 ± 0.6 pmol/mg protein, $n=6$ in panel B). Vertical bars represent S.E. of means ($n=4-8$). *Significantly different from control values ($P<0.05$).

and tissue cyclic GMP concentrations were increased by hydrogen peroxide (Fig. 3B) and sodium nitroprusside (58.6 ± 13.4 pmol/mg protein, $n=5$). Hydrogen peroxide for 15–20 min in the absence of norepinephrine did not alter basal vascular tone, but increased tissue cyclic GMP concentrations.

3.3. Effect of hydrogen peroxide on norepinephrine-induced MLC_{20} phosphorylation

Phosphorylation of MLC_{20} was determined in aorta strips, using Western blotting with anti MLC_{20} antibody (Figs. 4 and 5A) and anti phosphorylated MLC_{20} antibody (Fig. 5B and C). MLC_{20} phosphorylation was significantly increased 1 min after application of norepinephrine (10 μM) to the aorta strips ($5.9 \pm 1.0\%$, $n=7$ vs. $35.9 \pm 4.9\%$, $n=12$; Fig. 4A, lanes 4–6 in Fig. 5A and B, lanes 2–4 in Fig. 5C). Hydrogen peroxide (100 μM) for 15 min had no effect on MLC_{20} phosphorylation (lane 5 in Fig. 5C), but attenuated the increase by norepinephrine in MLC_{20} phosphorylation (Fig. 4A, lanes 6–8 in Fig. 5C). On the other hand, when the tissue was incubated with norepinephrine for 20 min, MLC_{20} phosphorylation was increased but to a lesser extent than in the tissue incubated with norepinephrine for 1 min ($3.7 \pm 1.7\%$, $n=5$ vs. $13.9 \pm 1.6\%$, $n=12$; Fig. 4B, lanes 7–9 in Fig. 5A and B), although force development was apparently greater at 20 min than at 1 min (Fig. 1). The

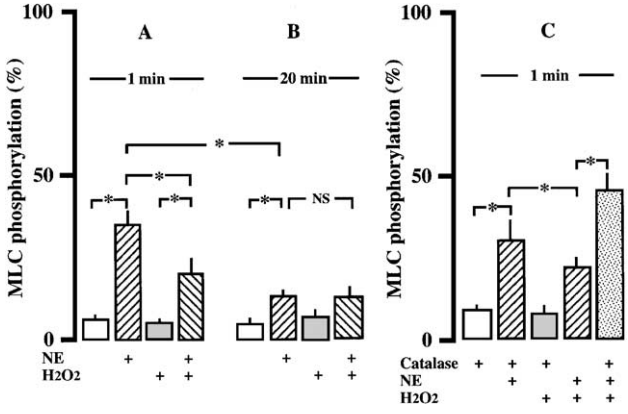

Fig. 4. Effects of hydrogen peroxide on norepinephrine-induced MLC_{20} phosphorylation in aortic smooth muscle. MLC_{20} phosphorylation was determined 1 (A and C) and 20 min (B) after application of norepinephrine (10 μM). Hydrogen peroxide (100 μM) and catalase (700 U/ml) were added to KHB buffer 15 and 30 min before, and during administration of norepinephrine, respectively. Ordinate, MLC_{20} phosphorylation is expressed as a percentage of phosphorylated forms to total MLC_{20}. Vertical bars represent S.E. of means ($n=4-8$). *Significant difference between both values ($P<0.05$). NS: not significantly different.

norepinephrine-induced increase in MLC_{20} phosphorylation was not attenuated by hydrogen peroxide, and hydrogen peroxide alone for 35 min did not alter MLC_{20} phosphorylation (Fig. 4B). When the aorta strips were treated with catalase (700 U/ml) for 30 min, MLC_{20} phosphorylation remained unchanged (Fig. 4C). Norepinephrine (10 μM) for 1 min in the presence of catalase increased MLC_{20} phosphorylation as did norepinephrine in the absence of catalase (Fig. 4C vs. A). The inhibitory effect of hydrogen peroxide on the norepinephrine-induced increase in MLC_{20} phosphorylation was abolished by catalase. Hydrogen peroxide did not alter MLC_{20} phosphorylation in the presence of catalase.

