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Mechanisms of hydroxyl radical-induced contraction of rat aorta

Jianfeng Li[a], Wenyan Li[a], Weimin Liu[b], Bella T. Altura[a,c], Burton M. Altura[a,c,d,*]

[a]*Department of Physiology and Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, NY 11203-2056, USA*
[b]*Department of Anatomy and Cell Biology, State University of New York, Downstate Medical Center, Brooklyn, NY 11203-2056, USA*
[c]*The Center for Cardiovascular and Muscle Research, State University of New York, Downstate Medical Center, Brooklyn, NY 11203-2056, USA*
[d]*Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, NY 11203-2056, USA*

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Abstract

The present study was designed to investigate the effects of hydroxyl radicals (•OH), generated via the Fe^{2+}-mediated Fenton reaction, on isolated rat aortic rings with and without endothelium. In the absence of any vasoactive agent, generation of •OH alone elicited an endothelium-independent contraction in rat aortic rings in a concentration-dependent manner. Hydroxyl radical-induced contractions of denuded rat aortic rings appeared, however, to be slightly stronger than those on intact rat aortic rings. The contractile responses to •OH were neither reversible nor reproducible in the same ring; even small concentrations of •OH radicals resulted in tachyphylaxis. Removal of extracellular calcium ions (Ca^{2+}) or buffering intracellular Ca^{2+} with 10 μM acetyl methyl ester of bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid (BAPTA-AM) significantly attenuated the contractile actions of •OH radicals. The presence of 1 μM staurosporine, 1 μM bisindolylmaleimide I, 1 μM Gö6976 [inhibitor of protein kinase C (PKC)], 2 μM PD-980592 (inhibitor of ERK), 10 μM genistein, and 1 μM wortmannin significantly inhibited the contractions induced by •OH. Proadifen (10 μM), on the other hand, significantly potentiated the hydroxyl radical-induced contractions. Exposure of primary cultured aortic smooth muscle cells to •OH produced significant, rapid rises of intracellular free Ca^{2+} ($[Ca^{2+}]_i$). Several, specific antagonists of possible endogenously formed vasoconstrictors did not inhibit or attenuate either hydroxyl radical-induced contractions or the elevation of $[Ca^{2+}]_i$. Our new results suggest that hydroxyl radical-triggered contractions on rat aortic rings are Ca^{2+}-dependent. Several intracellular signal transduction systems seem to play some role in hydroxyl radical-induced vasoconstriction of rat aortic rings.

Keywords: Hydroxyl radical; Aortic ring, rat; Contraction; Vascular smooth muscle; Signal transduction pathway

1. Introduction

Reactive oxygen species comprise a group of molecules such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radicals (•OH). Reactive oxygen species are continuously generated in vivo, but an excessive level of these species is considered to be responsible for several pathophysiological processes partly by affecting vascular tone (for recent review, see Fattman et al., 2003). A large body of studies on blood vessels has shown that reactive oxygen species can modulate vascular tone directly or indirectly leading to vasodilation or vasoconstriction (Rubanyi, 1988; Yang et al., 1998, 1999; Karas, 1999; Silveira et al., 2003). Individual reactive oxygen species may exert different and sometimes complex actions on the tone of blood vessels via different mechanisms (Rubanyi, 1988; Yang et al., 1998, 1999; Li et al., 2004). The hydroxyl radical is one of the most aggressive species of reactive oxygen species which can be generated by the interaction of $O_2^{\bullet-}$ and H_2O_2, especially in the presence of catalytic metal ions. The •OH radical is known to play important roles in both normal and pathological conditions. It has been suggested that •OH radicals can produce both relaxant and contractile actions on isolated arteries from different

* Corresponding author. Box 31, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, New York 11203, USA. Tel.: +1 718 270 2194; fax: +1 718 270 3103.
E-mail address: baltura@downstate.edu (B.M. Altura).

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animals, which may be dependent upon vessel and species (Bharadwaj and Prasad, 1997; Pieper et al., 1997; Shen et al., 2000; Zeitz et al., 2002). Previous investigations have suggested that the relaxant effects of ·OH may be due to the synthesis of prostaglandins, the stimulation of guanylate cyclase, and activation of K^+-ATP channels as well (Mittal and Murad, 1977; Deby and Deby-Dupont, 1981; Zeitz et al., 2002). However, as for the contractile action of ·OH, the available information is relatively scarce and imprecise.

