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Mechanisms of hydrogen peroxide-induced contraction of rat aorta

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Abstract

It has been suggested that reactive oxygen species may be involved in the regulation of vascular tone. However, the underlying mechanisms remain to be elucidated. The present studies were designed to investigate the contractile effects of hydrogen peroxide (H_2O_2), one of the reactive oxygen species, on isolated ring segments of rat aorta with and without endothelium. H_2O_2 induced an endothelium-independent contraction in isolated rat aorta ring segments in a concentration-dependent manner at concentrations from 5×10^{-6} to 5×10^{-3} M. H_2O_2-induced contractions of denuded rat aorta rings were stronger than those on intact rat aorta segments. The contractile effects of H_2O_2 were inhibited completely by 1200 u/ml catalase. The presence of 1.0 μM Fe^{2+} or 10 μM proadifen, a cytochrome P450 monooxygenase inhibitor, potentiated the contractile effect of H_2O_2 on isolated rat aorta segments. 1 mM deferoxamine (a Fe^{2+} chelator) or 100 μM dimethyl sulfoxide (a hydroxyl radical scavenger) significantly attenuated the vessel contractions induced by hydrogen peroxide plus Fe^{2+} or hydrogen peroxide itself. Removal of extracellular Ca^{2+} ($[Ca^{2+}]_0$), addition of 5 μM verapamil, administration of a protein kinase C inhibitor (staurosporine), treatment with an inhibitor of protein tyrosine phosphorylation (genistein) or employment of 5.0 μM indomethacin resulted in a significant attenuation of the contractile responses of the vessels to H_2O_2. Pharmacological antagonists (e.g. a muscarinic acetylcholine receptor antagonist (atropine), an antagonist of histamine H_1 receptors (diphenhydramine), an antagonist of histamine H_2 receptors (cimetidine), an α-adrenoceptor antagonist (phentolamine), a β-adrenoceptor antagonist (propranolol) and an antagonist of serotonin receptor (methysergide)) did not inhibit or attenuate the contractions induced by H_2O_2. Exposure of primary aortic smooth muscle cells to H_2O_2 (5×10^{-6} to 5×10^{-3} M) produced significant rises of intracellular Ca^{2+} ($[Ca^{2+}]_i$) within 20 s. Employment of 1.0 μM Fe^{2+} markedly enhanced the increment in $[Ca^{2+}]_i$ in the smooth muscle cells. 10 μM proadifen treatment failed to alter the hydrogen peroxide-induced increment in $[Ca^{2+}]_i$ of the smooth muscle cells. However, the presence of 5 μM indomethacin significantly attenuated the rise in $[Ca^{2+}]_i$ in smooth muscle cells. The present results suggest that H_2O_2 can induce contractions of rat aorta segments, at pathophysiological concentrations, which are Ca^{2+}-dependent. Hydroxyl radicals ($^{\cdot}OH$), cyclooxygenase products, protein kinase C and products of protein tyrosine phosphorylation appear to play some role in hydrogen peroxide-induced contractions. Metabolites catalyzed by cytochrome P450-dependent enzymes (upon treatment with hydrogen peroxide) appear to exert a vasodilator effect on rat aorta segments. Lastly, some unidentified mediators, produced by a cytochrome P450 inhibitor (proadifen), during hydrogen peroxide treatment, appear to play some role in contraction of vascular smooth muscle of rat aorta segments in vitro.

Keywords: Hydrogen peroxide (H_2O_2); Ca^{2+}; Prostaglandin; Aorta, rat; Protein kinase C; Cytochrome P450 product

1. Introduction

It is accepted widely that intracellular calcium ions (Ca^{2+}) have an important function in modulating the contractility of all kinds of muscle, including vascular smooth muscle cells. Activation of Ca^{2+}-mobilizing receptors has been shown to stimulate phosphatidylinositol diphosphate breakdown, catalyzed by phospholipase C. This reaction results in an increased formation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which has been suggested to contribute to vascular smooth muscle contraction by releasing Ca^{2+} from the sarcoplasmic reticulum (Berridge, 1989), stimulating Ca^{2+} entry (Litten et al., 1987) and sensitizing the contractile apparatus by activating protein kinase C (Rasmussen et al., 1987; Karaki, 1989; Ruzycky and Morgan, 1989).

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Reactive oxygen species have been shown to elevate systemic and pulmonary vascular resistance (Rhoades et al., 1988; Prasad et al., 1989, 1990). Hydrogen peroxide, one of the reactive oxygen species, being the two-electron reduction product of oxygen, is an important byproduct of oxidative metabolism. It can be formed by the vascular endothelium (Panus et al., 1993) and smooth muscle cells (Gutteridge, 1995). H_2O_2 generated by glucose and glucose oxidase or H_2O_2 itself has been demonstrated to produce contraction in isolated rabbit aorta (Mehta et al., 1986), canine coronary (Rubanyi and Vanhoutte, 1986), rat pulmonary artery (Rhoades et al., 1990) and bovine pulmonary artery (Wolin et al., 1985). In addition, H_2O_2 has been shown to induce concentration-dependent increases in contraction in the above-mentioned vessels (Rhoades et al., 1988, 1990). It has been reported that H_2O_2 can stimulate the metabolism of arachidonic acid via the cyclooxygenase pathway in canine basilar artery (Katusic et al., 1993), rabbit pulmonary artery (Robert et al., 1984) and human placental arteries and veins (Omar et al., 1992).

These studies provide evidence that H_2O_2 is vasoactive on a variety of arteries. It is, thus, possible that H_2O_2-induced contractions may be mediated through a second messenger system. However, the ability of H_2O_2 to alter vascular tone may be much more complex and the underlying mechanisms of H_2O_2's effect remain to be elucidated. The present work was undertaken to investigate the contractile effect of H_2O_2 on isolated ring segments of rat aorta with and without endothelium and to obtain insight into the relationship between tone of the vessel, H_2O_2 and mediators generated by hydrogen peroxide.

2. Materials and methods

2.1. General procedures

Male adult Wistar rats (350–450 g) were sacrificed by stunning and subsequent decapitation. The thoracic aortae were removed carefully, so as to protect the endothelial lining and placed in normal Krebs–Ringer bicarbonate solution containing (in mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; dextrose, 10 and $NaHCO_3$, 25 (Altura and Altura, 1974; Zhang et al., 1992a). The rings of rat thoracic aorta were immersed in cold normal Krebs–Ringer bicarbonate solution. They were cleaned of adhering fat and loose connective tissue, with special care taken not to touch the luminal surface. In some preparations, the endothelium was removed by gently rubbing against the teeth of a pair of forceps (Zhang et al., 1992a). These segments were mounted on stainless-steel pins under 2 g resting tension in an organ bath, attached to force transducers (Grass Model FT 03) and connected to Grass Model 7 polygraphs. The organ bath containing normal Krebs–Ringer bicarbonate solution was gassed continuously with 95% O_2 and 5% CO_2 and warmed to 37°C (pH 7.4). Tissues were allowed to equilibrate for at least 90 min before data collection. Incubation media were routinely changed every 15 min as a precaution against interfering metabolites (Altura and Altura, 1970). Stimulation of rings with 80 mM KCl was repeated every 30–45 min 2–3 times until responses were stable. The successful removal of endothelium was assessed by performing successive dose–response curves to acetylcholine (10^{-7}–10^{-4} M) and showing that acetylcholine failed to relax segments precontracted by 1.0 μM phenylephrine, while acetylcholine did relax the endothelium-intact segments (Zhang et al., 1992b; Zheng et al., 1994).

