Hypertension Increases Contractile Responses to Hydrogen Peroxide in Resistance Arteries through Increased Thromboxane A₂, Ca²⁺, and Superoxide Anion Levels

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ABSTRACT

This study investigated the mechanisms underlying the response to hydrogen peroxide (H₂O₂) in mesenteric resistance arteries from spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto (WKY) rats. Arteries were mounted in microvascular myographs for isometric tension recording and for simultaneous measurements of intracellular Ca²⁺ concentration ([Ca²⁺]), superoxide anion (O₂⁻) production was evaluated by dihydroethidium fluorescence and confocal microscopy, and thromboxane A₂ (TXA₂) production was evaluated by enzyme immunocassay. H₂O₂ (1–100 μM) induced biphasic responses characterized by a transient endothelium-dependent contraction followed by relaxation. Simultaneous measurements of tension and Ca²⁺ showed a greater effect of H₂O₂ in arteries from hypertensive than normotensive rats. The cyclooxygenase (COX) inhibitor, indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1-H-indole-3-acetic acid] (1 μM), the COX-1 inhibitor, SC-58560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole] (1 μM); the thromboxane (TXA₂) synthase inhibitor, furegrelate [5-(3-pyridinylmethyl)-2-benzofurancarboxylic acid, sodium salt] (10 μM); and the TXA₂/prostaglandin H₂ receptor antagonist, SQ 29,548 ([1S-1-[1.α,2.α,2.β,3.α,3.α,4.α]-7-3-[2-[[(phenylamino) carbonyl] hydrazino] methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid]) (1 μM) abolished H₂O₂ contraction in arteries from WKY rats but only reduced it in SHRs. The O₂⁻ scavenger, tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) (1 mM), and the NADPH oxidase inhibitor, apocynin (4′-hydroxy-3′-methoxycacetophenone) (0.3 mM), decreased H₂O₂ contraction in arteries from SHRs but not in WKY rats. H₂O₂ induced TXA₂ and O₂⁻ production that was greater in SHRs than in WKY rats. The TXA₂ analog, U46619 [9,11-di-deoxy-11α,9α-epoxymethano prostaglandin F₂α (0.1 nM–1 μM)], also increased O₂⁻ production in SHR vessels. H₂O₂-induced TXA₂ production was decreased by SC-58560. H₂O₂-induced O₂⁻ production was decreased by tiron, apocynin, and SQ 29,548. In conclusion, the enhanced H₂O₂ contraction in resistance arteries from SHRs seems to be mediated by increased TXA₂ release from COX-1 followed by elevations in vascular smooth muscle [Ca²⁺]i levels and O₂⁻ production. This reveals a new mechanism of oxidative stress-induced vascular damage in hypertension.

Reactive oxygen species (ROS) like superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) have been suggested as mediators of vascular structural and functional alterations observed in hypertension (Lacy et al., 2000; Paravicini and Touyz, 2006; Alvarez et al., 2007). Several sources of O₂⁻ have been described. Among them, xanthine oxidase, uncoupled nitric-oxide synthase, and cyclooxygenase (COX) can produce O₂⁻ in different conditions (Touyz, 2003). However, it is well established that at the vascular level, NADPH oxidase is the main source of O₂⁻ (Touyz, 2003; Lyle and Griendling, 2006). Dismutation of O₂⁻ by superoxide dismutase produces H₂O₂, a main source of O₂⁻ that can also be produced by COXs, contributing to the oxidative stress in vascular disease.
more stable reactive oxygen specie that is considered an important second messenger in smooth muscle cell signaling and hypertrophy. However, its role in vascular tone is controversial (Ardanaz and Paganò, 2006). Several studies have reported vasodilator, vasoconstrictor, or both vasodilator and vasoconstrictor effects of H$_2$O$_2$ depending on the species, vascular bed, and contractile state. Thus, in contracted conductance vessels, H$_2$O$_2$ induces vasorelaxation through activation of guanylate cyclase (Burke-Wolin et al., 1991) or through opening of voltage-dependent (Gao et al., 2003), Ca$^{2+}$-activated (Iida and Katusic, 2000), or ATP-sensitive (Wei et al., 1996) K$^+$ channels. However, H$_2$O$_2$ also induces contraction in a variety of vessels (Jin and Rhoades, 1997; Rodríguez-Martínez et al., 1998; Yang et al., 1998, 1999; Pelaez et al., 2000; Gao and Lee, 2001; Hernanz et al., 2003; Gil-Longo and Gonzalez-Vazquez, 2005). Other investigators have shown that H$_2$O$_2$ induces biphasic responses characterized by a contractile response followed by relaxation (Leffler et al., 1990; Gao et al., 2003; Gil-Longo and Gonzalez-Vazquez, 2005). The mechanisms involved in H$_2$O$_2$-induced vasoconstriction seem to depend on smooth muscle Ca$^{2+}$ influx (Ardanaz and Paganò, 2006), and in vascular smooth muscle cells, it has been described that H$_2$O$_2$ increases the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) (Yang et al., 1999; Tabet et al., 2004). The intracellular pathways proposed to be involved in H$_2$O$_2$ vasoconstriction include activation of COX (Rodríguez-Martínez et al., 1998; Gao and Lee, 2001), protein kinase C (Yang et al., 1999), mitogen-activated protein kinases (Thakali et al., 2007), and tyrosine kinases (Jin and Rhoades, 1997). In addition, it has been described that both H$_2$O$_2$ and TXA$_2$ induce O$_2^•$ production (Li et al., 2001; Muzaffar et al., 2004; Cogollo et al., 2006). However, whether this O$_2^•$ contributes to the effect of H$_2$O$_2$ is unknown.