Up to now, the exact mechanism(s) by which ·OH exerts its vascular effects still remain to be elucidated. It is possible that the ·OH-induced vascular actions are mediated via a second messenger system. Previous studies from our lab have shown that protein kinase C, mitogen-activated protein kinase, and nonreceptor tyrosine kinases play essential roles in H_2O_2-tiggered signal transduction pathways in vascular smooth muscle cells (Yang et al., 1998, 1999). It is, however, unclear whether H_2O_2 and ·OH act on vascular tone via similar or different mechanisms. The current study was undertaken to investigate the effects of ·OH on isolated ring segments of rat aorta and to gain insight into the underlying mechanisms of its action, with special attention given to potential signal transduction mediators generated by ·OH.

2. Materials and methods

2.1. General procedures

Male adult Wistar rats (350–450 g) were sacrificed by stunning and subsequent decapitation. The aortas were isolated according to previously established methods (Zhang et al., 1992a). The vessels for the ring segments were carefully excised, cleaned, and the tissues cut into 3- to 4-mm segments. For intact tissue preparation, extreme care was taken to avoid damage of endothelial cells. For denuded arteries, the intima of the vessels were removed by gently rubbing against the teeth of a pair of forceps (Zhang et al., 1992b). The tissues were placed in normal Krebs–Ringer bicarbonate (NKRB) 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, 1980; Zhang et al., 1993). The segments were mounted on stainless-steel pins under 2 g resting tension in organ baths, attached to force transducers (Grass Model FT 03) and connected to Grass Model 7 polygraphs. The organ baths, containing NKRB solution, were 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). At the beginning of an experiment, rings were exposed for 30–45 min to 80 mM KCl and this was repeated every 30–45 min, until responses were stable (two to three times). Successful removal of endothelium was assessed by showing that acetylcholine (10^{-8} to 10^{-6} M) failed to relax the denuded segments. Segments precontracted by 5×10^{-7} M phenylephrine did relax ~90% in response to acetylcholine in endothelium-intact segments (Zhang et al., 1992a, 1998). ·OH were generated through the Fenton reaction using 100 μM $FeSO_4$ and 10, 50, 100, 200, and 400 μM H_2O_2. $FeSO_4$ and H_2O_2 were added directly into the bath simultaneously to ensure that generation of ·OH took place rapidly. Radical exposure was performed in a darkened chamber in order to avoid photobleaching (Zeitz et al., 2002 and Ren et al., 2001). Responses to the ·OH and other drugs were expressed as a percentage of the stable level of contraction induced by 80 mM KCl. All of the experiments and protocols in this study were approved by the Animal Use and Care Committee at our institution.

2.2. Extracellular calcium (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 NKRB solution containing 0.2 mM EGTA for ~90 min before initiation of the experiments (Yang et al., 1999). 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 (Yang et al., 1999). After obtaining stable conditions for the ring segments, experiments were begun (Whitney et al., 1995; Yang et al., 1999).

2.3. Intracellular Ca^{2+} measurements

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured in isolated primary cultured smooth muscle cells, as harvested and prepared previously (Zhang et al., 1992a,b), in the presence or absence of generated ·OH radicals, using the Ca^{2+}-sensitive membrane-permeant fluorescent dye, fura 2-AM (acetoxymethylester of 1-2-(5- carboxyoxazol-2-yl)-6-aminobenzofuran- 5-oxy-2-(2′-amino- 5′methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Tsien, 1980), according to previously established methods in our lab (Zhang et al., 1997). Monolayers of the aortic smooth muscle cells, 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

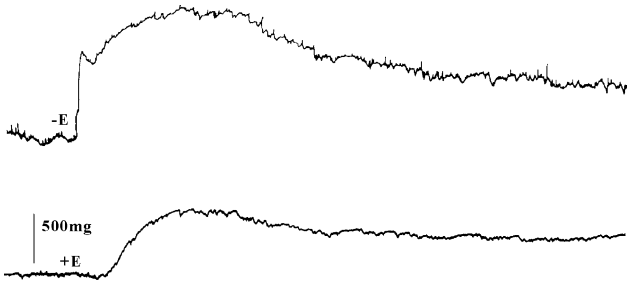

Fig. 1. Contractile response to $FeSO_4$ plus 200 μM H_2O_2 in isolated intact (+E) and endothelium-denuded (−E) rat aortic rings.