2.2. Extracellular Ca^{2+} and intracellular Ca^{2+} experiments

For the extracellular Ca^{2+}-free experiments, the rat aortic ring segments were equilibrated in Ca^{2+}-free normal Krebs–Ringer bicarbonate solution containing 0.2 mM EGTA for at least 90 min before initiation of the experiments. For intracellular Ca^{2+}-buffered experiments, the acetyl methyl ester of bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) (a membrane permeable Ca^{2+} chelator) was added to the bath medium, at a final bath concentration of 10 μM BAPTA-AM. After obtaining stable conditions of the ring segments, experiments were begun (Whitney et al., 1995). Responses to H_2O_2 and other drugs were expressed as a percentage of the stable level of contraction induced by 80 mM KCl.

2.3. Intracellular Ca^{2+} measurement

Intracellular Ca^{2+} in smooth muscle cells from rat aorta ($[Ca^{2+}]_i$), in the presence or absence of H_2O_2 or some pharmacological antagonists of different vasoactive agents were monitored using a Ca^{2+}-sensitive membrane permeant fluorescent dye, e.g. the acetoxymethyl ester of 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′methyl-phenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid(fura 2-AM) (Tsien, 1980), according to previously established methods (Zhang et al., 1992b). Primary rat aortic smooth muscle cells were isolated and cultured in Dulbecco's modified Eagle's medium at 37°C in a humidified atmosphere composed of 95% O_2 and 5% CO_2 according to previously established methods (Zhang et al., 1992b). Cells for image analysis experiments were seeded on glass coverslips (12 mm diameter; about 1×10^{-4} cells/coverslip) and used 2–3 days postseeding. Monolayers of smooth muscle cells from rat aorta grown on the coverslips were loaded with 2.0 μM fura 2-AM and 0.12% pluronic acid F-127 (60 min, 37°C). The monolayers were washed two to three times with phosphate-buffered saline and 20 mM HEPES (pH 7.4) and incubated with this buffer at room temperature until ready to use. The monolayers were inserted in a leakproof coverslip holder. Buffer was added to the monolayer on the coverslip. The cover-

slip holder was mounted onto the stage of a temperature-controlled Nikon fluorescence microscope and digitized by a TN8500 FluorPlex III Image Analyzer (Tracor Northern, Madison, WI). Buffer (control), H_2O_2 or pharmacological antagonists were added to the monolayers in the above-mentioned set-up. The cultured smooth muscle cell monolayers, preloaded with fura 2-AM, were excited alternatively, at 340 and 380 nm and the emission intensity was recorded at 510 nm, using a silicon intensified target camera. Background autofluorescence for both excitation wavelengths was acquired from blanks for each experiment and subtracted from each pair of images separately before ratioing. Fluorescence ratios (R) were obtained by dividing the 340 nm image by the 380 nm image. No image misalignments occurred when those two ratiometric images were superimposed. The resulting images were then used to calculate $[Ca^{2+}]_i$ in smooth muscle cells, using external standards containing 2.54 and 0 mM Ca^{2+} plus 10 mM EGTA for maximum (R_{max}) and minimum (R_{min}) fluorescence ratios of the 340 and 380 nm images. $[Ca^{2+}]_i$ was calculated according to the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times B \times (R - R_{min})/(R_{max} - R)$$

A K_d of 224 nM was used for the fura-2/Ca^{2+} complex. B is the ratio of the fluorescence intensity of fura-2 to the Ca^{2+}:fura-2 complex excited at 380 nm.

2.4. Drugs

The following pharmacological agents were purchased from Sigma Chemical Co. (St. Louis, MO): hydrogen peroxide (H_2O_2), acetylcholine, catalase (from bovine liver; 25 000 u/mg protein), deferoxamine, ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA), $FeSO_4$, methylene blue, proadifen, propranolol, staurosporine, genistein and verapamil. Atropine sulfate was bought from MANN Res. Lab. (New York). The acetyl methyl ester of bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) and fura 2-AM was purchased from Molecular Probes (Eugene, OR). Cimetidine and diphenhydramine were received from Smith Kline and French Laboratories (Welwyn Garden City, Herts, UK). Dimethyl sulfoxide (DMSO) was purchased from CALBIOCHEM corporation (La Jolla, CA). Phentolamine methanesulfonate was purchased from CIBA Pharmaceutical Company (Summit, NJ). Methysergide maleate was purchased from Sandoz Pharmaceuticals (Hanover, NJ). All other organic and inorganic chemicals were obtained from Fisher Scientific (Fair Lawn, NJ).

2.5. Calculations and statistical analysis

The percent contraction was expressed as the mean ± standard error of the mean (S.E.M.). Statistical evaluation of the results was carried out by analysis of Newman–Keuls test, which took into account that some vessels were from the same animal. The results were considered significant at a P-value < 0.05.

3. Results

3.1. Hydrogen peroxide induces endothelium-independent contraction in the isolated rat aorta

The isometric contraction technique was used in our experimental analysis. Fig. 1 shows that H_2O_2 elicits a concentration-dependent contraction of isolated rat aortic rings. The concentration of H_2O_2 needed to induce near-maximal contraction was $\sim 4.42 \pm 0.23$ mM. When cumulative dose–response curves were determined, there appeared to be a significantly greater developed tension in the denuded arteries by comparison to dose–response curves in the presence and absence of the endothelium ($P < 0.05$, all hydrogen peroxide concentration $> 4.41 \times 10^{-6}$ M). The effective concentration producing approximately 50% of the maximal contractile responses (ED_{50}) of H_2O_2 was $\sim 48.9 \pm 2.58$ μM. Since the contractile effects of hydrogen peroxide on the vessels is endothelium-independent, most of the following experiments were performed on endothelium-denuded aorta. Denuded aorta has been used by several investigators (Furchgott, 1991; Zembowicz et al., 1993; Main and Martin, 1995; Bharadwaj and Prasad, 1995) as a reasonable model for studying vessel damage induced by reactive oxygen species, even though it may not be better than endothelium-intact aorta. Although it is quite conceivable that H_2O_2 affects the endothelium, our study was not designed to address this possibility.

3.2. Effect of catalase on hydrogen peroxide-induced contraction of isolated rat aorta

Fig. 2 indicates that equilibration of denuded rat aortic ring segments with 1200 u/ml catalase, a scavenger of

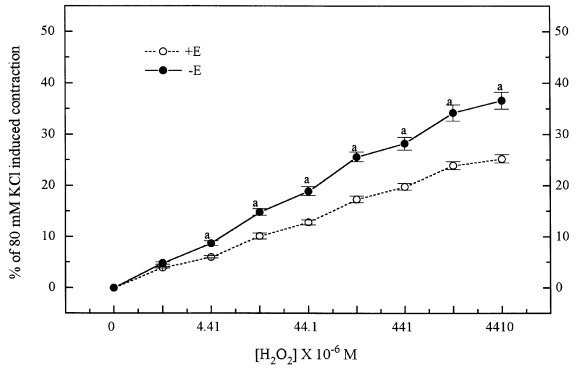

Fig. 1. Cumulative concentration-effects of H_2O_2 on rat aorta segments with (+E) or without (−E) endothelium. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. [a] denotes $P < 0.05$ compared to control. The number of experiments is 8 each.