Hypertension increases vasoconstrictor responses to H$_2$O$_2$ in rat aorta (Rodríguez-Martínez et al., 1998) and superior mesenteric artery (Gao and Lee, 2001). The enhanced H$_2$O$_2$ contraction was found to be mediated by augmented TXA$_2$ release (Gao and Lee, 2001). Increased vascular resistance plays an important role in hypertension, but it is unclear whether H$_2$O$_2$ vasoconstriction is changed in peripheral resistance arteries. In this study, we tested the hypothesis that hypertension increases H$_2$O$_2$ contraction in resistance arteries by mechanisms that involve increase in TXA$_2$ and O$_2^•$ production and changes in smooth muscle [Ca$^{2+}$]$_i$. Therefore, we examined in mesenteric resistance arteries (MRAs) from hypertensive and normotensive rats: 1) the effect of H$_2$O$_2$ on vascular tone, [Ca$^{2+}$]$_i$, and vascular TXA$_2$ and O$_2^•$ production; and 2) the participation of TXA$_2$ and O$_2^•$ in vasoactive responses induced by H$_2$O$_2$.

**Materials and Methods**

**Animals and Tissue Preparation.** Male spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto (WKY) rats were obtained from colonies maintained at the Animal Quarters of the Facultad de Medicina of the Universidad Autónoma de Madrid and from Møllegaard Breeding Center (Skensved, Denmark). All work with rats was performed by using the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and complies with the current Spanish and European laws (RD 223/88 MAPA and 609/96). Rats were euthanized by decapitation, and the mesenteric vascular bed was removed and placed in cold (4°C) Krebs-Henseleit solution (KHS) (115 mM NaCl, 25 mM NaHCO$_3$, 4.7 mM KCl, 1.2 mM MgSO$_4$, 7H$_2$O, 2.5 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 11.1 mM glucose, and 0.01 mM Na$_2$EDTA) bubbled with a 95% O$_2$/5% CO$_2$ mixture.

Segments of third-order branches of the mesenteric artery were dissected free of fat and connective tissue and used for reactivity, simultaneous measurements of [Ca$^{2+}$]$_i$, and tension, and O$_2^•$ production studies. The second- and third-order branches were used for TXA$_2$ production studies.

**Reactivity Experiments.** Ring segments, 2 mm in length, were mounted in a small-vessel dual chamber myograph for measurement of isometric tension. Two steel wires (40-μm diameter) were introduced through the lumen of the segments and mounted according to the method described by Mulvany and Halpern (1977).

After a 30-min equilibration period in oxygenated KHS at 37°C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference/wall tension ratio of the segments by setting their internal circumference, Lo, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mm Hg (Mulvany and Halpern, 1977). Segments were washed with KHS and left to equilibrate for 30 min; contractility of segments was then tested by an initial exposure to a high-K$^+$ solution (120 mM K$^+$-KHS), which was identical to KHS, except that NaCl was replaced by KCl on an equimolar basis.

The presence of endothelium was determined by the ability of 10 μM acetylcholine to induce relaxation in arteries precontracted with phenylephrine to achieve a contractile response of approximately 50% K$^+$-KHS contraction. In some segments, endothelium was removed as described previously (Osol et al., 1989). Endothelium removal was assessed by the inability of 10 μM acetylcholine to produce vasodilation.