the coverslip. The coverslip holder was mounted onto the stage of a temperature-controlled Nikon TMS inverted microscope with a long working distance Nikon Fluor objective (n.a. 0.5), attached to a 300-W xenon light source and the CCD camera for image acquisition. Buffer (control), and generated ·OH radicals were added to the monolayers. The primary cultured aortic smooth muscle cell monolayers, preloaded with fura 2-AM, were excited alternatively, at 340 nm and 380 nm, and the emission intensity was recorded at 510 nm, using a silicon intensified target camera (Zhang et al., 1997). 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 the smooth muscle cells, using external standards containing 2.54 mM Ca^{2+} 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 (Zhang et al., 1997). B is the ratio of fluorescence intensity of fura-2 to Ca^{2+}: fura-2 complex excited at 380 nm.

All of these measurements were performed in the dark so as to prevent photobleaching.

2.4. Reagents

The following pharmacological agents were purchased from Sigma (St. Louis, MO): $FeSO_4$, bisindolylmaleimide I HCl, acetylcholine HCl, phenylephrine HCl, *N*,*N*-tetra-acetic acid (EGTA), indomethacin, propranolol HCl, naloxone HCl, atropine sulfate, H_2O_2, phentolamine methanesulfonate, staurosporine, genistein, and proadifen. The BAPTA-AM and fura 2-AM was purchased from Molecular Probes Eugene, OR). Dimethyl sulfoxide (DMSO), Gö6976, PD-980592 and wortmannin were purchased from CALBIOCHEM (La Jolla, CA, USA). Cimetidine HCl and diphenhydramine HCl were gifts from Smith Kline and France Labs (Welwyn Garden City, Herts, UK). Methysergide maleate was a gift from Sandoz Labs (Hanover, NJ). All other organic and inorganic chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and were of the highest purity.

2.5. Calculations and statistical analyses

The contractile responses to ·OH were expressed as a percentage of the maximum response of that tissue to a 15-min exposure to 80 mM KCl and the results are expressed as the means±standard error of the means (S.E.M). Statistical evaluation of the results was carried out by analysis of paired t-tests and analysis of variance (ANOVA). The results were considered significant at a P-value of <0.05. All experiment protocols employed at least four to six to different animals.

3. Results

3.1. Contractile effects of ·OH on rat aortic rings

In the absence of any vasoactive agent, ·OH alone induced contractile effects on rat aortic rings. Fig. 1 shows a typical tracing of the vasoconstrictor effect of ·OH on rat aortic rings with and without endothelium. As shown in Fig. 2, ·OH radicals elicit concentration-dependent contractions of rat aortic rings at concentrations of from 10 to 400 μM. In addition, a comparison of the concentration–

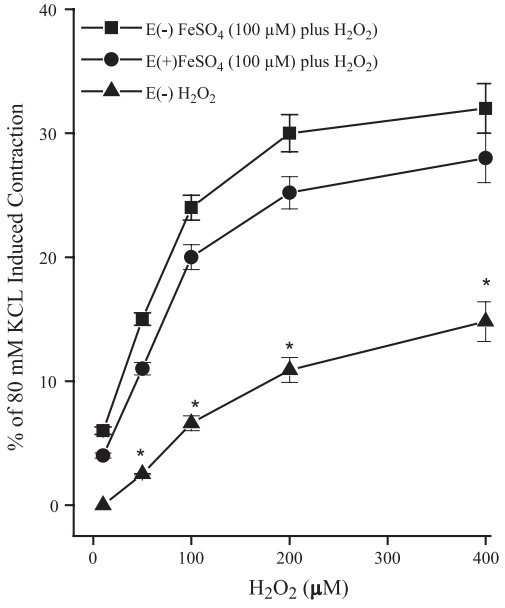

Fig. 2. Contractile concentration–effect curves to ·OH and H_2O_2 on rat aortic rings in the absence or presence of endothelium. Values are expressed as means±S.E. No paired points are significantly different from one another at p>0.05 (paired t-test). +EC=tissue with endothelial cells, −EC=tissue without endothelial cells.