3.4. Relaxant effect of hydrogen peroxide

The aorta strips were contracted with a submaximal concentration of norepinephrine to give an 85–75% maximal response. Hydrogen peroxide (0.3–100 μM) produced concentration-dependent relaxation with EC_{50} values of 5.85 ± 0.21 μM, $n = 6$ (Fig. 6). The concentration–response curves for hydrogen peroxide were partly shifted to the right and upwards by methylene blue (10 μM) and ODQ (10 μM), and to the left by zaprinast (5 μM).

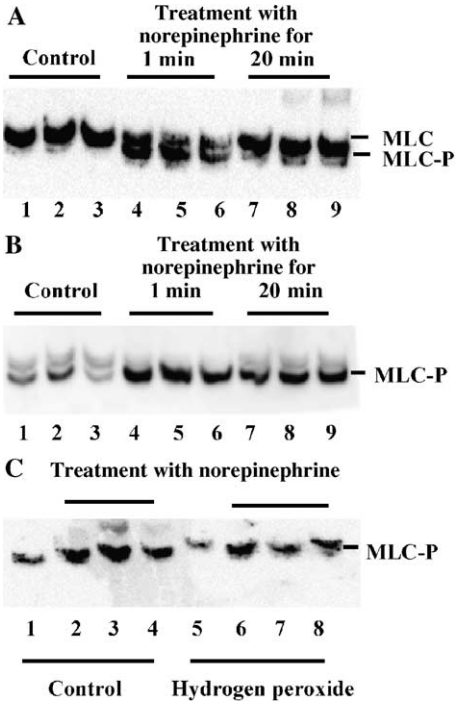

Fig. 5. Effects of hydrogen peroxide on norepinephrine-induced phosphorylation of MLC_{20}. (A) shows representative immunoblotting of MLC (nonphosphorylated MLC_{20}) and MLC-P (phosphorylated MLC_{20}) using anti MLC_{20} antibody. (B) and (C) show immunoblotting of MLC-P with anti-phosphorylated MLC_{20} antibody. In (A) and (B), lanes 1–3, controls; lanes 4–6 and lanes 7–9, norepinephrine (10 μM) for 1 and 20 min, respectively. In (C), the strips were pretreated with vehicle (lanes 1–4) and 100 μM hydrogen peroxide for 15 min (lanes 5–8) and then further incubated for 1 min with 10 μM norepinephrine in the presence (lanes 6–8) and absence of hydrogen peroxide (lanes 2–4).

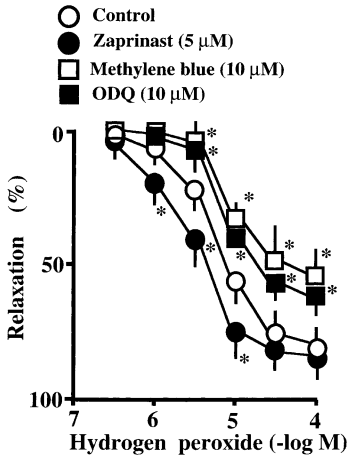

Fig. 6. Effects of drugs affecting cyclic GMP production and degradation on hydrogen peroxide-induced relaxation. The relaxant response to hydrogen peroxide was determined in aorta strips which had been treated with 5 μM zaprinast (●), 10 μM methylene blue (□) or 10 μM ODQ (■) for 20–50 min and then contracted with norepinephrine. Control (○). Papaverine (100 μM)-induced relaxation is expressed as 100% (ordinate). Vertical bars represent S.E. of means ($n = 5–7$). *Significantly different from appropriate controls ($P < 0.05$).

4. Discussion

The findings in the present study on guinea-pig aorta are as follows: (1) hydrogen peroxide inhibited norepinephrine-induced phasic and sustained contractions, (2) MLC_{20} phosphorylation was increased by exposing aorta strips to norepinephrine for 1 min and to a lesser extent for 20 min, (3) the former effect but not the latter was inhibited by hydrogen peroxide, (4) in norepinephrine-contracted aorta, hydrogen peroxide elicited a relaxation, and increased tissue concentrations of cyclic GMP significantly but less markedly than did sodium nitroprusside, and (5) the relaxant effect of hydrogen peroxide was changed by drugs affecting cyclic GMP production or degradation.

