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Hydrogen peroxide modulates phenylephrine-induced contractile response in renal hypertensive rat aorta

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ABSTRACT

Endothelium-derived factors play an important role in vascular tone control. This study aimed to evaluate how endothelium and reactive oxygen species (ROS) contribute to phenylephrine (PE)-induced contraction in renovascular hypertensive (2K–1C) and normotensive (2K) rats aortas. The effects of the superoxide scavenger Tiron (0.1 mM and 1 mM) or catalase (30 U/ml, 90 U/ml, 150 U/ml and 300 U/ml) on the PE-induced contraction were evaluated in both intact endothelium (E+) and denuded (E−) aortas. Endothelium removal increased the PE-induced contractions. The maximum contractile response decreased only in 2K–1C rat E+ aorta, and catalase (30 U/ml, 90 U/ml, 150 U/ml) partially reversed this effect. Endothelium increased the basal hydrogen peroxide (H_2O_2) production in 2K and 2K–1C rats aortas. PE-stimulated H_2O_2 production was higher in 2K–1C (E+/E−) than in 2K (E+/E−). Inhibition of the enzymes cyclooxygenase, NADPH-oxidase, xanthine-oxidase, and superoxide dismutase reduced the PE-stimulated H_2O_2 production in 2K–1C rat aorta. The decreased contraction to PE in 2K–1C rat aorta is partially due to endothelial H_2O_2 production; however, in denuded aorta, it contributes to maintaining the contractile response. Superoxide plays an important role on the PE-induced contraction in 2K rat denuded aorta, whereas in 2K–1C rat aorta, it is H_2O_2 that plays an important role in this effect.

1. Introduction

Vascular cells such as endothelial and smooth muscle cells produce reactive oxygen species originating from O_2 metabolism. Reactive oxygen species include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). However, under physiological conditions, nitric oxide (NO) is a recognized vasodilator nitrogen species that endothelial cells produce in a controlled manner (Touyz and Schiffrin, 2004). Together, these species control vascular smooth muscle contraction and relaxation.

Angiotensin II (Ang II) stimulates reactive oxygen species production mainly by activating the nicotinamide adenine dinucleotide phosphate enzymatic activity (NADPH Oxidase, Nox) in the vessel wall (Griendling et al., 1994; Pagano et al., 1995; Rajagopalan et al., 1996). Moreover, Nox seems to contribute to ROS production in spontaneously hypertensive rats (SHR) endothelial cells regardless of Ang II (Wind et al., 2010). Other oxidases such as xantine oxidase (Phan et al., 1989) and uncoupled NO-synthase (NOS) (Satoh et al., 2005) can also produce reactive oxygen species in the vascular system.

Independent of the source, $O_2^{\bullet -}$ seems to be the major vascular oxygen metabolism-derived reactive oxygen species (Rajagopalan et al., 1996); the antioxidant enzyme superoxide dismutase (SOD) rapidly dismutates $O_2^{\bullet -}$ to H_2O_2 (McCord and Fridovich, 1969). Following this reaction, the antioxidant enzyme catalase degrades H_2O_2 to water and molecular oxygen (Maral et al., 1977). The imbalance between pro- and antioxidant enzymes characterizes oxidative stress, which is implicated in the pathogenesis of hypertension. Attention is currently focused on reactive oxygen species enzymatic sources, because they seem to play an important role in developing and maintaining arterial hypertension in humans (Sagar et al., 1992) and in experimental models of hypertension such as SHR (Suzuki et al., 1995), Dahl salt-sensitive rats (Swei et al., 1997), and Ang II-infused rats (Laursen et al., 1997).

The $O_2^{\bullet -}$ anion is highly reactive and can reduce NO availability; NO is an important vasodilator that endothelial cells produce (Costa et al., 2009). Impaired endothelium-dependent vascular relaxation characterizes endothelial dysfunction, which is usually found in several hypertensive models. However, $O_2^{\bullet -}$ production can also contribute to sympathetic hyperactivity, besides decreasing endothelium-dependent vasodilation during renovascular hypertension (Costa et al., 2009; Nishi et al., 2010). The $O_2^{\bullet -}$ anion can also increase the arteriolar tone in SHR rats (Suzuki et al., 1998), and an

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adrenal pathway may partially mediate this effect. Adrenalectomy reduces xanthine oxidase expression, reactive oxygen species production, and arteriolar tone to normotensive levels (Delano et al., 2006). These findings reveal a close relationship between $O_2^{\bullet -}$ production and adrenergic pathways activity.

Although it is known that renovascular hypertension involves reduced endothelium-dependent relaxation (Callera et al., 2004), the role of reactive oxygen species on the contractile response induced by α_1-adrenoceptor agonists in this hypertensive model is not yet clear. Therefore, the present study aimed to evaluate the role of reactive oxygen species, $O_2^{\bullet -}$, and H_2O_2 on the contractile response induced by the α_1-adrenoceptor agonist phenylephrine in denuded (E−) or intact endothelium (E+) aortas from normotensive (2K) and hypertensive (2K–1C) rats.

2. Methods

2.1. Animals

All the procedures were performed in accordance with the standards and policies of the Ethics Committee on Animal Care and Use of the University of São Paulo. Male rats (180–200 g) were anesthetized with tribromoethanol (2.5 mg kg^{-1}, i.p.); after a midline laparotomy, a silver clip with 0.20 mm internal diameter was placed around the left renal artery. Normotensive two-kidney rats (2K) were submitted to laparotomy only. Animals were maintained on standard rat chow, as described previously (Callera et al., 2004). The systolic blood pressure (SBP) was measured by an indirect tail-cuff method six weeks after the surgery. Rats were considered hypertensive when SBP was higher than 180 mmHg.