response curves with and without endothelium demonstrates that most of the contractile effects exerted by the generated ·OH on rat aortic rings is endothelium-independent (Fig. 2). It is also shown in Fig. 2 that H_2O_2-produced contractions are significantly weaker than those produced by ·H. H_2O_2 can not account for more than 35% of the ·OH-induced contractions. Although it is quite conceivable that ·OH might slightly affect the endothelium (Fig. 1), the current study was not designed to address this possibility further. In the current study, therefore, most of the following experiments were carried out on endothelium-denuded aorta. Fig. 3 demonstrates that the Fenton reaction, used in the present study, elicited contractions that were impaired significantly in the presence of the hydroxyl radical scavenger sodium formate (Fig. 3). Furthermore, catalase did not influence the hydroxyl radical-induced contraction when it was added as the contractile effects reached stable levels (data not shown, n=4). These experiments indicate that the vascular contractions are mainly due to ·OH generation. It should be noted here that the vasoconstrictor effects of ·OH on rat aortic rings were neither reversible nor reproducible on the same ring, which were totally different from the effects of H_2O_2 (Fig. 4). Tachyphylaxis is seen even at threshold levels of ·OH generation (data not shown).

3.2. Effects of extra-and intracellular calcium on hydroxyl radical-induced vasoconstriction of denuded rat aortic rings

The relationship between ·OH-induced contraction and Ca^{2+} was next examined in this study. As shown in Fig. 5,

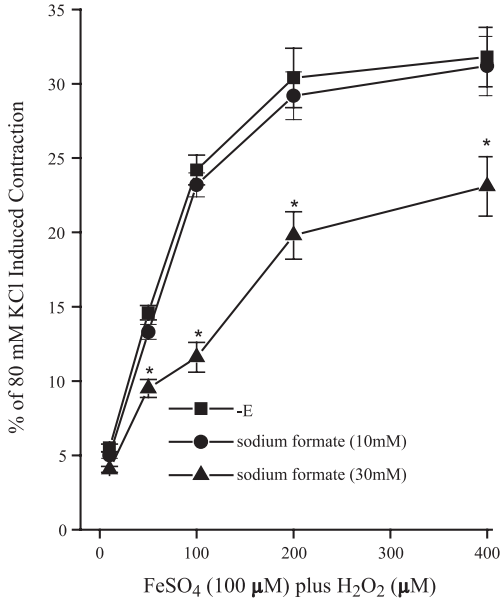

Fig. 3. Contractile concentration–effect curves to ·OH on rat aortic rings in the absence of endothelium, obtained in the presence of sodium formate. Values are expressed as means±S.E. as percentage of the tension developed by 80 mM KCl; n=4 each. *p<0.05 compared to control (paired t-test).

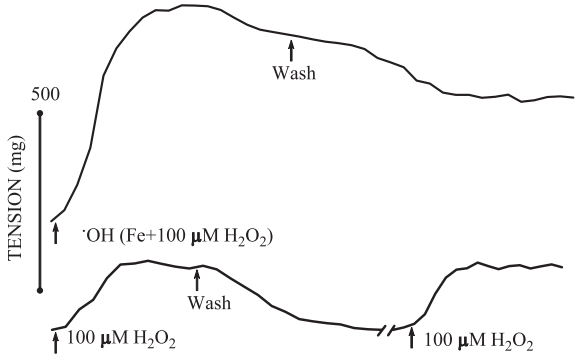

Fig. 4. A comparison of contractile responses of rat aortic rings to ·OH and H_2O_2. Note that it is impossible to fully relax contractions induced by ·OH, whereas those produced by H_2O_2 are relaxed and are completely reproducible.

removal of either extracellular Ca^{2+} from the bathing medium or use of buffered intracellular Ca^{2+}, by preincubation of the tissues in 10 μM BAPTA-AM, produced almost complete inhibition of the effects of hydroxyl radical-induced contraction on denuded rat aortic rings. These results suggest the hydroxyl radical-induced contractions on rat aortic rings are both extracellular- and intracellular calcium-dependent.