The relaxation response to hydrogen peroxide was dependent, partly dependent or independent of the presence of endothelial cells (see Introduction). Briefly, it was suggested that hydrogen peroxide relaxed blood vessels via both an enhanced release of endothelial nitric oxide resulting in an accumulation of cyclic GMP in smooth muscle (Zembowicz et al., 1993; Yang et al., 1998a, 1999a), and endothelial production of cytochrome *P*450 metabolites in rat aorta (Yang et al., 1999a). In addition, the relaxation was associated with release of acetylcholine in dog basilar artery (Yang et al., 1998a). We also found that hydrogen peroxide elicited relaxation and increased tissue concentrations of cyclic GMP in endothelium-denuded, norepinephrine-contracted aorta. Hydrogen peroxide alone also increased tissue cyclic GMP concentrations (Fig. 3B). The relaxation was

partly inhibited by methylene blue and ODQ (soluble guanylate cyclase inhibitors), and potentiated by zaprinast (an inhibitor of cyclic GMP-selective phosphodiesterase), supporting the previous findings with rabbit mesenteric artery (Fujimoto et al., 2001). In aorta which was not stretched at the resting tension, hydrogen peroxide increased the tissue concentration of cyclic GMP and also increased it 1 min after treatment with norepinephrine in the continuous presence of hydrogen peroxide (Fig. 3A). 8-Bromo cyclic GMP and glyceryl trinitrate (which relaxed vascular smooth muscle via cyclic GMP) as well as hydrogen peroxide attenuated the contractile response to norepinephrine. The present and previous results suggest that hydrogen peroxide acts directly on vascular smooth muscle to cause cyclic GMP-mediated relaxation (Burke and Wolin, 1987; Burke-Wolin et al., 1991; Zembowicz et al., 1993; Iesaki et al., 1994; Hayabuchi et al., 1998; Fujimoto et al., 2001), although others found that the drug dilated cat and dog cerebral arterioles without involving cyclic GMP (Wei et al., 1996; Iesaki et al., 1999; Iida and Katusic, 2000). We are of the opinion that the relaxant effect of hydrogen peroxide cannot be solely explained by an accumulation of cyclic GMP in aortic smooth muscles, since the hydrogen peroxide-induced increase in the tissue concentration of cyclic GMP was markedly less than that by sodium nitroprusside (2–3 vs. 30 times). The basal cyclic GMP concentration in the present study was close to the result usually found in endothelium-denuded aorta (Delpy et al., 1996).

It is generally accepted that the agonist-induced contraction is due both to Ca^{2+} influx from extracellular space, Ca^{2+} released from internal stores, and to an increase in Ca^{2+} sensitivity of contractile proteins. We previously found that, unlike verapamil, hydrogen peroxide inhibited the norepinephrine-induced, short-lived contraction of rabbit mesenteric artery in a Ca^{2+}-free solution, suggesting that the contraction initiated by Ca^{2+} released from its storage sites is sensitive to hydrogen peroxide (Fujimoto et al., 2001).

Norepinephrine increased MLC_{20} phosphorylation at 1 min after application of the agonist. Hydrogen peroxide failed to change MLC_{20} phosphorylation, but reduced the increase in MLC_{20} phosphorylation induced by norepinephrine, and the effect of hydrogen peroxide was abolished by catalase, which catalyzed the conversion of hydrogen peroxide to oxygen and water. Thus, the inhibitory effect of hydrogen peroxide on norepinephrine-induced phasic contraction is at least in part explained by decreased phosphorylation of MLC_{20}. Similarly, hydrogen peroxide reduced the acetylcholine-induced contraction and phosphorylation of regulatory myosin light chain in airway smooth muscle (Lorenz et al., 1999).

It has been shown that vascular contraction in response to some agonists is maintained despite decreases in MLC_{20} phosphorylation and in intracellular Ca^{2+}, suggesting an increase in Ca^{2+} sensitivity of contractile proteins (Himpens et al., 1990). Furthermore, it seemed that myosin light chain kinase could stimulate the adenosine triphosphatase activity of smooth muscle myosin without phosphorylating MLC_{20} (Kishi et al., 2000). In spite of its marked relaxant effect on vascular and tracheal smooth muscles, hydrogen peroxide produced a rapid (transient), followed by a sustained, increase in intracellular Ca^{2+} concentration (Krippeit-Drews et al., 1995; Iesaki et al., 1996; Lorenz et al., 1999). It was reported that hydrogen peroxide and cyclic GMP attenuated Ca^{2+} sensitivity of contractile proteins in rabbit aorta, rat mesenteric artery, ovine cranial artery and guinea-pig portal vein (Iesaki et al., 1996; Kawada et al., 1997; Sauzeau et al., 2000; Nauli et al., 2001). The vasorelaxation in response to hydrogen peroxide and 8-bromo cyclic GMP was more marked in agonist-stimulated arteries than in arteries depolarized by high-K^+ solution (Burke and Wolin, 1987; Iesaki et al., 1996; Fujimoto et al., 2001). High K^+-induced contraction is produced by increased concentrations of intracellular Ca^{2+} without a significant increase in Ca^{2+} sensitivity. Although we did not directly evaluate the effect of cyclic GMP on Ca^{2+} sensitization, it is possible that the relaxant effect of hydrogen peroxide is accounted for by cyclic GMP-mediated inhibition of agonist-induced Ca^{2+} sensitization.