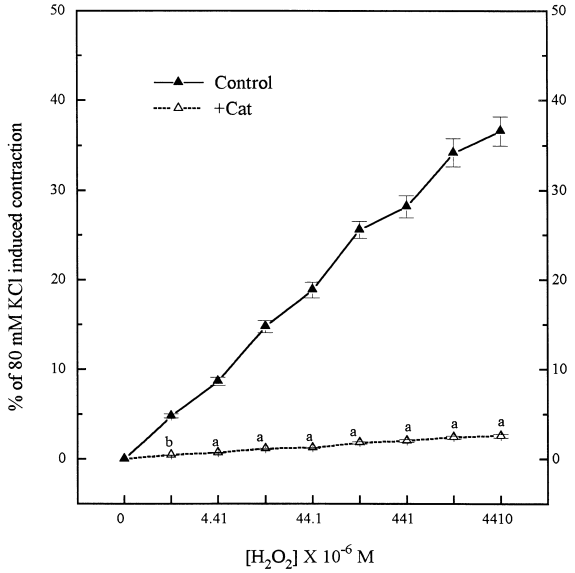

Fig. 2. Concentration–effect curves to H_2O_2 obtained in the absence and in the presence of catalase (Cat, 1200 u/ml) in denuded rat aorta segments. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. [a] denotes $P < 0.01$ and [b] denotes $P < 0.05$ compared to control. $n = 8$ each.

H_2O_2, for 15 min, results in almost a complete disappearance of the contractile response of denuded rat aorta rings to H_2O_2 as compared with the response in the absence of catalase. Application of catalase after a submaximal concentration of 100 μM H_2O_2 led to a relaxation of the vessels to baseline levels in the organ bath (not shown).

3.3. Involvement of Ca^{2+} in hydrogen peroxide-induced vasoconstriction of rat aorta

The requirement of calcium for the endothelium-independent contraction, produced by certain concentrations of H_2O_2, was substantiated in this study. As shown in Fig. 3A and B, responses of denuded rat aortic rings to H_2O_2 were assessed after removing Ca^{2+} from the organ bath for at least 90 min or after administration of 5.0 μM verapamil (a Ca^{2+} channel antagonist) to our system. Removal of Ca^{2+} from the extracellular medium (Fig. 3A) or addition of 5.0 μM verapamil to the bath media (Fig. 3B) had an appreciable inhibitory effect on the contraction caused by each concentration of H_2O_2 tested. The contractile effects of H_2O_2 on denuded rat aortic rings were

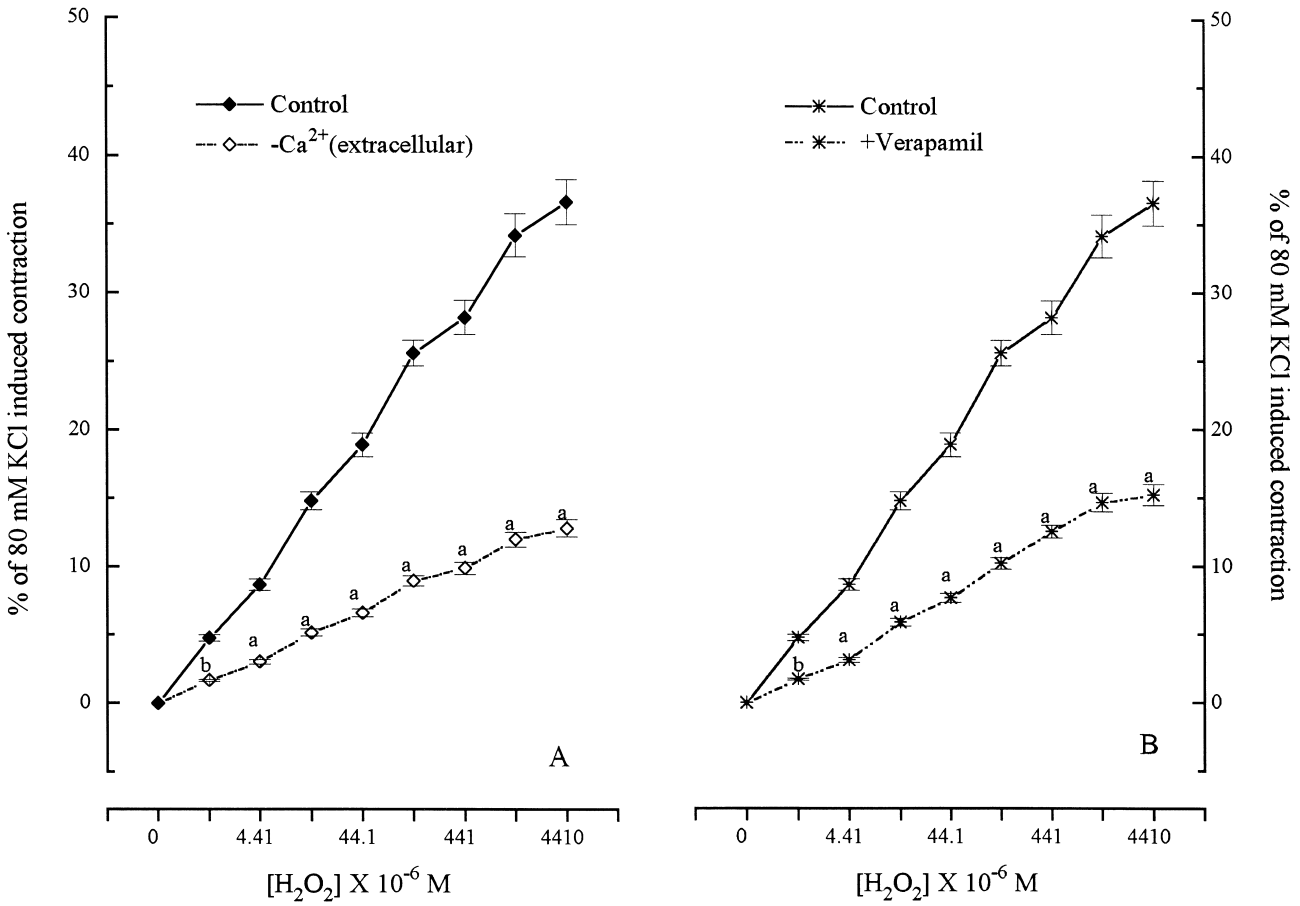

Fig. 3. Cumulative-effect curves to H_2O_2 obtained in the absence and in the presence of extracellular Ca^{2+} (A) or 1.0 μM verapamil (B) in denuded rat aorta segments. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. [a] denotes $P < 0.01$ and [b] denotes $P < 0.05$ compared to control. $n = 6$ each.

inhibited almost completely when the intracellular Ca^{2+} in smooth muscle cells was chelated by 10 μM membrane-permeable BAPTA-AM (data not shown). These results confirm that the contraction induced by H_2O_2 in rat aorta ring segments is Ca^{2+}-dependent.

