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Original Contribution

Signaling pathways involved in the H_2O_2-induced vasoconstriction of rat coronary arteries

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ABSTRACT

Hydrogen peroxide (H_2O_2) is an endogenous endothelium-derived hyperpolarizing factor released by flow and involved in the regulation of coronary blood flow. Because opposing vasoactive effects have been reported for H_2O_2 depending on the vascular bed and experimental conditions, the aim of this study was to assess whether H_2O_2 may act as a coronary vasoconstrictor and if so to determine the underlying signaling mechanisms. Intramyocardial arteries from male Wistar rats were mounted on microvascular myographs for simultaneous measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and tension. On coronary arteries precontracted with the thromboxane A_2 (TxA_2) analogue U46619, H_2O_2 (1–300 μM) elicited further moderate contractions in the proximal arterial segments and relaxed the more distal coronary branches, the contractions being markedly augmented in arteries depolarized by raising extracellular K^+. H_2O_2-elicited vasoconstriction on K^+30-precontracted coronary arteries was blunted by catalase and significantly reduced by endothelial cell removal and by inhibitors of cyclooxygenase (COX) and of the TxA_2 receptor (TP). H_2O_2 (50 μM) increased by about 10-fold basal superoxide anion ($O_2^{\bullet -}$) production in coronary arteries measured by lucigenin-enhanced chemiluminescence, and H_2O_2-elicited contractions were reduced by the superoxide dismutase mimetic tempol and by NADPH oxidase inhibition. Furthermore, blockade of the ERK and p38 mitogen-activated protein (MAP) kinases significantly reduced the contractions elicited by high and low concentrations of peroxide, respectively, whereas Rho kinase inhibition nearly abolished these responses. H_2O_2 (50 μM) elicited simultaneous and similar sustained increases in $[Ca^{2+}]_i$ and tension that were blunted by blockade of voltage-dependent L-type channels, but resistant to the nonselective Ca^{2+} channel blocker 2-aminoethoxydiphenyl borate. Moreover, endothelial cell removal reduced the increases in $[Ca^{2+}]_i$ and contraction elicited by peroxide. The present data demonstrate that H_2O_2 is an endothelium-dependent vasoconstrictor in rat coronary arteries that activates smooth muscle Ca^{2+} entry through L-type and non-L-type channels and various intracellular signaling pathways including the release of a COX-derived TP agonist, stimulation of the MAP and Rho kinase pathways, and production of NADPH oxidase-derived superoxide.

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Reactive oxygen species (ROS) including superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are important second-messenger molecules involved in multiple signaling pathways within the vascular wall under both physiological and pathophysiological conditions. Dysregulated ROS production and/or metabolism–oxidative stress–promotes enhanced vasoconstriction, growth, and inflammation associated with cardiovascular diseases such as hypertension, diabetes, and atherosclerosis [1,2]. ROS derived from several intracellular sources including NADPH oxidase, the mitochondrial electron transport chain, xanthine oxidase, cyclooxygenase (COX), and uncoupled nitric oxide (NO) synthase are involved in the regulation of arterial tone in the pulmonary and systemic circulation and also in the activation of redox-sensitive gene transcription to promote proliferation, migration, and survival of vascular smooth muscle cells [3,4].

H_2O_2 is the most likely ROS involved in signaling in the vasculature because it is inherently more stable than other ROS and has been reported to act as both a vasoconstrictor and a

Abbreviations: K^+30, 30 mM K^+ solution; ACh, acetylcholine; COX, cyclooxygenase; EDHF, endothelium-derived hyperpolarizing factor; Fura2-AM, Fura-2 acetoxymethyl ester; KPSS, high (124 mM) K^+ solution; $[Ca^{2+}]_i$, intracellular Ca^{2+}; BK_{Ca}, large conductance calcium-activated K^+ channel; LAD, left anterior descending; MAP, mitogen-activated protein; SOC, nonselective store-operated Ca^{2+}; PSS, physiological saline solution; ROS, reactive oxygen species; $O_2^{\bullet}-$, superoxide anion; SOD, superoxide dismutase; TXA_2, thromboxane A_2; TP, thromboxane A_2/PHG_2 receptor; K_V, voltage-dependent K^+ channels

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vasodilator [3]. H_2O_2-induced contraction is coupled to various intracellular pathways including stimulation of COX and prostaglandins and thromboxane A_2 (TXA_2) release, stimulation of protein kinase C, MAP and Rho kinase activation, and/or elevation of $[Ca^{2+}]_i$ in vascular myocytes [3,5–9]. On the other hand, H_2O_2-induced vasodilatation has been associated with the release of endothelial mediators such as NO and prostacyclin, but most importantly, H_2O_2 itself has been shown to be an endothelium-derived hyperpolarizing factor (EDHF) in arterioles from various vascular beds [10,11].