We found that norepinephrine increased MLC_{20} phosphorylation less at 20 min than at 1 min (Fig. 4) in spite of the greater amplitude of contraction at 20 min than at 1 min after norepinephrine and that the norepinephrine-induced contraction and MLC_{20} phosphorylation was reduced and was not reduced by hydrogen peroxide, respectively. The MLC_{20} phosphorylation-independent mechanisms may involve phosphorylation (or dephosphorylation) of certain proteins including caldesmon and calponin (Walsh et al., 1994; Throckmorton et al., 1998). Rembold et al. (2000) suggested that cyclic GMP-mediated phosphorylation of heat shock proteins regulates force development (vasorelaxation) independent of MLC_{20} phosphorylation. It will be of interest to determine if the phosphorylation of these proteins can be modulated by norepinephrine, hydrogen peroxide or both, in guinea-pig aorta.

It has been shown that hydrogen peroxide is produced in human artery and rat aorta smooth muscles (Berry et al., 2000; Torrecillas et al., 2000). Although cat cerebral arterioles did respond by relaxation to hydrogen peroxide at a concentration as low as 10 nM (Wei et al., 1996), the minimum concentrations (about 1 μM) of hydrogen peroxide required for relaxation in the guinea-pig aorta (present study) were close to those found for rabbit mesenteric small artery and canine cerebral artery (Yang et al., 1998a,b; Fujimoto et al., 2001). The endothelium may act as a physical and biochemical barrier to hydrogen peroxide via direct inactivation of hydrogen peroxide by catalase (Walia et al., 2000). Consequently, the concentrations of hydrogen peroxide used in the present experiment may be similar to its concentrations acting on smooth muscle cells, unless catalase is present extracellularly. Since human leukocytes at their normal plasma concentration can generate 80–480 μM hydrogen peroxide/h (Liu and Zweier, 2001), the

present results suggest that hydrogen peroxide is physiologically or pathophysiologically important as a vasodilator.

In conclusion, increases in force development and MLC_{20} phosphorylation occur after 1-min exposure to norepinephrine, and both are reduced by hydrogen peroxide. When the aorta strips are stimulated for 20 min by norepinephrine, the increase in force development is greater than that after 1-min stimulation and the increase in MLC_{20} phosphorylation is less than that after 1 min. At that time, hydrogen peroxide reduces force development without affecting MLC_{20} phosphorylation. It is suggested that the relaxant effect of hydrogen peroxide is in part mediated by cyclic GMP.

References

Barlow, R.S., White, R.E., 1998. Hydrogen peroxide relaxes porcine coronary arteries by stimulating BK_{Ca} channel activity. Am. J. Physiol. 275, H1283–H1289.

Barlow, R.S., El-Mowafy, A.M., White, R.E., 2000. H_2O_2 opens BK_{Ca} channels via the PLA_2-arachidonic acid signaling cascade in coronary artery smooth muscle. Am. J. Physiol. 279, H475–H483.

Bass, A.S., Berk, B.C., 1995. Differential activation of mitogen-activated protein kinases by H_2O_2 and O_2^- in vascular smooth muscle cells. Circ. Res. 77, 29–36.

Bény, J.-L., Von der Weid, P.-Y., 1991. Hydrogen peroxide: an endogenous smooth muscle cell hyperpolarizing factor. Biochem. Biophys. Res. Commun. 176, 378–384.

Berry, C., Hamilton, C.A., Brosnan, M.J., Magill, F.G., Berg, G.A., McMurray, J.J.V., Dominiczak, A.F., 2000. Investigation into the sources of superoxide in human blood vessels. Angiotensin II increases superoxide production in human internal mammary arteries. Circulation 101, 2206–2212.

Burke, T.M., Wolin, M.S., 1987. Hydrogen peroxide elicites pulmonary arterial relaxation and guanylate cyclase activation. Am. J. Physiol. 252, H721–H732.