3.4. Effects of indomethacin and specific pharmacological antagonists of various vasoconstrictor agents on hydrogen peroxide-induced contraction of isolated rat aorta

Several pharmacological reagents were used to characterize the mechanisms of H_2O_2's vascular contractile action. As shown in Fig. 4, the contractile response of intact rat aortic rings to H_2O_2 were antagonized significantly by 5.0 μM indomethacin, an inhibitor of cyclooxygenase. The inhibitory effect of indomethacin on the vasoconstriction caused by H_2O_2 establishes a possible involvement of cyclooxygenase products in the mechanism of the H_2O_2-induced responses. A muscarinic acetylcholine receptor antagonist (atropine, 5.0 μM), an antagonist of histamine H_1 receptors (diphenhydramine, 5.0 μM), an antagonist of histamine H_2 receptors (cimetidine, 5.0 μM), an α-adrenoceptor antagonist (phentolamine 5.0 μM), a β-adrenoceptor antagonist (propranolol, 5.0 μM) and an antagonist of serotonin receptor (methysergide, 5.0 μM), did not modify the response of denuded rat aortic rings to H_2O_2 (data not shown, $n = 8$ each). Although both propranolol and cimetidine are thought to have some antioxidant properties, antioxidant effects of propranolol are significant at 50 μM or higher (Sugawara et al., 1994) and $^{\cdot}OH$ scavenging effects of cimetidine are significant only at concentration of at least 28 μM (Lapenna et al., 1994). So, such antioxidant actions, in our present study, seem unlikely. Overall, these results indicate that the contractile response of denuded rat aorta ring segments to H_2O_2 is not dependent upon a release of vasoconstrictors such as acetylcholine, histamine, noradrenaline or serotonin from nerve endings of the smooth muscle cells.

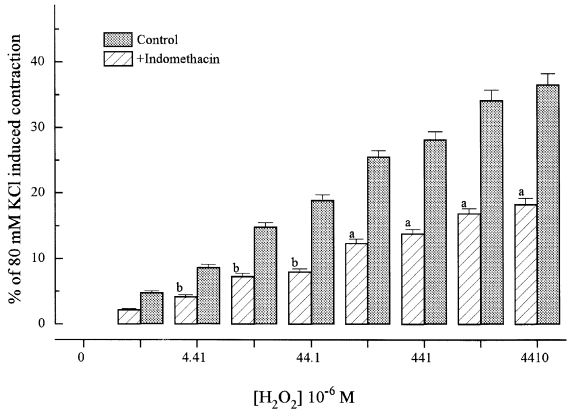

Fig. 4. Concentration–contractile effects to H_2O_2 obtained in the absence and in the presence of indomethacin (5.0 μM) in denuded rat aortic segments. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. [a] denotes $P < 0.01$ and [b] denotes $P < 0.05$ compared to control. $n = 8$ each.

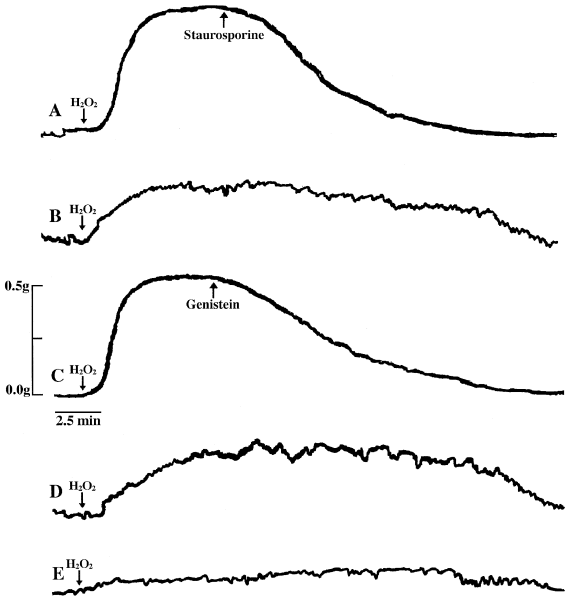

Fig. 5. Contractile responses to 100 μM H_2O_2 obtained after the addition of H_2O_2 (A and C), after the preaddition (15 min incubation) of 5×10^{-7} M staurosporine (B) and 7.5×10^{-5} M genistein (D) or after the preaddition of both 5×10^{-7} M staurosporine and 7.5×10^{-5} M genistein (E) in denuded rat aorta segments.

3.5. Contribution of protein kinase C and protein tyrosine phosphorylation to hydrogen peroxide-induced contraction of isolated rat aorta ring

To assess the involvement of protein kinase C or/and protein tyrosine phosphorylation in H_2O_2-induced contraction of isolated rat aortic rings, the effects of staurosporine, a protein kinase C inhibitor (Burke and Hablitz, 1996) and genistein, an inhibitor of protein tyrosine kinase (Albrecht and Tidball, 1997), were employed in this study. It is interesting to point out that treatment of rat aortic segments with 5×10^{-7} M staurosporine or 7.5×10^{-5} M genistein for 15 min significantly suppressed the contractile responses of the arteries to 100 μM hydrogen peroxide. The fast phase of the H_2O_2-induced contractions was attenuated and slowed in the presence of 5×10^{-7} M staurosporine or 7.5×10^{-5} M genistein, whereas the relative tonic or slow component remained unaltered (Fig. 5B and D). The suppression of staurosporine on the contractions was a little stronger than that of genistein (Fig. 5B and D). As shown in Fig. 5A and C, the contractions of the vessel segments to hydrogen peroxide were almost relaxed completely by adding the concentrations of staurosporine or genistein at the plateau of the H_2O_2-produced contractions. Equilibration of denuded rat aorta with both 5×10^{-7} M staurosporine and 7.5×10^{-5} M genistein for 15 min results in almost a complete disappearance of the contrac-

tile responses of the arteries to hydrogen peroxide as compared with control (Fig. 5E). The data strongly suggest that protein kinase C activity and protein tyrosine phosphorylation are important steps in H_2O_2-induced contraction of rat aorta rings in vitro.

3.6. Possible involvement of metabolites generated by cytochrome P450 in hydrogen peroxide-induced contraction of isolated rat aorta

We tested the effects of proadifen, which is an inhibitor of cytochrome P450 monooxygenase (Oyekan et al., 1994), on isolated rat aortic segments in order to investigate the possibility that metabolites of arachidonic acid, generated via cytochrome P450-dependent enzymes, are involved in the contractile responses to H_2O_2. Fig. 6 represents six experiments. The presence of proadifen (10 μM) surprisingly potentiated contractile responses of the vessels to all concentrations of H_2O_2 tested. The greatest augmentation (40%) occurred at the highest concentration of H_2O_2 tested.

3.7. Hydroxyl radical is involved in hydrogen peroxide-induced contraction of isolated rat aorta

To examine for a possible role of iron-catalyzed hydroxyl radical ($^{\cdot}OH$) formation in the aforementioned contraction effects of H_2O_2 on the vessels, experiments were carried out in the presence of iron ion donors by using $FeSO_4$. As shown in Fig. 7, with the addition of 1.0 μM $FeSO_4$ to the organ bath, after equilibration for 15 min, the contractile responses of the arteries to H_2O_2 were significantly augmented. In the presence of 1.0 μM Fe^{2+}, H_2O_2 induced approximately 140% of the contraction of the arteries compared to the control ($P < 0.05$). The presence

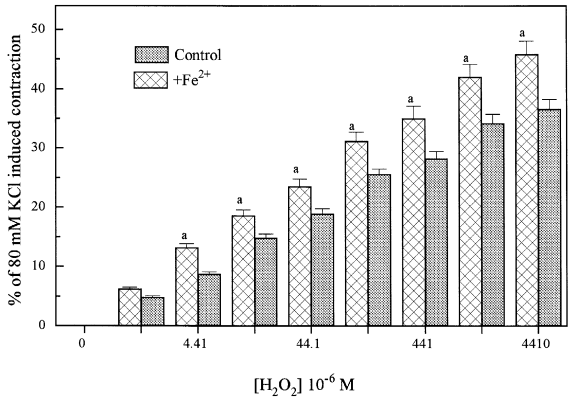

Fig. 7. Cumulative concentration–contractile effects to H_2O_2 obtained in the absence and in the presence of Fe^{2+} (1.0 μM) in denuded rat aorta segments. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. [a] denotes $P < 0.05$ compared to control. $n = 8$ each.

of 1 mM deferoxamine (a Fe^{2+} chelator) or 100 μM DMSO (a scavenger of hydroxyl radical) remarkably suppressed the contractile effect of H_2O_2 plus Fe^{2+} (Fig. 8A) or H_2O_2 alone (Fig. 8B) on the aorta segments. These results suggest that the contractile responses of the vessels to hydrogen peroxide are associated with the production of $^{\cdot}OH$.