In previous experiments, we have observed that, in MRAs, H$_2$O$_2$ does not induce vascular responses in quiescent arteries. Therefore, we performed the experiments in phenylephrine-contracted vessels, as described previously (Gao et al., 2003). Thus, once the presence of endothelium was determined, a single cumulative concentration-response curve for H$_2$O$_2$ (1–100 μM) was performed in segments contracted with phenylephrine to achieve a contractile response of approximately 50% K$^+$-KHS contraction. The participation of COX-derived prostanooids was analyzed using indomethacin, SC-58560, SQ 29,548, or furegrelate. The participation of O$_2^•$ was evaluated by preincubation of the arteries with tiron or apocynin. All drugs were added 30 min before the H$_2$O$_2$ concentration-response curve. At the end of the experiment, the viability of the segments was tested by its resistance to a high-K$^+$ solution (120 mM K$^+$-KHS, data not shown).

**Measurements of TXA$_2$ Production.** Arteries from WKY rats and SHRs were incubated in oxygenated KHS (37°C) and equilibrated for 30 min. Phenylephrine (1 μM) was added for 4 min, and then cumulative doses of H$_2$O$_2$ (1–100 μM, 4 min each dose) were added to the incubation medium in the absence or in the presence of SC-58560 or NS-398. At the end, the medium was collected and frozen (−80°C) for further analysis. The levels of the metabolite of TXA$_2$, TBX$_2$, were determined using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

**Simultaneous Measurements of [Ca$^{2+}$]$_i$ and Tension.** Simultaneous measurements of [Ca$^{2+}$]$_i$ and tension were performed in intact arterial segments by Fura 2-AM fluorescence, as described previously (Jensen et al., 1992). In brief, the arteries were loaded in the dark in PSS (119 mM NaCl, 4.7 mM KCl, 1.18 mM KH$_2$PO$_4$, 1.17 mM MgSO$_4$, 1.5 mM CaCl$_2$, 24.9 mM NaHCO$_3$, 0.027 mM EDTA, 11 mM glucose, pH 7.4) containing 8 μM Fura 2-AM and 0.05% Cremophor EL for 60 min at 37°C. Thereafter, arteries were washed three times in PSS, and the solution was changed to PSS with fresh Fura 2-AM for 60 min.

The myograph chamber was mounted on a Zeiss inverted microscope equipped for dual-excitation wavelength fluorometry (DeltaScan, Photon Technology International, Lawrenceville, NJ). The ar-
teries were illuminated with alternating 340 and 380 nm of light, and the intensity of the emitted fluorescence was collected at a wavelength of 510 nm using a photomultiplier and monitored together with the tension recording (Prieto et al., 2000; Villalba et al., 2007). At the end of each experiment, Ca\(^{2+}\)-insensitive signals were determined after quenching with Mn\(^{2+}\), and the values obtained were subtracted from those obtained during the experiment. The ratio (R) of fluorescence at 340 and 380 (F\(_{340}\)/F\(_{380}\)) corrected for autofluorescence, was taken as a measurement of [Ca\(^{2+}\)]\(_i\).

The experimental protocol was as follow. Arteries were first stimulated with K\(^+\)-KHS; once the presence of endothelium was determined, a cumulative concentration-response curve for H\(_2\)O\(_2\) (1–100 \(\mu\)M) was performed in segments contracted with phenylephrine. In another set of experiments, a concentration-response curve for the TXA\(_2\)/prostaglandin H\(_2\) analog, U46619 (0.1 nM–1 \(\mu\)M), was performed in phenylephrine-contracted arteries. The net contraction and [Ca\(^{2+}\)]\(_i\), increase induced by H\(_2\)O\(_2\) were measured at each concentration. In the case of U46619, the cumulative contraction and [Ca\(^{2+}\)]\(_i\), increase were measured.