Burke-Wolin, T., Abate, C.J., Wolin, M.S., Gurtner, G.H., 1991. Hydrogen peroxide-induced pulmonary vasodilation: role of guanosine 3′,5′-cyclic monophosphate. Am. J. Physiol. 261, L393–L398.

Delpy, E., Coste, H., de Gouville, A.-C.M., 1996. Effects of cyclic GMP elevation on isoprenaline-induced increase in cyclic AMP and relaxation in rat aortic smooth muscle: role of phosphodiesterase 3. Br. J. Pharmacol. 119, 471–478.

Fraile, M.L., Conge, M.V., Sanz, L., Moreno, M.J., Marco, E.J., Lopez De Pablo, A.L., 1994. Different influence of superoxide anions and hydrogen peroxide on endothelial function of isolated cat cerebral and pulmonary arteries. Gen. Pharmacol. 25, 1197–1205.

Fujimoto, S., Itoh, T., 1997. Role of nitric oxide and nitric oxide-independent relaxing factor in contraction and relaxation of rabbit blood vessels. Eur. J. Pharmacol. 330, 177–184.

Fujimoto, S., Asano, T., Sakai, M., Sakurai, K., Takagi, D., Yoshimoto, N., Itoh, T., 2001. Mechanisms of hydrogen peroxide-induced relaxation in rabbit mesenteric small artery. Eur. J. Pharmacol. 412, 291–300.

Guyton, K.Z., Liu, Y., Gorospe, M., Xu, Q., Holbrook, N.J., 1996. Activation of mitogen-activated protein kinase by H_2O_2. J. Biol. Chem. 271, 4138–4142.

Hayabuchi, Y., Nakaya, Y., Matsuoka, S., Kuroda, Y., 1998. Hydrogen peroxide-induced vascular relaxation in porcine coronary arteries is mediated by Ca^{2+}-activated K^+ channels. Heart Vessels 13, 9–17.

Hearse, D.J., Maxwell, L., Saldanha, C., Gavin, J.B., 1993. The myocardial vasculature during ischemia and reperfusion: a target for injury and protection. J. Mol. Cell. Cardiol. 25, 759–800.

Himpens, B., Kitazawa, T., Somlyo, A.P., 1990. Agonist-dependent modulation of Ca^{2+} sensitivity in rabbit pulmonary artery smooth muscle. Pflügers Arch. 417, 21–28.

Hirai, T., Tsuru, H., Tanimitsu, N., Takumida, M., Watanabe, H., Yajin, K., Sasa, M., 2000. Effect of hydrogen peroxide in guinea pig mucosa vasculature. Jpn. J. Pharmacol. 84, 470–473.

Iesaki, T., Okada, T., Yamaguchi, H., Ochi, R., 1994. Inhibition of vasoactive amine induced contractions of vascular smooth muscle by hydrogen peroxide in rabbit aorta. Cardiovasc. Res. 28, 963–968.

Iesaki, T., Okada, T., Shimada, I., Yamaguchi, H., Ochi, R., 1996. Decrease in Ca^{2+} sensitivity as a mechanism of hydrogen peroxide-induced relaxation of rabbit aorta. Cardiovasc. Res. 31, 820–825.

Iesaki, T., Gupte, S.A., Kaminski, P.M., Wolin, M.S., 1999. Inhibition of guanylate cyclase stimulation by NO and bovine arterial relaxation to peroxynitrite and H_2O_2. Am. J. Physiol. 277, H978–H985.

Iida, Y., Katusic, Z.S., 2000. Mechanisms of cerebral arterial relaxations to hydrogen peroxide. Stroke 31, 2224–2230.

Jiang, M.Y., Morgan, K.G., 1987. Intracellular calcium levels in phorbol ester-induced contractions of vascular smooth muscle. Am. J. Physiol. 253, H1365–H1371.

Jin, N., Rhoades, R.A., 1997. Activation of tyrosine kinases in H_2O_2-induced contraction in pulmonary artery. Am. J. Physiol. 272, H2686–H2692.

Jin, N., Packer, C.S., Rhoades, R.A., 1991. Reactive oxygen-mediated contraction in pulmonary arterial smooth muscle: cellular mechanisms. Can. J. Physiol. Pharm. 69, 383–388.