3.8. Influence of Fe^{2+}, proadifen, indomethacin, extracellular Ca^{2+} or verapamil on the hydrogen peroxide-induced contractile patterns of the arteries

The H_2O_2-induced contractile patterns of denuded rat aortic ring segments in the absence and presence of 1.0 μM Fe^{2+}, 10 μM proadifen, 5 μM indomethacin, extracellular Ca^{2+} or 5 μM verapamil are shown in Fig. 9. The

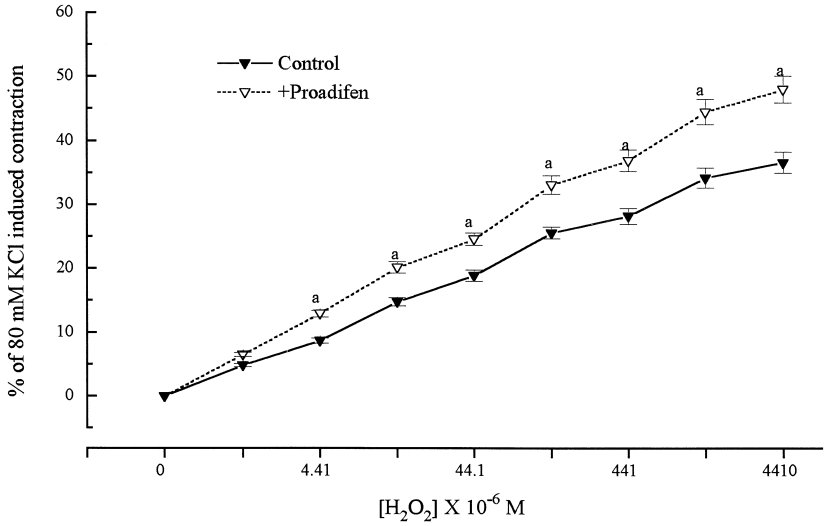

Fig. 6. Dose–response curves to H_2O_2 obtained in the absence and in the presence of proadifen (10 μM) in denuded rat aorta segments. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. [a] denotes $P < 0.05$ compared to control. $n = 6$ each.

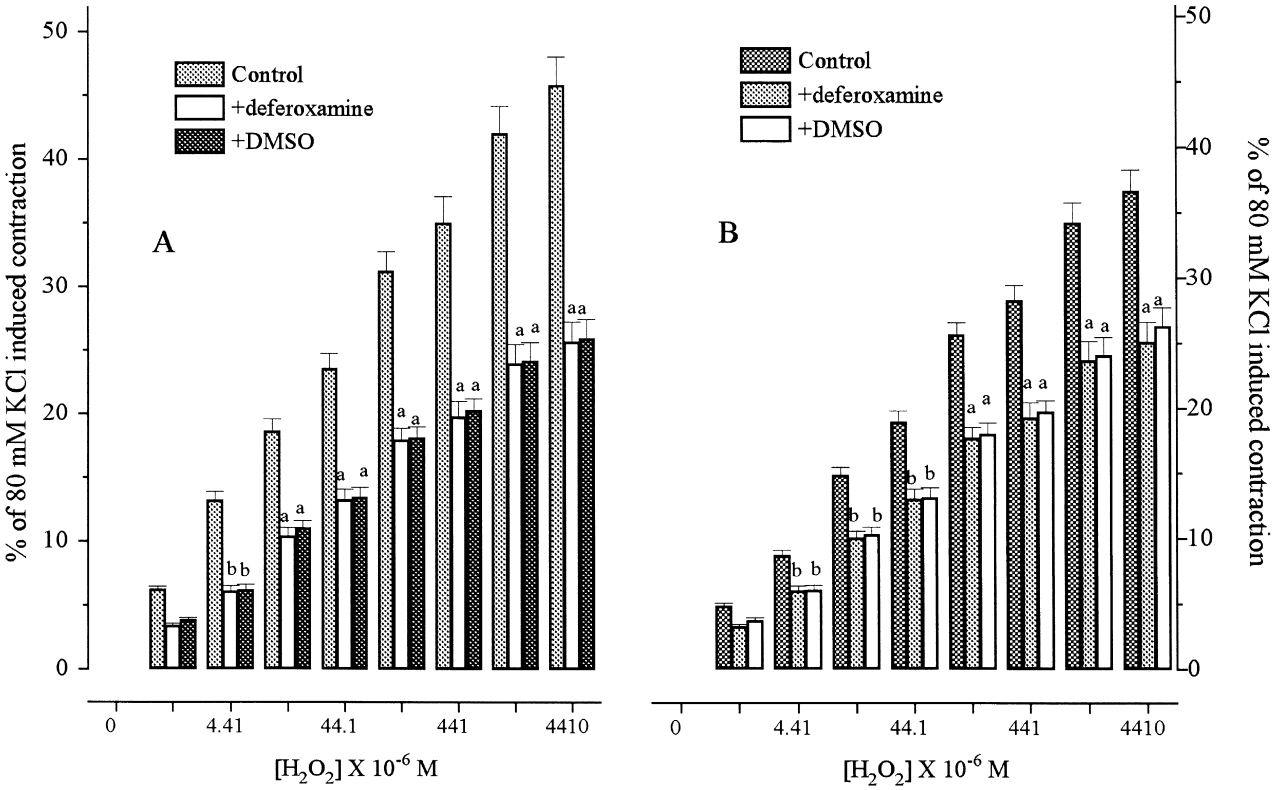

Fig. 8. Concentration–contractile effects to H_2O_2 plus Fe^{2+} (A) or H_2O_2 (B) obtained in the absence and in the presence of deferoxamine (1.0 mM) or dimethyl sulfoxide (100 μM) in denuded rat aortic segments. Each point represents the mean ± S.E. expressed as percentage of the tension developed by 80 mM KCl. [a] denotes $P < 0.01$ and [b] denotes $P < 0.05$ compared to control. $n = 8$ each.