Detection of Superoxide Anion Production. The oxidative fluorescent dye dihydroethidium was used to evaluate in situ O\(_2^-\) production. Intact MRAs from WKY rats and SHRs were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (130 mM NaCl, 5.6 mM KCl, 2 mM CaCl\(_2\), 0.24 mM MgCl\(_2\), 8.3 mM HEPES, 11 mM glucose, pH 7.4). Afterward, dihydroethidium (2 \(\mu\)M, 30 min, 37°C) was added to the incubation buffer in a light-protected atmosphere. Arteries were then mounted in a glass-bottomed microwell dish and viewed with a fluorescent laser scanning confocal microscope (Leica TCS SP2; Leica, Wetzlar, Germany). In some experiments, arteries were incubated with tiron, apocynin, or SQ 29,548 for 30 min before imaging. Stacks of images (7.5-\(\mu\)m-thick serial optical slices) were taken from the adventitia to the lumen in basal conditions. Next, phenylephrine (1 \(\mu\)M) was added for 2 min to the arteries, and a second stack of images was captured. Thereafter, cumulative doses of H\(_2\)O\(_2\) (1–100 \(\mu\)M, 2 min each dose and 5 min last dose) were added, and images were collected for each dose. All images were taken using the same imaging settings in each case. Vessels from untreated and tiron-, apocynin-, or SQ 29,548-treated arteries were imaged every day in parallel. In another set of experiments, cumulative doses of U46619 (10 nM–3 \(\mu\)M, 2 min each dose) were added to phenylephrine-contracted SHR arteries in the absence and in the presence of SQ 29,548. Images were collected as described above. Fluorescence was detected with a 568-nm-long pass filter. For quantification, maximal intensity projections were obtained, and the H\(_2\)O\(_2\)- or U46619-induced fluorescence above the phenylephrine fluorescence levels was calculated.

Western Blot Analysis. Arteries from WKY rats and SHRs were incubated in oxygenated KHS (37°C) and equilibrated for 30 min. Phenylephrine (1 \(\mu\)M) was added for 4 min, and then cumulative doses of H\(_2\)O\(_2\) (1–100 \(\mu\)M, 4 min each dose) were added to the incubation medium. At the end, arteries were frozen at −80°C. COX-1 protein expression was determined in homogenates from these arteries (35 \(\mu\)g of protein), as described previously (Alvarez et al., 2005). In brief, proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinyl difluoride membranes overnight. Membranes were incubated with rabbit polyclonal antibody for COX-1 (1:100; Cayman Chemical) and mouse polyclonal antibody for \(\alpha\)-actin (1:250,000; Sigma-Aldrich, St. Louis, MO). After washing, membranes were incubated with anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:5,000; Bio-Rad, Hercules, CA). The immunocomplexes were detected using an enhanced horseradish peroxidase-luminol chemilu-

Fig. 1. A, typical tracings showing the contractile effects of H\(_2\)O\(_2\) in mesenteric resistance arteries from SHRs. B, concentration-response curves for H\(_2\)O\(_2\) in mesenteric resistance arteries from WKY rats and SHRs. Results are expressed as a percentage of the maximal response. ANOVA (two-way); *, \(p < 0.0001\) versus WKY rats. Number of animals, 54 to 61.
minescence system (ECL Plus; GE Healthcare, Chalfont St. Giles, UK) and subjected to autoradiography (Hyperfilm ECL; Amersham). Signals on the immunoblot were quantified using the NIH Image computer program (version 1.56).

**Drugs and Solutions.** H2O2, indomethacin, acetylcholine chloride, phenylephrine hydrochloride, furegrelate, U46619, tiron, and apocynin were obtained from Sigma-Aldrich. NS-398 was obtained from Calbiochem (San Diego, CA). Fura 2-AM was obtained from Invitrogen (Carlsbad, CA). SQ 29,548 was obtained from ICN Iberica (Barcelona, Spain). SC-58560 was donated by Laboratorios Menarini (Barcelona, Spain). Indomethacin was dissolved in NaHCO3 (5%), and U46619 and SC-58560 were dissolved in ethanol. In both cases, further dilutions were in distilled water.

**Data Analysis and Statistics.** Contractile responses for each dose are expressed as a percentage of contraction to K+-KHS. The maximal response (E\text{max}) and pD\textsubscript{2} values for H2O2 contraction were calculated by a nonlinear regression analysis of each individual concentration-response curve using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). All data are expressed as mean values ± S.E.M. Results were analyzed using Student's t test or two-way ANOVA. A p value below 0.05 was considered significant.

**Results**

The effective lumen diameter, calculated as Lo/π, was smaller in segments from hypertensive rats (248 ± 6 µm, n = 61, p < 0.05) than from normotensive rats (272 ± 8 µm, n = 54). The maximal response to K+-KHS was similar in arteries from both strains (WKY rats, 2.9 ± 0.2 mN/mm, n = 54; SHRs, 3.1 ± 0.2 mN/mm, n = 61). The acetylcholine relaxation was smaller (p < 0.001) in arteries from SHRs (76 ± 2%, n = 61) compared with WKY rats (88 ± 2%, n = 54).