3.3. Effects of protein kinase C (PKC) antagonists on hydroxyl radical-induced vasoconstriction of denuded rat aortic rings

Fig. 6 shows that pretreatment of denuded rat aortic rings with staurosporine, or bisindolylmaleimide I (both potent antagonists of PKC), or Gö6976 (a PKC_{α}-and $PKC_{\beta 1}$-selective antagonist), for 20 min, significantly decreased hydroxyl radical-induced vasoconstriction. These three PKC antagonists were all dissolved in DMSO. The DSMO concentrations applied in these experiments were <0.1 μM

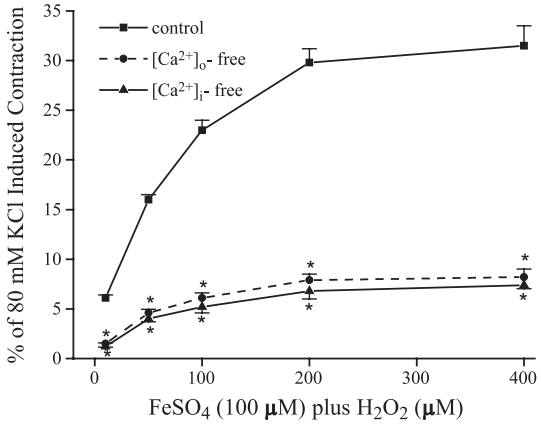

Fig. 5. Contractile effects to ·OH radicals on rat aortic rings in the absence of endothelium, obtained in the absence of extracellular Ca^{2+} and intracellular Ca^{2+}. Values are expressed as means±S.E. as percentage of the tension developed by 80 mM KCl; n=4 each. *p<0.05 compared to control (paired t-test).

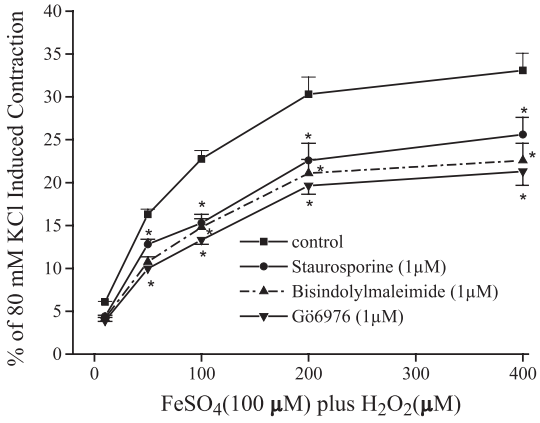

Fig. 6. Contractile effects to •OH on rat aortic rings in the absence of endothelium, obtained in the presence of staurosporine, Gö6976 or bisindolylmaleimide I HCl. Values are expressed as means±S.E. as percentage of the tension developed by 80 mM KCl; n=4 each. *p<0.05 compared to control (paired t-test).

for staurosporine, and bisindolylmaleimide I and around 7.5 μM for Gö6976. In control experiments, equivalent amounts of DMSO were employed and exerted no effects on hydroxyl radical-induced vasoconstriction (n=4, data not shown).

3.4. Effects of PD-980592, genistein, and wortmannin on hydroxyl radical-induced vasoconstriction of denuded rat aortic rings

Fig. 7 illustrates that pretreatment of denuded rat aortic rings with PD-980592 (an antagonist of mitogen-activated protein kinase), genistein (an antagonist of protein tyrosine kinase) or wortmannin [an antagonist of phosphatidylinositol 3-kinases (PI_3Ks)] for 20 min significantly suppressed hydroxyl radical-induced vasoconstriction of denuded rat aortic rings.

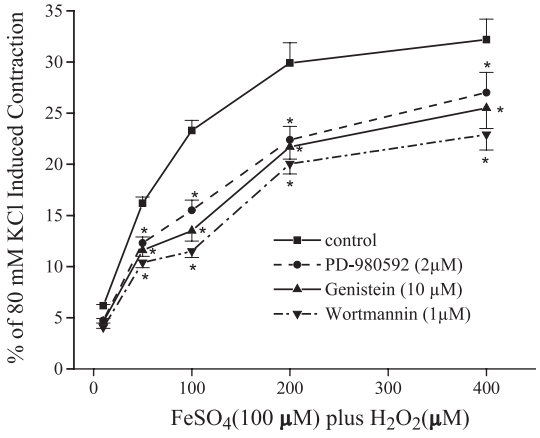

Fig. 7. Contractile effects to •OH radicals on rat aortic rings in the absence of endothelium, obtained in the presence of PD-980592, genistein and wortmannin. Values are expressed as means±S.E. as percentage of the tension developed by 80 mM KCl; n=4 each. *p<0.05 compared to control (paired t-test).