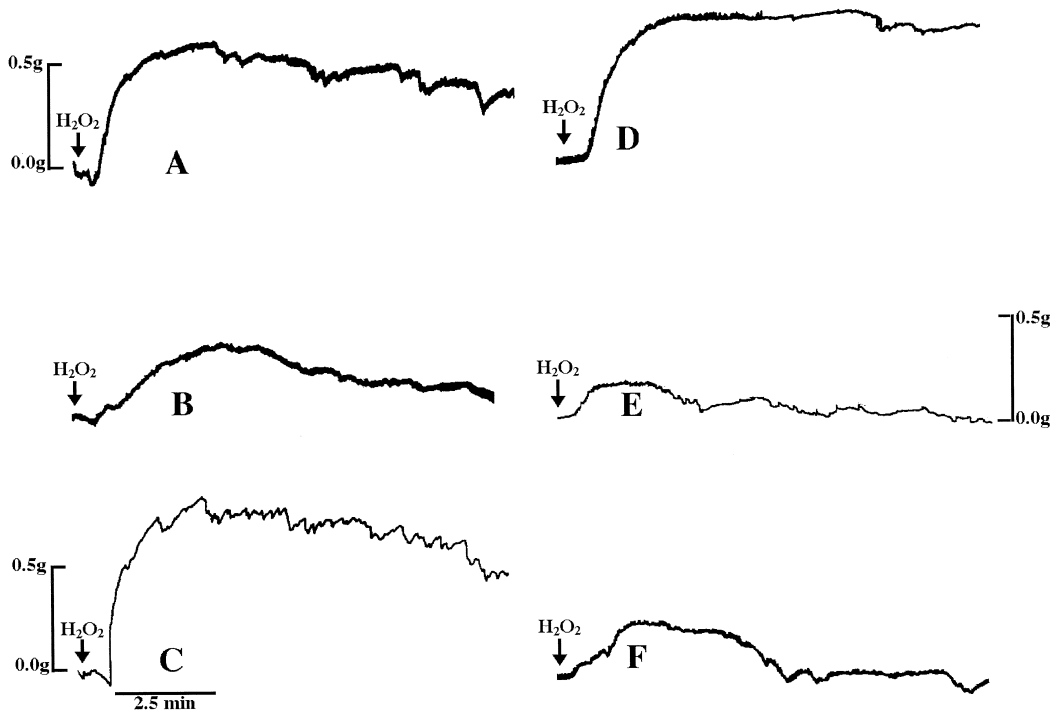

Fig. 9. Contractile-responses to 100 μM H_2O_2 obtained in the absence (A) and in the presence of 5 μM indomethacin (B), 1 μM Fe^{2+} (C), 10 μM proadifen (D), extracellular Ca^{2+} (E) or 5 μM verapamil (F) in denuded rat aorta segments.

Table 1
Percent maximal responses of intracellular Ca^{2+} concentration in vascular smooth muscle cells to hydrogen peroxide in the absence and presence of 1200 u/ml catalase, 5 μM indomethacin, 1 μM Fe^{2+} or 10 μM proadifen

Treatment	$[H_2O_2]\ 10^{-6}$ M				
	0	4.41	44.1	441	4410
Control	100 ± 4.3	188.5 ± 9.3[a]	245.8 ± 9.8[a]	356.5 ± 15.4[a]	485.2 ± 18.6[a]
+ Cat (1200 u/ml)	99.8 ± 3.9	123.6 ± 4.9	134.8 ± 5.3	138.2 ± 5.6	142.5 ± 6.3[b]
+ Indo (5 μM)	98.2 ± 4.0	145.3 ± 6.8[b]	190.6 ± 8.0[a]	250.2 ± 14.6[a]	335.8 ± 8.6[a]
+ Fe^{2+} (1 μM)	185.6 ± 5.3[a]	256.9 ± 8.9[a]	369.6 ± 12.5[a]	496.8 ± 23.8[a]	626.9 ± 35.6[a]
+ Proadifen (10 μM)	103.8 ± 4.8	191.8 ± 7.9[a]	256.9 ± 12.9[a]	369.6 ± 21.7[a]	491.6 ± 13.3[a]

The data was obtained after 20 s of the reagent treatments. Each result indicates the mean ± S.E. ($n = 10–15$) expressed as percentage with reference to $[Ca^{2+}]_i$ at the resting state.
[a] Denotes $P < 0.01$ and [b] denotes $P < 0.05$ compared to control (no treatment $[Ca^{2+}]_i$).

vessel's contraction elicited by 100 μM H_2O_2 consisted of an initial fast response (phasic component, which is related to intracellular Ca^{2+} release) followed by a slower increase in tension (tonic component, which is dependent on extracellular Ca^{2+} ($[Ca^{2+}]_0$) (Fig. 9A) (Bolton, 1979; Zhang et al., 1991). On addition of 1.0 μM Fe^{2+} to Krebs–Ringer bicarbonate solution or with exposure of our experimental solution to 10 μM proadifen, the maximum amplitudes of the H_2O_2-induced contractions in the arteries were potentiated, while the phasic and tonic components of the contractile curves were not altered (Fig. 9C and D). In the presence of 5 μM indomethacin, in our experimental system, the H_2O_2-induced maximum contraction in denuded rat aorta ring segments was significantly depressed and the fast component of H_2O_2 contraction was attenuated and slowed, whereas the relative tonic or slow component remained unaltered (Fig. 9B). Absence of Ca^{2+} in the extracellular medium (Fig. 9E) or administration of 5.0 μM verapamil (Fig. 9F) to the bath media produced significant attenuation of the maximal amplitudes of H_2O_2-induced contractions in the arteries coupled with a reduction in the rising slope of the fast component followed by relaxation to the basal resting level.

3.9. Hydrogen peroxide induces concentration-dependent increments in $[Ca^{2+}]_i$ in single smooth muscle cells of isolated rat aorta

The effects of H_2O_2 on $[Ca^{2+}]_i$ of smooth muscle cells from rat aorta were studied by using the direct technique of Ca^{2+} visualization in single cells as revealed by the digital imaging microscope using fura 2-AM. The control $[Ca^{2+}]_i$ was 112 ± 6.84 nM. As shown in Table 1, H_2O_2 elicited a concentration-dependent increase in $[Ca^{2+}]_i$ within 20 s. Preincubation with 1200 u/ml catalase before H_2O_2 challenge prevented the H_2O_2-induced rise in $[Ca^{2+}]_i$ in the smooth muscle cells. In the presence of 1.0 μM Fe^{2+}, H_2O_2 produced a potentiation of the increases in $[Ca^{2+}]_i$ in the treated cells compared to control. The employment of 10 μM proadifen in our cell system did not alter the increment in $[Ca^{2+}]_i$ produced by the various concentrations of H_2O_2. With the addition of 5 μM indomethacin to the system, H_2O_2 induced a smaller rise in $[Ca^{2+}]_i$ in the cells compared to control.

4. Discussion

The mechanisms whereby reactive oxygen species elicit vasoconstriction are poorly understood. Our present study attempts to define the effects of H_2O_2 on isolated rat aorta ring segments and to gain insight into the relationship between contractility of the vessels induced by H_2O_2, Ca^{2+} and some metabolites generated by H_2O_2 treatment. The present results demonstrate that H_2O_2 produces a concentration-dependent contraction on isolated rat aorta ring segments. The presence of a stronger contractile effect of hydrogen peroxide observed on artery segments after removal of the endothelium suggests that the maximum contractile responses of the vessels to H_2O_2 are endothelium-independent. The concentration of hydrogen peroxide used in this study as well as others (Wolin and Belloni, 1985; Rhoades et al., 1990), that resulted in contraction, could conceivably occur in-vivo with leukocyte activation and adherence with vascular injury (Rhoades et al., 1990). Such high, localized H_2O_2 concentrations could result, initially, in vasoconstriction and in subsequent arterial smooth muscle dysfunction. Since cell surface receptors for hydrogen peroxide have not been identified, we suspect that H_2O_2 may be acting through an intracellular mechanism. H_2O_2 can easily cross lipid bilayer membranes of cells (Salvemini and Botting, 1993; Salvemini et al., 1993a,b). It has been reported that the bovine pulmonary artery can metabolize H_2O_2 at an appreciable rate, that millimolar concentrations of the peroxide are used to cause detectable oxidation of the glutathione pool, and that oxidation of the tissue's glutathione pool is probably not involved in the relaxant responses to micromolar concentrations of H_2O_2 (Burke and Wolin, 1987). These observations imply that an intracellular mechanism for hydrogen peroxide's action is most likely.