In phenylephrine-contracted MRAs, H2O2 (1–100 µM) induced a biphasic response characterized by a transient contraction at each concentration, followed by relaxation (Fig. 1A). The contractile response induced by H2O2 was greater in arteries from hypertensive than normotensive rats (Fig. 1B). H2O2 contraction was reduced after endothelium removal in arteries from both WKY rats and SHRs (Table 1).

**Role of COX-Derived Prostanoids in H2O2 Contraction.** In arteries from WKY rats, the nonselective COX inhibitor, indomethacin (1 µM), and the selective COX-1 inhibitor, SC-58560 (1 µM), almost fully abolished the contractile response to H2O2. However, in arteries from SHRs, both indomethacin and SC-58560 only reduced H2O2 contraction (Fig. 2A). These results suggest that a COX-1-derived contractile prostanoid contributes to H2O2 contraction. To determine whether TXA\textsubscript{2} is the prostanoid involved in the H2O2 contraction, the arteries were incubated with the TXA\textsubscript{2} synthase inhibitor, furegrelate (10 µM), or with the TXA\textsubscript{2}/prostaglandin H\textsubscript{2} receptor antagonist, SQ 29,548 (1 µM). Similarly to indomethacin and SC-58560, both drugs almost...
abolished the contractile response to H$_2$O$_2$ in arteries from WKY rats and reduced H$_2$O$_2$ contraction in arteries from SHRs (Fig. 2B). The removal of endothelium did not modify the inhibitory effect of SQ 29,548 on H$_2$O$_2$ contraction in arteries from WKY rats (inhibition of E$_{\text{max,E}}$, 72.3 ± 4.3%, n = 11; E$_{-}$, 75.8 ± 8.0%, n = 4; p > 0.05) and from SHRs (inhibition of E$_{\text{max,E}}$, 55.3 ± 10.8%, n = 13; E$_{-}$, 58.4 ± 9.8%, n = 4, p > 0.05).

Basal TXB$_2$ levels were greater in arteries from SHRs (185.2 ± 29.5 ng/μg protein, n = 12) compared with WKY rats (74.2 ± 21 ng/μg protein, n = 10, p < 0.01). H$_2$O$_2$ increased TXB$_2$ production more in arteries from SHRs than WKY rats (Fig. 3A). This increase was abolished by incubation of the arteries with SC-58560 but was not modified by the COX-2 inhibitor, NS-398 (1 μM) (Fig. 3A). COX-1 protein expression levels were similar in both strains, and this expression was not modified by H$_2$O$_2$ (Fig. 3B).

Simultaneous Measurements of [Ca$^{2+}$]$_i$ and Tension. Either K$^+$-KHS or phenylephrine increased [Ca$^{2+}$]$_i$ and tension to similar levels in arteries from WKY rats and SHRs (Fig. 4A). Cumulative application of H$_2$O$_2$ (1–100 μM) induced a concentration-dependent transient increase in [Ca$^{2+}$]$_i$ coupled to the transient increase in tension (Fig. 4A). Both responses were greater in arteries from SHRs compared with WKY rats (Fig. 4B). Because we have found that H$_2$O$_2$-induced vasoconstriction is mainly mediated by TXA$_2$, we aimed to determine whether an alteration in the TXA$_2$ effect is responsible for the observed differences in the H$_2$O$_2$ effects. For this, the
effects of the TXA2 mimetic U46619 on tension and [Ca2+]i were evaluated in arteries precontracted with phenylephrine. U46619 (0.1 nM–1 μM) induced a contractile response coupled to increases in [Ca2+]i (Fig. 5A), which were similar in vessels from both rat strains (Fig. 5B). These results suggest that the greater increase in [Ca2+]i and tension induced by H2O2 in arteries from SHRs is not due to alterations in the TXA2 effect and suggest the existence of an additional mediator contributing to H2O2 contraction.