Kawada, T., Toyosato, A., Islam, M.O., Yoshida, T., Imai, S., 1997. cGMP-kinase mediated cGMP- and cAMP-induced Ca^{2+} desensitization of skinned rat artery. Eur. J. Pharmacol. 232, 75–82.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Iwamatsu, A., Kaibuchi, K., 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science (Wash. D.C.) 273, 245–248.

Kishi, H., Mikawa, T., Seto, M., Sasaki, Y., Kanayasu-Toyoda, T., Yamaguchi, T., Inamura, M., Ito, M., Karaki, H., Bao, J., Nakamura, A., Ishikawa, R., Kohama, K., 2000. Stable transfectants of smooth muscle cell line lacking the expression of myosin light chain kinase and their characterization with respect to the actomyosin system. J. Biol. Chem. 275, 1414–1420.

Kitazawa, T., Masuo, M., Somlyo, A.P., 1991. G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. Proc. Natl. Acad. Sci. U. S. A. 88, 9307–9310.

Krippeit-Drews, P., Haberland, C., Fingerle, J., Drews, G., Lang, F., 1995. Effects of H_2O_2 on membrane potential and $[Ca^{2+}]i$ of cultured rat arterial smooth muscle cells. Biochem. Biophys. Res. Commun. 209, 139–145.

Lacy, F., O'Connor, D.T., Schmid-Schoenbein, G.W., 1998. Plasma hydrogen peroxide production in hypertensive and normotensive subjects at genetic risk of hypertension. J. Hypertens. 16, 291–303.

Liu, X., Zweier, J.L., 2001. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: evaluation in human polymorphonuclear leukocytes. Free Radic. Biol. Med. 31, 894–901.

Lorenz, R.R., Warner, D.O., Jones, K.A., 1999. Hydrogen peroxide decreases Ca^{2+} sensitivity in airway smooth muscle by inhibiting rMLC phosphorylation. Am. J. Physiol. 277, L816–L822.

Matoba, T., Shimokawa, H., Nakashima, M., Hirakawa, Y., Mukai, Y., Hirano, K., Kanaide, H., Takeshita, A., 2000. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. J. Clin. Invest. 106, 1521–1530.

Mori, M., Tsushima, H., 2000. Activation of Rho signaling contributes to lysophosphatidic acid-induced contraction of intact ileal smooth muscle of guinea-pig. Can. J. Physiol. Pharm. 78, 729–736.

Nauli, S.M., Zhang, L., Pearce, W.J., 2001. Maturation depresses cGMP-mediated decreases in $[Ca^{2+}]i$ and Ca^{2+} sensitivity in ovine cranial arteries. Am. J. Physiol. 280, H1019–1028.

Okatani, Y., Watanabe, K., Sagara, Y., 1997. Effect of nitric oxide, prostacyclin, and thromboxane on the vasospastic action of hydrogen peroxide on human umbilical artery. Acta Obstet. Gynecol. Scand. 76, 515–520.

Pelaez, N.J., Braun, T.R., Paul, R.J., Meiss, R.A., Packer, C.S., 2000a.

H_2O_2 mediates Ca^{2+}- and MLC_{20} phosphorylation-independent contraction in intact and permeabilized vascular muscle. Am. J. Physiol. 279, H1185–H1193.

Pelaez, N.J., Osterhaus, S.L., Mak, A.S., Zhao, Y., Davis, H.W., Packer, C.S., 2000b. MAPK and PKC activity are not required for H_2O_2-induced arterial muscle contraction. Am. J. Physiol. 279, H1194–H1200.

Raimondi, L., Banchelli, G., Sgromo, L., Pirisino, R., Ner, M., Parini, A., Cambon, C., 2000. Hydrogen peroxide generation by monoamine oxidase in rat white adipocytes: role on cAMP production. Eur. J. Pharmacol. 395, 177–182.

Rao, G.N., Runge, M.S., Alexander, R.W., 1995. Hydrogen peroxide activation of cytosolic phospholipase A_2 in vascular smooth muscle cells. Biochim. Biophys. Acta 1265, 67–72.

Rembold, C.M., Foster, D.B., Strauss, J.D., Wingard, C.J., Van Eyk, J.E., 2000. cGMP-mediated phosphorylation of heat shock protein 20 may cause smooth muscle relaxation without myosin light chain dephosphorylation in swine carotid artery. J. Physiol. 524, 865–878.