The endothelium-independent contractions of the vessels to H_2O_2 were inhibited, significantly, in the presence of the cyclooxygenase inhibitor, indomethacin (Fig. 4). Fig. 9B demonstrates that with the exposure to 5 μM indomethacin in our experimental set-up, the hydrogen peroxide-induced maximal contraction in the vessels was attenuated and the fast component (phasic stage related to intracellular Ca^{2+} release) of H_2O_2 contraction curve was reduced and slowed. It is unlikely that the observed effect of the cyclooxygenase inhibitor, indomethacin, was due to a direct chemical-interaction of indomethacin with H_2O_2, since a recent report demonstrates that the total protein prostanoid-receptor antagonist GR-32191B significantly inhibited the contractile effects of H_2O_2 in denuded human airway smooth muscle (Rate et al., 1995), indicating the involvement of prostanoid-receptor activation in H_2O_2-induced contraction. Thus, it is logical to conclude that the contractile effects of H_2O_2 are mediated, at least in part, by the increased metabolism of arachidonic acid via the cyclooxygenase pathway in vascular smooth muscle cells. This conclusion is reinforced by the fact that H_2O_2 is known to stimulate the production of prostaglandin D_2, prostaglandin I_2 in human airway smooth muscle (Rate et al., 1995) and increases release of both prostaglandin $F_{2\alpha}$ and thromboxane A_2 in smooth muscle of guinea pig trachea (Gao and Vanhoutte, 1993). The present study also shows that with exposure of rat aortic rings to 5.0 μM indomethacin, H_2O_2 addition results in an attenuated rise in $[Ca^{2+}]_i$ in rat aorta smooth muscle cells, compared to control. It has been reported that eicosanoids, e.g. prostaglandin $F_{2\alpha}$ and (15)*S*-hydroxy-11$_\alpha$,9$_\alpha$-(epoxymethano)-5Z,13E-dienoic acid (U46619) can induce increases in $[Ca^{2+}]_i$ of smooth muscle cells from rat aorta (Kurata et al., 1993; Zhang et al., 1997) probably by inositol 1,4,5-trisphosphate via phosphatidylinositol diphosphate breakdown and possibly by an increase in the sensitivity of the contractile apparatus. Collectively, these findings support our proposal that hydrogen peroxide-elicited vascular smooth muscle contractions are mediated, at least partly, by cyclooxygenase products.

The primary trigger of smooth muscle contraction is a rise in cytoplasmic $[Ca^{2+}]_i$. Changes in intracellular Ca^{2+} homeostasis are thought to play important roles in smooth muscle cell responses to oxidants (Sandirasegarane and Gopalakrishnan, 1995). In the present study, it was demonstrated that removal of extracellular Ca^{2+} or administration of the voltage-gated L-type Ca^{2+} channel antagonist, verapamil, significantly depressed the contractile effects of hydrogen peroxide on the vessel segments. Although verapamil is thought to exhibit some antioxidant properties, it does not affect H_2O_2 generation from alloxan-treated pancreatic islets until millimolar concentrations are used (Kim et al., 1994). Its antioxidant action can, thus, probably be neglected in our study, since we employed 5 μM verapamil. When the intracellular Ca^{2+} in smooth muscle cells was entirely buffered by 10 μM BAPTA-AM, a membrane-permeable Ca^{2+} chelator, the contractile responses of the vessels to H_2O_2 disappeared almost completely (data not shown, $n = 8$). Our present study demonstrates, also, that H_2O_2 produced a rapid, significant concentration-dependent increase in $[Ca^{2+}]_i$ of smooth muscle cells from rat aorta, which is consistent with observations reported in other types of smooth muscle cells (Schachter et al., 1989; Roveri et al., 1992). In view of our new data, we propose that both extracellular and intracellular Ca^{2+} are required in vessel contractions caused by hydrogen peroxide. Since the prolonged absence of extracellular Ca^{2+} or employment of the L-type Ca^{2+} channel blocker, verapamil, inhibited approximately 70% of the contractile effects of H_2O_2 on the arteries (Fig. 3A and B) and the initial phase of the smooth muscle contraction induced by hydrogen peroxide was transient (followed by a well sustain tonic response), it is probable that H_2O_2 acts directly to provide a pathway for Ca^{2+} entry from the extracellular fluid via voltage-gated membrane Ca^{2+} channels as well as producing a Ca^{2+}-induced Ca^{2+} release from intracellular stores (e.g. sarcoplasmic reticulum). The influx of extracellular Ca^{2+} appears to play a principle role in Ca^{2+}-dependent vessel contraction caused by hydrogen peroxide. Recently, it has been reported that hydrogen peroxide can potentiate the voltage-dependent influx of Ca^{2+} of smooth muscle cells (Sasaki and Okabe, 1993). In addition, it has been reported that influx of Ca^{2+}, after exposure to oxidants, appears to be responsible for the increase in $[Ca^{2+}]_i$ of smooth muscle cells (Sandirasegarane and Gopalakrishnan, 1995). Krippeit-Drews et al. (1995) and Vollrath et al. (1995) have, indeed, recently proposed that hydrogen peroxide produces an early intracellular Ca^{2+} increment in some smooth muscle cells, which at least partly results from the mobilization of Ca^{2+} from the sarcoplasmic reticulum or even possibly mitochondria (Roychoudhury et al., 1996a,b). Whether or not Ca^{2+} channel antagonists, like verapamil, have antioxidant properties on rat aorta at around 10^{-6} M concentration remains to be seen.

Our present results also demonstrate that a protein kinase C inhibitor (staurosporine, 5×10^{-7} M) or 7.5×10^{-5} M genistein, an inhibitor of protein tyrosine kinase, which is an enzyme for catalyzing protein tyrosine phosphorylation, significantly suppressed the H_2O_2-produced contraction of rat aortic rings, which suggests the probable involvement of protein kinase C activity and protein tyrosine phosphorylation in the artery contraction caused by hydrogen peroxide. Despite the possibility that genistein may exhibit antioxidant effects in certain cells, e.g. in J774 cells (at a concentration of 100 μM) (Murio et al., 1994), we believe that this property can be neglected in the present study, although it remains a possibility. Protein kinase C is an ubiquitous enzyme that plays a pivotal role in the control of a variety of cellular processes (Nishizuka, 1986). Activation of protein kinase C is an important mechanism by which vasoconstrictors act (Exton, 1988).