H_2O_2 plays a major role as an endogenous EDHF in coronary microvessels, where it is involved along with NO and adenosine in the coronary autoregulation [12] and released by shear stress in the flow-mediated dilation [13]. Furthermore, H_2O_2 is also involved in the pacing-induced metabolic coronary vasodilatation [14], and H_2O_2-derived from $O_2^{\bullet -}$ produced from cardiac metabolism dilates coronary vessels to couple coronary blood flow to myocardial O_2 consumption [15]. However, opposing vasoactive effects have been reported for H_2O_2 depending on the vascular bed and experimental conditions, and the ability of H_2O_2 to consistently act either as an endothelial relaxant factor or as a vasoconstrictor seems to depend on the polarization of the membrane and functional activity of the K^+ channels [7,16,17], the pathological condition [5,8,18], and the redox status of the arterial wall [19]. In fact, blockade of the H_2O_2 vasodilator pathway has been reported to unmask a prostaglandin-mediated vasoconstrictor effect of peroxide in coronary arteries [20]. The aim of this study was to assess whether, in addition to its well-established role as coronary endothelial vasodilator, H_2O_2 may also have vasoconstrictor actions in coronary arteries and to determine the signaling pathways underlying H_2O_2 contractile effects in rat coronary arteries in vitro.

Material and methods

Animal and tissue preparation

All animal protocols conformed to the European Union *Guidelines for the Care and the Use of Laboratory Animals* (European Union Directive 2010/63/EU) and were approved by the Institutional Animal Care and Use Committee at Complutense University (Madrid, Spain). The heart and the mesentery were removed from 12- to 14-week-old male Wistar rats and placed in cold (4 °C) physiological saline solution (PSS) of the following composition (mM): 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 1.5 $CaCl_2$, 24.9 $NaHCO_3$, 0.027 EDTA, and 11 glucose; pH 7.4. Second- or third-order branches of the left anterior descending (LAD) coronary artery and third-order branches of mesenteric arteries were dissected as previously described [21] and mounted in microvascular myographs (Danish Myotechnology, Denmark) for isometric tension recording. The arteries were equilibrated for 30 min in PSS at 37 °C and then the relationship between passive wall tension and internal circumference was determined in each artery. From this, the internal circumference l_{100} corresponding to a transmural pressure of 100 mm Hg for a relaxed vessel in situ was calculated. The arteries were set to an internal circumference l_1 equal to 0.9 × l_{100}, because force development is close to maximal at this internal circumference [21].

Experimental procedure for the functional experiments

The contractile ability of the arteries was tested at the beginning of each experiment by measuring the vasoconstrictor responses to a high K^+ solution (KPSS), equivalent to PSS except that NaCl was exchanged for KCl on an equimolar basis, giving a final concentration of 123.7 mM K^+. To determine the vasoactive responses to H_2O_2 in coronary arteries, increasing concentrations (1–100 μM) of peroxide were added on arteries precontracted with either the TXA_2 analogue U46619 (1–3 μM) or a 30 mM K^+ solution (K^+30) and compared with the responses evoked by peroxide in mesenteric arteries precontracted with either U46619 (1–3 μM) or K^+30.

The mechanisms involved in the contractile responses to H_2O_2 were assessed by constructing cumulative concentration–response curves in arteries precontracted with K^+30 in the absence and the presence of an inhibitor of COX (indomethacin, 1 μM) and the TP antagonist ICI 192 (3 μM); a free radical scavenger, the SOD mimetic tempol (30 μM); an inhibitor of NADPH oxidase (apocynin, 100 μM); inhibitors of the MAP kinases ERK MAP kinase (PD 98059, 3 μM) and p38 MAP kinase (SB 239063, 1 μM); and an inhibitor of the Rho kinase (Y-27632, 3 μM). The endothelial integrity was tested in each artery by examining the relaxant effect of 10 μM acetylcholine (ACh). The role of endothelial cells in the vasoactive response to H_2O_2 was tested in arteries in which the endothelium was mechanically removed by guiding a human hair inside the vessel lumen and gently moving it forward and back several times. The absence of functional endothelium was confirmed by the lack of relaxation in response to ACh.

In the experiments aimed at assessing the Ca^{2+} mechanisms involved in the H_2O_2-induced contraction, the contractile responses and changes in $[Ca^{2+}]_i$ elicited by a single concentration of H_2O_2 (50 μM) were assessed in both endothelium-intact and endothelium-denuded arteries, in the absence and presence of the selective voltage-dependent L-type Ca^{2+} channel blocker nifedipine (1 μM). The effects of the nonselective store-operated Ca^{2+} (SOC) channel inhibitor 2-aminoethoxydiphenyl borate (2-APB; 50 μM) were tested on the non-L-type Ca^{2+} entry induced by H_2O_2.

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 acetoxymethyl ester (Fura2-AM) fluorescence as previously described [22]. The myograph was mounted on an inverted microscope (Axiovert S100 TV) equipped for dual excitation wavelength microfluorimetry (Deltascan; Photon Technology International). Coronary arteries were loaded in the dark in PSS containing 4 μM Fura2-AM for 2 h at 37 °C, and the solution was washed and changed to PSS with fresh Fura2-AM after 1 h. Arteries were then illuminated with alternating 340- and 380-nm light using a monochromator-based system, and the intensity of the emitted fluorescence was collected at a wavelength of 510 nm using a photomultiplier and monitored together with the tension. Coronary arteries were stimulated with KPSS at the beginning of each experiment to test vessel viability. 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 recorded during the experiment. The ratio of fluorescence at 340 and 380 nm (F340/F380) corrected for autofluorescence was taken as a measure of $[Ca^{2+}]_i$.

Measurement of superoxide production by chemiluminescence

The level of production of $O_2^{\bullet -}$ by coronary arteries and myocardial tissue under basal conditions and upon stimulation with H_2O_2 (50 μM) was detected by lucigenin-enhanced chemiluminescence as previously described in intact small arteries [23]. Briefly, segments of coronary arteries and samples of myocardial tissue

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