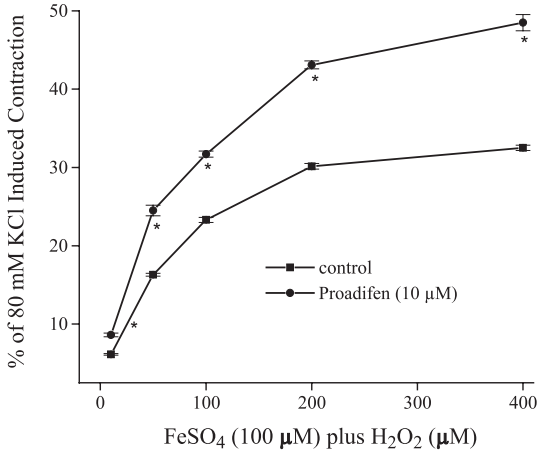

Fig. 8. Contractile effects to •OH on rat aortic rings in the absence of endothelium, obtained in the presence of proadifen. Values are expressed as means±S.E. as percentage of the tension developed by 80 mM KCl; n=4 each. *p<0.05 compared to control (paired t-test).

3.5. Effects of proadifen on hydroxyl radical-induced vasoconstriction of denuded rat aortic rings

Fig. 8 indicates that preincubation of the vessels with proadifen for 20 min significantly potentiated the hydroxyl radical-induced vasoconstrictions of denuded rat aortic rings by shifting the concentration–response curves to the left.

3.6. Effects of •OH on $[Ca^{2+}]_i$ in isolated aortic smooth muscle cells

The effects of hydroxyl radical generation on $[Ca^{2+}]_i$ in primary cultured aortic smooth muscle cells, isolated from rat aorta, were examined by means of the direct technique of Ca^{2+} visualization in single cells as revealed by the digital imaging microscope using fura 2-AM. As shown in Fig. 8, the control basal level for $[Ca^{2+}]_i$ was 100±3.2 nM. Generation of •OH in the tissue baths triggered a rapid

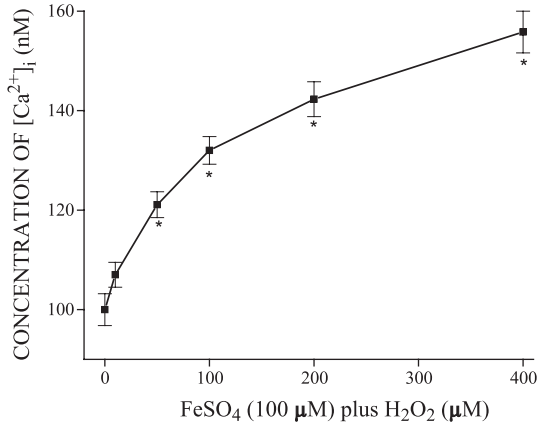

Fig. 9. Generation of •OH radicals results in rapid elevation in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration in rat aortic smooth muscle cells. The data were obtained 30 s after treatment with •OH. Each result indicates the mean±S.E. (n=8–10).

·OH concentration-dependent increase in $[Ca^{2+}]_i$. These responses for elevation in $[Ca^{2+}]_i$ were initiated quite rapidly, i.e., within 10–30s after addition of Fe^{2+} plus H_2O_2 (Fig. 9).

3.7. Variety of pharmacologic antagonists fail to attenuate or inhibit the contractile action of ·OH radicals on rat aortic rings

Incubation of rat aortic rings with a wide variety of specific amine, opiate and prostanoid antagonists (i.e., 10^{-6} M diphenhydramine, 10^{-5} M cimetidine, 10^{-6} M phentolamine, 10^{-6} M methysergide, 10^{-5} M propranolol, 10^{-6} M atropine, 10^{-5} M naloxone and, 10^{-5} M indomethacin) failed to either attenuate or interfere with contractions induced by ·OH radicals (data not shown, n=4–6 each). Likewise, these antagonists failed to attenuate the rises in $[Ca^{2+}]_i$ produced by ·OH radicals (n=30–60 cells each, data not shown).

4. Discussion

Various sources of electrons in vivo, such as the thiol group of cysteine or the reduced form of vitamin C, can rapidly reduce oxygen to form oxygen radicals, i.e., one-, two-, and three-electron reductions of O_2 yield the superoxide anion ($O_2^{\bullet -}$), H_2O_2, and ·OH, respectively. Incompletely reduced forms of oxygen are extremely reactive. These molecules constitute powerful oxidizing agents that possess high toxicity for living systems. The subject of this study, i.e., the hydroxyl radical, represents the most potent oxidizing agent known and the most active mutagen produced by ionizing radiation.