Protein kinase C is thought to phosphorylate smooth muscle myosin and myosin light-chain kinase in vitro (Ikebe et al., 1985), increase the Ca^{2+} sensitivity of the contractile apparatus (Drenth et al., 1989; Xie et al., 1996) and influence the sustained phase of agonist-induced contraction (Rasmussen et al., 1987; Andrea and Walsh, 1992). Protein kinase C plays a prominent role in regulating the smooth muscle membrane associated phospholipase A_2 and subsequent liberation of arachidonic acid under exposure of the cells to hydrogen peroxide (Chakraborti and Michael, 1993; Rao et al., 1995). Several lines of evidence indicate the involvement of oxidants, such as H_2O_2, in protein kinase C mobilization in vascular smooth muscle cell membranes (Stauble et al., 1994; Baas and Berk, 1995) and other cells (Chen and Chan, 1993; Zor et al., 1993). H_2O_2 can increase 1,2-diacylglycerol and inositol 1,4,5-trisphosphate production in porcine pulmonary artery through the hydrolysis of phosphatidylinositol diphosphate by phospholipase C activation (Shasby et al., 1988), which would lead to activation of protein kinase C and to a liberation of Ca^{2+} from intracellular stores. Recently, it has been reported that hydrogen peroxide by itself is capable of increasing the activity of phospholipase Cγ, through its direct stimulatory effect on tyrosine phosphorylation in human leukemic T-cells (Secrist et al., 1993). Tyrosine phosphorylation of phosphatidylinositol 3-kinase leads to activation and synthesis of phosphatidylinositol 3,4,5-trisphosphate, which has been shown to be a selective activator of ξ-protein kinase C (Nakanishi et al., 1993). This is one possible route by which hydrogen peroxide could activate membrane protein kinase C. Treatment of vascular smooth muscle cells with oxidants including hydrogen peroxide has been reported to enhance protein tyrosine phosphorylation, and the enhancement has been shown to be a result of both a sustained activation of protein tyrosine kinase and a suppression of protein tyrosine phosphatase in vascular smooth muscle cells (Zor et al., 1993; Baas and Berk, 1995). All of these studies could be invoked to support our proposal that Ca^{2+} is necessary in the vessel contraction elicited by hydrogen peroxide and that the process of the contraction may involve several different, as yet, unidentified signaling pathways.

The significant, incremental contraction of H_2O_2 on the arteries, noted herein, in the presence of 1 μM Fe^{2+}, in our experimental system (Fig. 5), coupled to the observed enhancement of a H_2O_2-elicited increase in $[Ca^{2+}]_i$ of aortic smooth muscle cells (Table 1) together with experiments showing that Fe^{2+} chelator, deferoxamine (1 mM) and hydroxyl radical scavenger, dimethyl sulfoxide (100 μM), produced significant inhibitory effects on the contractions induced by each concentration of hydrogen peroxide plus Fe^{2+} or hydrogen peroxide, itself, suggests that H_2O_2 initiates vascular smooth muscle contraction, in part, by hydroxyl radical ($^{\cdot}OH$) generation; hydrogen peroxide can react with Fe^{2+} to produce $^{\cdot}OH$ through the Fenton reaction. Our results are in agreement with several reports demonstrating that hydroxyl radicals are probably involved in oxidant-induced contractions and increases in $[Ca^{2+}]_i$ in smooth muscle cells. Using rabbit lingual arterial smooth muscle, Sasaki and Okabe (1993) have shown that hydroxyl radicals can potentiate the voltage-dependent influx of Ca^{2+} in smooth muscle cells. Other investigations have been reported recently indicating that hydrogen peroxide-mediated Ca^{2+} release from mitochondria of bovine pulmonary vascular smooth muscle tissue is through the involvement of $^{\cdot}OH$ radicals (Roychoudhury et al., 1996a,b). Furthermore, it is known that hydroxyl radicals can induce contraction of rabbit tracheal smooth muscles, which appears to be similar to our results and to be mediated, in part, by arachidonic acid metabolites (Prasad and Gupta, 1993).

Turning to our experiments with 10 μM proadifen (an inhibitor of cytochrome P450 monooxygenase which is an arachidonic acid metabolizing enzyme), rat aortic contractions caused by hydrogen peroxide were potentiated markedly. From Fig. 9D, it can be seen that exposure of rat aortic rings to 10 μM proadifen significantly enhanced the maximum amplitude of hydrogen peroxide-induced contractions. These results provide suggestive evidence for a role for release of cytochrome P450-dependent metabolites in response to hydrogen peroxide. It is possible that hydrogen peroxide may stimulate not only the synthesis and release of cyclooxygenase products but also the production of metabolites catalyzed by cytochrome P450-dependent enzymes. But the latter does not seem to so much mediate the contraction of the artery segments as it appears to be quite important in relaxation of the vessels (unpublished findings). It has been reported that cGMP stimulation by organic nitrates requires bioactivation of these compounds by a cytochrome P450-dependent enzyme system (Schroder, 1992). The former is necessary for initiating relaxation of vascular smooth muscle (Oyekan et al., 1994). The latter report indicates also that the prototypical inhibitor of cytochrome P450, proadifen, impairs the expression of relaxation of vascular smooth muscle to acetylcholine, isoproterenol and diazoxide (Oyekan et al., 1994). Our present study demonstrates that the employment of 10 μM proadifen does not alter the increase in $[Ca^{2+}]_i$ in aortic smooth muscle cells produced by various concentrations of hydrogen peroxide. So, it seems that the proadifen augmentation of H_2O_2-induced contraction in the aortic segments is not the result of changes in $[Ca^{2+}]_i$ in vascular smooth muscle cells. The exact reason for the enhancement of contraction by proadifen remains to be determined. The most likely explanation, however, for this potentiation is that there may be some Ca^{2+}-independent substances produced by proadifen which may mediate the contraction of vascular smooth muscle of rat aorta on hydrogen peroxide administration. Recently, it has been suggested, that vascular smooth muscle contraction can occur under conditions where the $[Ca^{2+}]_i$ is low and fixed and that these contractions may be mediated by protein

kinase C (Whitney et al., 1995). A few new and atypical protein kinase C's, such as δ, ϵ and ξ-protein kinase C subspecies, have been shown to be Ca^{2+}-independent (Zor et al., 1993). So, it still remains questionable whether cytochrome P450 inhibitors can stimulate production of Ca^{2+}-independent protein kinase C, alter the activity of Ca^{2+}-independent protein kinase C in vascular smooth muscle or initiate production and release of other mediators which affect contractility of vascular smooth muscle. In the absence of additional data, it could be that some of the effects of proadifen might be due to actions on the oxidase or peroxidase activities of the enzyme. Further study will be necessary to identify and elucidate the mechanism of cytochrome P450 inhibitor-induced contraction in rat aortic smooth muscle.

Although the possible physiological role of hydrogen peroxide is poorly understood, it has been proposed that peroxide generation is related to the metabolic state of the tissue and oxygen tension (Ignarro and Kadowitz, 1985). Thus, cellular generation of peroxide and cell metabolism via P450 enzymes could be involved in mechanisms for sensing oxygen tension, in a manner that would reflect the specialized metabolic properties of vascular tissues. Rat aortic smooth muscle contraction, induced by hydrogen peroxide, caused production of metabolites (inhibited by indomethacin herein) catalyzed by cyclooxygenase which could be viewed as a potential mechanism in control of vascular tone by oxygen tension.

In summary, our present study suggests that hydrogen peroxide can induce contractions of isolated rat aortic ring segments at appreciable pathophysiological concentrations in-vitro, which are obviously Ca^{2+}-dependent (both extra- and intracellular), involve probably hydroxyl radicals (products of the reaction between Fe^{2+} and H_2O_2) and are mediated, at least in part, by protein kinase C's, cyclooxygenase products and tyrosine phosphorylated products generated by hydrogen peroxide. The H_2O_2-induced metabolites catalyzed by cytochrome P450-dependent enzymes appears to exert relaxant effects on rat aorta. Lastly, there appears to be a mediator produced by the cytochrome P450 inhibitor proadifen (after hydrogen peroxide treatment) which may be Ca^{2+}-independent and which elicits contraction of vascular smooth muscle.

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