Rhoades, R.A., Packer, C.S., Roepke, D.A., Jin, N., Meiss, R.A., 1990. Reactive oxygen species alter contractile properties of pulmonary arterial smooth muscle. Can. J. Physiol. Pharm. 68, 1581–1589.

Rodríguez-Martînez, M.A., García-Cohen, E.C., Baena, A.B., González, R., Salaíces, M., Marín, J., 1998. Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanism involved. Br. J. Pharmacol. 125, 1329–1335.

Ross, R., 1993. The pathogenesis of atherosclerosis: a perspectove for the 1990's. Nature 362, 801–809.

Rubanyi, G.M., Vanhoutte, P.M., 1986. Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle. Am. J. Physiol. 250, H815–H821.

Sauzeau, V., LeJeune, H., Cario-Toumaniantz, C., Smolenski, A., Lohmann, S.M., Bertoglio, J., Chardin, P., Pacaud, P., Loirand, G., 2000. Cyclic GMP-dependent protein kinase signaling pathway inhibits Rho A-induced Ca^{2+} sensitization of contraction in vascular smooth muscle. J. Biol. Chem. 275, 21722–21729.

Sheehan, D.W., Giese, E.C., Gugino, S.F., Russell, J.A., 1993. Characterization and mechanisms of H_2O_2-induced contractions of pulmonary arteries. Am. J. Physiol. 264, H1542–H1547.

Sobey, C.G., Heistad, D.D., Faraci, F.M., 1997. Mechnisms of bradykinin-induced cerebral vasodilatation in rats. Evidence that reactive oxygen species activate K^+ channels. Stroke 28, 2290–2295.

Somlyo, A.P., Somlyo, A.V., 1994. Signal transduction and regulation in smooth muscle. Nature 372, 231–236.

Throckmorton, D.C., Packer, C.S., Brophy, C.M., 1998. Protein kinase C activation during Ca^{2+}-independent vascular smooth muscle contraction. J. Surg. Res. 78, 48–53.

Torrecillas, G., Boyano-Adánez, M.D.C., Medina, J., Parra, T., Griera, M., Lopez-Ongil, S., Arilla, E., Rodríguez-Puyol, M., Rodríguez-Puyol, D., 2000. The role of hydrogen peroxide in the contractile responses to angiotensin II. Mol. Pharmacol. 59, 104–112.

Vanhoutte, P.M., 2001. Endothelium-derived free radicals: for worse and for better. J. Clin. Invest. 107, 23–25.

Walia, M., Sormaz, L., Samson, S., Lee, R.M.K.W., Grover, A.K., 2000. Effects of hydrogen peroxide on pig coronary artery endothelium. Eur. J. Pharmacol. 400, 249–253.

Walsh, M.P., Andrea, J.E., Allen, B.G., Clément-Chomienne, O., Collins, E.M., Morgan, K.G., 1994. Smooth muscle protein kinase C. Can. J. Physiol. Pharm. 72, 1392–1399.

Wei, E.P., Kontos, H.A., Beckman, J.S., 1996. Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. Am. J. Physiol. 271, H1262–H1266.

Yang, Z.-W., Zhang, A., Altura, B.T., Altura, B.M., 1998a. Endothelium-dependent relaxation to hydrogen peroxide in canine basilar artery: a potential new cerebral dilator mechanism. Brain Res. Bull. 47, 257–263.

Yang, Z.-W., Zheng, T., Zhang, A., Altura, B.T., Altura, B.M., 1998b. Mechanisms of hydrogen peroxide-induced contraction of rat aorta. Eur. J. Pharmacol. 344, 169–181.

Yang, Z.-W., Zhang, A., Altura, B.T., Altura, B.M., 1999a. Hydrogen peroxide-induced endothelium-dependent relaxation of rat aorta. Involvement of Ca^{2+} and other cellular metabolites. Gen. Pharmacol. 33, 325–336.

Yang, Z.-W., Zheng, T., Wang, J., Zhang, A., Altura, B.T., Altura, B.M., 1999b. Hydrogen peroxide induces contraction and raises $[Ca^{2+}]i$ in canine cerebral arterial smooth muscle: participation of cellular signaling pathways. Naunyn-Schmeideberg's Arch. Pharmacol. 360, 646–653.

Zembowicz, A., Hatchett, R.J., Jakubowski, A.M., Gryglewski, R.J., 1993. Involvement of nitric oxide in the endothelium-dependent relaxation induced by hydrogen peroxide in the rabbit aorta. Br. J. Pharmacol. 110, 151–158.