The present study demonstrates that generation of ·OH produces vasoconstriction of isolated rat aortic rings in a concentration-dependent manner. The ·OH-elicited contraction was significantly impaired in the presence of the specific hydroxyl radical scavenger sodium formate, suggesting that most of the contraction is due to generation of ·OH (Shen et al., 1997). The fact that almost full contractile actions on the rat aortic rings are observed, after removal of the endothelium, indicates that most, if not all, of the ·OH-induced contractile are independent of endothelial cell-derived mediators. Moreover, the current work also demonstrates that the ·OH-produced contractions are not reproducible and elicit tachyphylaxis in any one ring (with or without endothelium). What is of singular interest, here, is that even generation of threshold levels of ·OH radicals results in tachyphylaxis. This indicates the action of ·OH is not reversible and probably leads to permanent vascular damage. This is currently under investigation in our lab.

At present, the underlying mechanisms whereby ·OH triggers vasoconstriction are poorly understood. The current work was designed to better define the effects of ·OH on rat aortic rings and to probe the relationships between contractility of vessels induced by generation of ·OH, Ca^{2+}, possible signaling pathways and some metabolites generated by ·OH exposure. It was also of interest to design the current study in a way to be able to compare potential mechanisms of action of ·OH and H_2O_2 on rat aortic smooth muscle.

It has been well documented that cytosolic calcium increases during contraction of vascular smooth muscles either as a consequence of influx of Ca^{2+} from the extracellular space or release of Ca^{2+} from intracellular stores. Conversely, relaxation is usually accompanied by a decrease in the cytosolic Ca^{2+} level in smooth muscle cells (Horowitz et al., 1996). In this study, removal of extracellular Ca^{2+} as well as buffering intracellular Ca^{2+} with the membrane-permeable Ca^{2+} chelator BAPTA-AM both inhibited most of the contractile actions of ·OH on rat aortic rings. In addition, our new experimental data also show that ·OH radicals induced a rapid, significant concentration-dependent increase in $[Ca^{2+}]_i$ in the primary cultured smooth muscle cells. This suggests that the passage of extracellular calcium ions across the vascular smooth membranes as well as a release of intracellular free calcium ions from intracellular stores probably play an essential role in the ·OH-induced contractile effects. This is in agreement with a previous conclusion that ·OH can enhance the voltage-dependent influx of Ca^{2+} in rabbit lingual smooth muscle cells (Sasaki and Okabe, 1993) and compares favorably to the actions of H_2O_2 on rat aortic rings (Yang et al., 1998) and cerebral arterial smooth muscle cells (Yang et al., 1999).

Multiple cellular signal pathways are known to participate in mechanisms of vasoconstriction, such as activation of protein kinase C (PKC), protein tyrosine kinase, mitogen-activated protein kinase (MAPK kinase), and PI3Ks. Vascular smooth muscle cells after exposure to ·OH may result in release of a variety of vasoactive substances that in turn modulate vascular tone. To assess the involvement of such possible substances in ·OH-induced contraction, several inhibitors as well signaling pathway inhibitors were employed in the present study. A variety of pharamacologic amine antagonists, opiate antagonist and a cox-I inhibitor failed to inhibit the contractile action of ·OH radicals, suggesting that endogenous formation or release of catecholamines, histamine, serotonin, acetylcholine, opiates, or prostanoids do not play any role in ·OH-induced contractile actions.

PKC, a family of Ca^{2+}-sensitive and Ca^{2+}-insensitive phospholipid-dependent protein kinases, that are present in high concentrations in vascular smooth muscle, have been shown to play important roles in cellular signal transduction (Kariya and Takai, 1987; Yang et al., 2001a,b). However, up until the present study, no experimental evidence has appeared on the possible role of PKC in ·OH-induced contractile effects on rat aortic rings. In the present work, ·OH-induced vasoconstrictions were found to be significantly reduced in the

presence of two potent PKC antagonists (bisindolylmaleimide I and staurosporine) and one selective PKC_{α} and $PKC_{\beta 1}$ antagonist (Gö6976). This suggests that PKC isoforms, probably PKC_{α} (possibly $PKC_{\beta 1}$ as well) may be a pivotal signaling mechanism by which ·OH acts on the aortic smooth muscle cells to promote vasoconstriction. This is, thus, similar to that found for H_2O_2 in rat aortic rings and cerebral arteries (Yang et al., 1998, 1999).