Role of O2\textsuperscript{\textcircled{2}} in the H2O2-Induced Contractile Responses. To analyze the role of O2\textsuperscript{\textcircled{2}} in the H2O2 contractile responses, arteries were incubated with the NADPH oxidase inhibitor, apocynin, or with the O2\textsuperscript{\textcircled{2}} scavenger, tiron. Both apocynin (0.3 mM) and tiron (1 mM) reduced the contractile response induced by H2O2 in arteries from hypertensive rats (Fig. 6). However, neither apocynin nor tiron modified the H2O2 effect in MRAs from WKY rats (Fig. 6). The removal of endothelium did not modify the inhibitory effect of tiron on H2O2 contraction in arteries from SHRs (inhibition of E\textsubscript{max}, E\textsubscript{max}, 35.6 ± 4.7%, n = 7; E\textsubscript{max}, 67.3 ± 18.9%, n = 4, p > 0.05). These results suggest that O2\textsuperscript{\textcircled{2}} participates in the H2O2 contraction only in arteries from SHRs. In accordance, in isolated MRAs, cumulative application of H2O2 (1–100 μM) induced a concentration-dependent increase in O2\textsuperscript{\textcircled{2}} production. This increase was greater (p < 0.01) in arteries from SHRs than WKY rats (Fig. 7A). The incubation of the arteries with either apocynin (0.3 mM) or tiron (1 mM) abolished the H2O2-induced O2\textsuperscript{\textcircled{2}} production (Fig. 7A).

It has been described that TXA2 might induce O2\textsuperscript{\textcircled{2}} production (Muzaffar et al., 2004; Cogolludo et al., 2006). Figure 7B shows that SQ 29,548 (1 μM) reduced H2O2-induced O2\textsuperscript{\textcircled{2}} production in MRAs from SHRs. Moreover, cumulative application of U46619 (10 nM–3 μM) induced a concentration-dependent increased in O2\textsuperscript{\textcircled{2}} production in arteries from SHRs that was abolished by SQ 29,548 (1 μM) (Fig. 7B). On the other hand, coincubation of the arteries with tiron plus SQ 29,458 induced a similar inhibitory effect on H2O2 contraction compared with SQ 29,548 alone (data not shown). All together, these results suggest that TXA2-derived O2\textsuperscript{\textcircled{2}} participates in H2O2 contraction.

Discussion

In the present study, we found that H2O2 induced endothelium-dependent contraction in MRAs that was greater in vessels from hypertensive than normotensive rats. An increase in TXA2 release derived from COX-1, [Ca2+]i levels, and O2\textsuperscript{\textcircled{2}} production derived, at least in part, from TXA2 in arteries from hypertensive rats may explain this increased response.

H2O2 can produce contraction, relaxation, or biphasic responses dependent on the vascular bed, animals, or experimental conditions (Ardanaz and Pagano, 2006). In our study, H2O2 induced a biphasic response, characterized by a transient contractile component followed by a relaxation. A similar pattern of H2O2 response was described in phenylephrine-precontracted rat superior mesenteric artery (Gao et al., 2003) and in isolated arterioles of rat gracilis muscle without precontraction (Cseko et al., 2004).

Plasmatic H2O2 levels are increased in humans with essential hypertension (Lacy et al., 2000). We observed that H2O2 induced a greater contraction in MRAs from hypertensive compared with normotensive rats. This difference seems not to be due to alterations in contractile mechanisms because K\textsuperscript{+}-KHS and phenylephrine responses were similar in arteries from both strains. In agreement, some studies in the superior mesenteric artery (Gao and Lee, 2001), aorta (Rodríguez-Martínez et al., 1998), and resistance arteries (Tabet et al., 2004) have demonstrated that H2O2 induces greater contractile responses in hypertensive than normotensive rats. Endothelium removal reduced the contractile re-
response to H$_2$O$_2$, suggesting the involvement of contractile factors released from endothelium in the response to H$_2$O$_2$, in agreement with that found in canine basilar artery (Yang et al., 1999). In contrast, in rat aorta (Rodríguez-Martínez et al., 1998) and superior mesenteric artery (Gao and Lee, 2001), endothelium removal increased H$_2$O$_2$ response.