2.2. Functional study by vascular reactivity

Male rats (400–450 g) were killed by decapitation under anesthesia. The thoracic aorta was quickly removed and cut into rings (4 mm length). In some rings, the endothelium was mechanically removed by gently rolling the vessel lumen on a thin wire. The aortic rings were placed between two stainless-steel stirrups and connected to an isometric force transducer (Letica Scientific Instruments, Barcelona, Spain), to measure tension in the vessels. The rings were placed in the organ chamber containing Krebs solution with the following composition (mM): 130.0 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 14.9 $NaHCO_3$, 5.5 glucose, and 1.6 $CaCl_2$. The solution was maintained at pH 7.4 gassed with 95% O_2 and 5% CO_2 at 37 °C. The rings were initially stretched to a basal tension of 1.5 g; then, they were allowed to equilibrate for 60 min. Endothelial integrity was qualitatively assessed by the degree of relaxation induced by acetylcholine (1 μM) in the presence of a contractile tone induced by phenylephrine (PE, 0.1 μM). For studies in endothelium–intact artery, the ring was discarded if relaxation with acetylcholine was not 80% or greater; in the case of endothelium–denuded artery, the ring was discarded if there was any degree of relaxation. Concentration-effect curves were constructed for PE in the case of 2K and 2K–1C denuded (E−) or intact endothelium (E+) rats aortas. These curves were obtained in the absence of or after 30 min incubation with the antioxidant agents $O_2^{\bullet -}$ scavenger Tiron (0.1 mM or 1 mM) or Catalase (extracted from bovine liver at concentrations of 30, 90, 150, and 300 U/ml), to degrade H_2O_2 to H_2O and O_2. The PE potency (pD_2) and maximum effect (ME) in inducing contraction were evaluated.

2.3. Measurement of basal reactive oxygen species levels in aortic rings and plasma

The aortic rings were initially stretched to a basal tension of 1.5 g; next, they were allowed to equilibrate for 60 min in the organ chamber. Endothelial integrity was assessed qualitatively as previously described. After intact-endothelium aortas were vertically embedded in tissue-tek, they were frozen and cut in serial 4 μm sections. Unfixed cryo-sections were incubated with10 μl of DHE (10 μM) for 30 min at room temperature, in the dark, to evaluate the in situ reactive oxygen species production (Castro et al., 2009). Some experiments were performed in the absence or in the presence of Tiron (1 mM) or Peg Catalase (300 U/ml). Sections were examined by fluorescence microscopy (Leica Imaging Systems Ltd., Cambridge-England), and the image was captured at ×400. Red fluorescence from 20 fields around the vessel was evaluated by using the ImageJ software (http://rsbweb.nih.gov/ij/), as described before (Montenegro et al., 2009). The arithmetic means of the fluorescence were calculated for each slide.

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid-reactive substances using a previously described fluorimetric method (Castro et al., 2009). This technique requires excitation at 515 nm, emission at 553 nm, and 1,1,3,3-tetramethoxypropane as standard (Yagi, 1998). The lipoperoxide levels were expressed in terms of malondialdehyde (nmol/ml).

2.4. Measurement of H_2O_2 production in aortic rings

2K and 2K–1K rats E+ and E− aortic rings samples were initially stretched to a basal tension of 1.5 g in the organ bath in order to reproduce the tension to which the preparations were submitted in the vascular reactivity studies. The effectiveness of endothelial removal (E−) or its integrity (E+) was assessed qualitatively. To this end, the aortic rings were stimulated with PE (0.1 μM) until they reached the maximum contractile response; another group of aortic rings was not stimulated (basal). Then, the aortas were immediately removed from the organ chamber and frozen in liquid nitrogen. Another group of 2K and 2K–1C rats intact aortas (E+) was incubated or not (control) with non-selective enzyme inhibitors of nitric oxide synthase (NOS) (L-NAME, 100 μM), cyclooxygenase (COX) (ibuprofen, 10 μM), NADPH oxidase (apocynin, 100 μM), xanthine oxidase (allopurinol, 100 μM), or superoxide dismutase (SOD) (DDC, 10 μM) for 30 min. After that, aortas were stimulated with PE (0.1 μM) until they reached maximum contractile response, followed by their immediate removal from the organ chamber and freezing in liquid nitrogen. Next, all the frozen samples of wet tissue were macerated in 200 μl of Krebs solution and centrifuged at 14.770 × *g*. The H_2O_2 production was measured by using the Amplex red H_2O_2 assay kit (Molecular Probes, Invitrogen, Carlsbad, CA-USA) according to the manufacturer's protocol. A H_2O_2 solution used to construct the standard curve on the same 96-well plate was incubated with Ultra red working solution (100 μM) at 37 °C together with the supernatant of the tissues; it was used to determine the H_2O_2 concentrations in the samples. Fluorescence emission was measured at excitation of 530 nm and emission of 590 nm on the Biotek Synergy HT plate reader. The results are expressed as the mean ± SEM of the absolute detected H_2O_2 concentration (μmol/l), without correction for tissue mass or total protein, because the aim of this experimental protocol was to evaluate total (absolute) H_2O_2 concentration produced by 2K and 2K–1C rats aortic rings in the absence or presence of PE and to verify possible sources of H_2O_2 production through enzymes inhibition.

2.5. Drugs and solutions

Phenylephrine, acetylcholine, Tiron, catalase from bovine liver, Peg-Catalase, L-NAME (L-N^G-Nitroarginine methyl ester), apocynin, indomethacin, allopurinol, and sodium diethyldithiocarbamate