It has been reported that PI3Ks are a group of enzymes that act directly to provide biochemical links between a novel phosphatidylinositol pathway and non-receptor tyrosine kinases that appear to modulate Ca^{2+} channels through activity of these PI3Ks (Blair and Marshal, 1997). Up to now, there has been no information regarding PI3Ks and ·OH-induced vasoconstriction on any vessel type. In this paper, we report that one potent antagonist of PI3Ks (wortmannin) markedly attenuated ·OH-induced contractions, suggesting the possible involvement of products of PI3Ks in such responses. Recently, H_2O_2-induced contractions were found to be somewhat dependent on PI3Ks as well (Yang et al., 1998, 1999, 2001a).

Previous studies have shown that protein tyrosine phosphorylation is an essential component in signal transduction pathways in smooth muscle cells (Malarkey et al., 1995; Albrecht and Tidball, 1997). Our present data also demonstrates that genistein, an inhibitor of protein tyrosine kinase (Albrecht and Tidball, 1997), which is an enzyme for catalyzing protein tyrosine phosphorylation, significantly suppressed the ·OH-induced vasoconstriction, which indicates the likely involvement of protein tyrosine phosphorylation in contractions caused by ·OH. The conclusion is supported by other investigators who have demonstrated that oxidative stress can promote protein tyrosine phosphorylation in smooth muscle cells (Baas and Berk, 1995; Yang et al., 1998, 1999, 2000, 2001a,b).

Several recent studies have been reported which indicate MAPK responds to diverse stimuli, including reactive oxygen species, and can transduce signals from the cell membrane to the nucleus (Blumer et al., 1994; Post and Brown, 1996; Yang et al., 1998, 1999, 2000, 2001b; Li et al., 2003; Touyz and Yao, 2003). Activation of MAPK plays an important role in modulating vascular tone (Yang et al., 1998, 1999, 2000, 2001b). In this investigation, the specific MAPK antagonist, PD-980592 (Rao, 1997), significantly prevented ·OH-induced contractions in rat aortic rings. This suggests that activation of MAPK is probably an important pathway in ·OH-mediated action on rat aortic smooth muscle cells.

It is known that ·OH can induce contraction of rabbit tracheal smooth muscle cells, which is thought to be mediated in part by arachidonic acid metabolites (Prasad and Gupta, 1993). Observations in the current work show that indomethacin (an inhibitor of prostanoid generation) failed to inhibit ·OH-induced contractions, but that proadifen, an inhibitor of cytochrome *P*450 monooxygenase, which is an arachidonic acid-metabolizing enzyme (Oyekan et al., 1994), significantly augmented ·OH-induced contractions. This result provides suggestive evidence that release of cytochrome *P*450-dependent metabolites may contribute to the ·OH-induced events seen herein. This appears to be similar to our previous results obtained with H_2O_2-induced contractile effects on rat aortic rings (Yang et al., 1998). Although the exact reason for the enhancement of contraction by proadifen remains to be determined, it is likely that ·OH radicals may stimulate not only the synthesis and release of cyclooxygenase products but also the production of metabolites catalyzed by cytochrome *P*450-dependent enzymes. The precise mechanism(s) whereby inhibition of cytochrome *P*450 augment ·OH-induced contraction in rat aortic smooth muscle needs to be studied further.

In summary, the data from the present investigation suggest that ·OH radicals induce contractile actions on rat aortic rings, but not relaxation on rat aortic rings. These ·OH-induced contractions are mediated via the activation of several well-known signal transduction pathways, and elevation of intracellular free Ca^{2+} levels, and are independent of the endothelium and release of endogenous amines, prostanoids or opiates. Production of, and contact, of at least rat aorta with this most highly reactive oxygen species, even in low concentration, appears to result in irreversible damage of the aortic smooth muscle cells. Such findings are thus quite different from those observed for H_2O_2, $O_2^{\bullet -}$, or peroxynitrite (Baas and Berk, 1995; Rubanyi, 1988; Yang et al., 1998, 1999; Shen et al., 2000; Li et al., 2004) and requires further investigation. Lastly, if our new findings hold for the peripheral microvasculature, they could be suggestive of a very important role for generation of ·OH radicals in regulation of the microvasculature and its pathophysiology.

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