**Role of Thromboxane A$_2$ in H$_2$O$_2$ Contraction.** COX activation has been suggested to be one of the mechanisms responsible for H$_2$O$_2$ vasoconstriction (Rodríguez-Martínez et al., 1998; Gao and Lee, 2001). In arteries from WKY rats, H$_2$O$_2$ contraction was almost abolished by the nonselective COX inhibitor, indomethacin, the TXA$_2$ receptor antagonist, SQ 29,548, and the TXA$_2$ synthase inhibitor, furegrelate. However, in arteries from SHRs, the contractile response to H$_2$O$_2$ was diminished, but not abolished, by any of these inhibitors. This is in contrast with findings reported in conductance arteries from hypertensive rats where COX inhibitors fully abolished H$_2$O$_2$ responses (Rodríguez-Martínez et al., 1998; Gao and Lee, 2001). The selective COX-1 inhibitor SC-58560 produced the same effect as indomethacin, SQ 29,548, and furegrelate. In accordance, H$_2$O$_2$-induced TXB$_2$ production was abolished by SC-58560. This effect on TXA$_2$ release was probably dependent of increased COX-1 activity because H$_2$O$_2$ did not alter COX-1 expression. These results suggest, for the first time, that vasoconstrictor responses to H$_2$O$_2$ are mediated by COX-1-derived TXA$_2$ in resistance arteries. The selective COX-2 inhibitor, NS-398, failed to modify the effect of H$_2$O$_2$ on TXA$_2$ release, discarding the involvement of this isoform. In contrast, in the cat aorta, tert-butyl hydroperoxide induced COX-2-dependent vasoconstrictor responses (Garcia-Cohen et al., 2000). It has been described that both endothelial and vascular smooth muscle cells contain cyclooxygenases (DeWitt et al., 1983; Kawka et al., 2007). However, the removal of endothelium did not modify the inhibitory effect of SQ 29,548 on H$_2$O$_2$ contraction, suggesting that H$_2$O$_2$ also leads to TXA$_2$ formation in the smooth muscle cells.

Basal and H$_2$O$_2$-stimulated TXB$_2$ levels were greater in MRAs from SHRs than WKY rats and could explain the greater contractile effect of H$_2$O$_2$ observed in SHRs. Aortic endothelial cells from SHRs express more COX-1 than WKY rats (Tang and Vanhoutte, 2008). However, we found a similar COX-1 protein expression in homogenates of mesenteric arteries from both strains. The fact that any of the COX pathway inhibitors fully abolished H$_2$O$_2$ contraction in arteries from SHRs might indicate that this mechanism is not entirely responsible for the greater contractile effect induced by H$_2$O$_2$. Other mechanisms that might be responsible for the greater H$_2$O$_2$ contraction observed in MRAs from SHRs compared with WKY rats are: 1) a greater Ca$^{2+}$ entry or sensitization of the contractile apparatus in response to H$_2$O$_2$ and 2) additional H$_2$O$_2$-derived mediators.

**Smooth Muscle Calcium and H$_2$O$_2$ Contraction.** In vascular smooth muscle cells, H$_2$O$_2$ increases [Ca$^{2+}$]$_i$ levels (Yang et al., 1999; Tabet et al., 2004), but to our knowledge there are no studies analyzing alterations in [Ca$^{2+}$]$_i$, in response to H$_2$O$_2$ in ex vivo arteries. We observed that, in MRAs, H$_2$O$_2$ induced an increase in [Ca$^{2+}$]$_i$, that was greater in arteries from SHRs than WKY rats, in agreement with findings in cultured VMSC (Tabet et al., 2004). The increase in [Ca$^{2+}$]$_i$, levels in arteries from hypertensive rats has a functional physiological consequence because the coupled vasoconstrictor response to H$_2$O$_2$ was also increased. This increased response seems to be specific for H$_2$O$_2$ because both K$^+$-KHS- and phenylephrine-induced increases in [Ca$^{2+}$]$_i$, were similar in arteries from both strains. As described above, we found that H$_2$O$_2$ vasoconstriction is mainly mediated by TXA$_2$; therefore, we aimed to determine whether an alteration in the TXA$_2$ effect is responsible for the observed differences in the H$_2$O$_2$ contraction and [Ca$^{2+}$]$_i$, in arteries from SHRs. Therefore, the effect of the TXA$_2$ mimetic U46619 on tension and [Ca$^{2+}$]$_i$, was evaluated in phenylephrine precontracted arteries. In these experimental conditions, U46619 induced a concentration-dependent increase in vascular tone coupled to increases in [Ca$^{2+}$]$_i$, that was similar in arteries from both strains, excluding alterations in TXA$_2$ signaling as responsible for the increased H$_2$O$_2$ effect in SHRs and points to the participation of an additional mediator released by H$_2$O$_2$ in this strain. Moreover, the changes in [Ca$^{2+}$]$_i$, to U46619 were different from those observed with H$_2$O$_2$; thus, only an increase in Fura fluorescence was observed for U46619, whereas H$_2$O$_2$ induced a transient increase followed by a decrease in fluorescence.

**Role for O$_2^•$ in H$_2$O$_2$ Contraction.** In rat aortic vascular smooth muscle cells, mouse aortic fibroblasts, and human coronary arteries, H$_2$O$_2$ induces NADPH oxidase activation and O$_2^•$ production (Li et al., 2001). Moreover, Seshiah et al. (2002) described that there is an ROS-associated self-sustained activation loop. Thus, O$_2^•$, generated by NADPH oxidase is converted in H$_2$O$_2$, which in turn activates signaling pathways and comes back to activate NADPH oxidase. The O$_2^•$ release in response to H$_2$O$_2$ in MRAs, which was fully blocked by tiron, was much greater in SHRs than WKY rats, and it was probably dependent on NADPH oxidase activation because it was abolished by apocynin. Heumüller et al. (2008) and Riganti et al. (2008) have reported recently that apocynin-
nin reduces ROS availability through its antioxidant properties independently of its ability to inhibit NADPH oxidase. We do not know the mechanism of apocynin in our experimental model. The important finding is that 

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O_2^-
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participates in the contractile response to H$_2$O$_2$ because this response was reduced by both tiron and apocynin only in arteries from SHRs. The removal of the endothelium did not modify the inhibitory effect of tiron on H$_2$O$_2$ contraction in arteries from SHRs; this suggests that both smooth muscle and endothelial cells would be responsible for the production of O$_2^-$ in response to H$_2$O$_2$, as described above for the TXA$_2$ production. It has been demonstrated that TXA$_2$ activates O$_2^-$ production from NADPH oxidase in pulmonary arteries (Muzaffar et al., 2004; Cogolludo et al., 2006). Because H$_2$O$_2$ responses are mediated by TXA$_2$ and O$_2^-$ participates in H$_2$O$_2$ contraction only in MRAs from SHRs, we aimed to determine the role of TXA$_2$ in H$_2$O$_2$-induced O$_2^-$ release in this strain. SQ 29,548 reduced H$_2$O$_2$-derived O$_2^-$ production. Moreover, in these arteries, U46619 induced an increase of O$_2^-$ production that was blocked by SQ 29,548. All together, these results

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**Fig. 7.** A, representative fluorescent microphotographs and quantitative analysis of confocal microscopy images of mesenteric resistance arteries from WKY rats and SHRs incubated without (basal) or with H$_2$O$_2$ (1–100 μM) in the absence (control) and in the presence of tiron (1 mM) or apocynin (0.3 mM). Image size, 375 × 375 μm. Images were captured with a fluorescence confocal microscope (×40 oil immersion objective, zoom 1). Number of animals, five to six. B, quantitative analysis of confocal microscopy images of mesenteric resistance arteries from SHRs incubated without (basal) or with H$_2$O$_2$ (1–100 μM) or U46619 (10 nM–3 μM) in the absence (control) or in the presence of SQ 29,548. Number of animals, 5 to 6.
indicate that, in SHR s, H₂O₂ induces TXA₂ release, which in turn would activate NADPH oxidase and O₂⁻ production, which modulate vascular responses. This reveals a new mechanism of oxidative stress-induced vascular damage in hypertension. The fact that coinubcation with tiront did not show an additional effect of SQ 29,548 supports this hypothesis. However, we cannot exclude that O₂⁻ production might be facilitated by an increased constitutive NADPH oxidase activity/expression and/or by diminished antioxidant mechanisms associated with hypertension (Lassègue and Griendling, 2004). H₂O₂-induced contraction was not fully abolished by any of the inhibitors used. This suggests that TXA₂- and O₂⁻-independent contractile mechanisms also participate in the H₂O₂ response in SHR s. Further experiments are required to evaluate these additional mechanisms.

In conclusion, we found that H₂O₂ produces a COX-1-derived TXA₂ release that induces contractile responses in MRAs from WKY rats and SHR s. We also observed that this contractile effect is greater in arteries from SHR s and is coupled to greater TXA₂ production and [Ca²⁺] levels. Moreover, the greater effect of H₂O₂ in SHR s seems to be associated with O₂⁻ production mediated, at least in part, by TXA₂ release. The fact that H₂O₂ might activate NADPH oxidase in this strain, thus producing more O₂⁻ and therefore facilitating increased H₂O₂ levels, supports the concept of a vicious circle associated with oxidative stress-induced vascular damage in hypertension. Future studies will be needed to evaluate the potential implications of the effect of H₂O₂ in human arteries from hypertensive patients.

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References
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