Tetrahydrobiopterin Alters Superoxide and Nitric Oxide Release in Prehypertensive Rats

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Abstract

Constitutive nitric oxide synthase (cNOS) with insufficient cofactor (6R)-5,6,7,8-tetrahydrobiopterin (H_4B) may generate damaging superoxide (O_2^-). This study was designed to determine whether cNOS-dependent generation of O_2^- occurs in spontaneously hypertensive rats (SHR) before the onset of hypertension. Aortas from 4-wk-old SHR and Wistar-Kyoto rats were used. cNOS was stimulated by calcium ionophore A23187. In situ measurements of nitric oxide and hydrogen peroxide by electrochemical sensors and O_2^- production by chemiluminescence method were performed. Isometric tension was continuously recorded. H_4B by high performance liquid chromatography and [^{3}H]citrulline assay were determined in homogenized tissue. The A23187-stimulated production of O_2^- and its superoxide dismutase product hydrogen peroxide were significantly higher, whereas nitric oxide release was reduced in SHR aortas, with opposite results in the presence of exogenous H_4B. Furthermore, N^G-monomethyl-L-arginine inhibited the generation of cNOS-dependent O_2^- by $\sim$ 70%. Natural H_4B levels were similar in both strains; however, equivalent cNOS activity required additional H_4B in SHR. The endothelium-dependent relaxations to A23187 were significantly inhibited by catalase, and enhanced by superoxide dismutase, only in SHR; however, these enzymes had no effect in the presence of H_4B. Thus, dysfunctional cNOS may be a source of O_2^- in prehypertensive SHR and contribute to the development of hypertension and its vascular complications. (*J. Clin. Invest.* 1998. 101:1530–1537.) Key words: nitric oxide synthase • hydrogen peroxide • SOD • catalase • aorta

Introduction

The endothelium modulates vascular tone through the release of nitric oxide (NO),[1] a potent vasodilator that regulates blood pressure and regional blood flow (1–4). Existing evidence suggests that the enzymatic pathway for the production of NO is impaired in the vascular endothelium of spontaneously hypertensive rats (SHR) or patients with essential hypertension (5–8). In normotensive mammalian tissue, constitutive nitric oxide synthase (cNOS) in the presence of sufficient cofactor (6R)-5,6,7,8-tetrahydrobiopterin (H_4B) receives and stores enough electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to transform the cosubstrates O_2 and L-arginine into the products NO and L-citrulline (9). It has been demonstrated in vitro (9, 10) that H_4B-deficient isolated cNOS, when activated, cannot catalyze the five electron oxidation of L-arginine into NO. However, the same H_4B-deficient cNOS can still receive electrons from NADPH and store them in its bound flavins, and then can donate them one-at-a-time to its other substrate, O_2, resulting in one electron reduction to form superoxide anion (O_2^-; $pK_a = 4.8$). The precise role of H_4B in regulation of cNOS catalytic activity is still not completely understood (11, 12). However, these findings suggest that H_4B is allosterically involved in the coupling of the oxidase and reductase domains of cNOS within the active dimer.

Interestingly, an impaired synthesis of H_4B occurs in adrenal cortex of SHR (13). This metabolic dysfunction was detected in prehypertensive animals suggesting that it may contribute to the development of hypertension and its complications. Recently, we reported that H_2O_2 formed via cNOS becomes a mediator of endothelium-dependent relaxations in intact coronary arteries depleted of H_4B (14). Whereas O_2^- is a potent mediator of endothelium-dependent contraction (15), H_2O_2 is a potent vasodilator (14). Since aortic tissue naturally contains superoxide dismutase (SOD; $1–5 \times 10^{-6}$ mol/liter), an enzyme that rapidly converts O_2^- ($k = 2 \times 10^9$ L mol^{-1}s^{-1}) into O_2 and H_2O_2 (16), as well as catalase ($1–5 \times 10^{-6}$ mol/liter), an enzyme that converts H_2O_2 into water and O_2 at a relatively moderate rate ($k = 10^7$ L mol^{-1}s^{-1}; reference 16). We hypothesized that a dysfunctional cNOS may trigger oxygen-derived free radical production in prehypertensive SHR (Fig. 1). We tested this hypothesis by examining the mediator(s) of endothelium-dependent relaxations and by directly measuring the NO, O_2^-, and H_2O_2 production from the aortic endothelium in prehypertensive SHR and normotensive Wistar-Kyoto (WKY) rats.

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1. *Abbreviations used in this paper:* cNOS, constitutive nitric oxide synthase; HOONO, peroxynitrous acid; HPLC, high performance liquid chromatography; H_4B, (6R)-5,6,7,8-tetrahydrobiopterin; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; O_2^-, superoxide; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; SSCE, silver/silver chloride reference electrode; WKY, Wistar-Kyoto.

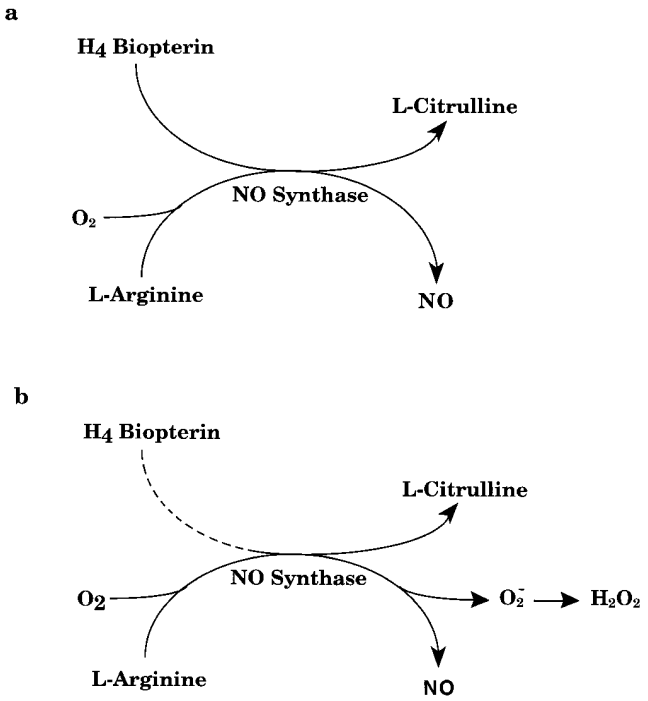

Figure 1. (*a*) Schematic representation of nitric oxide synthase (*NO Synthase*) reaction leading to L-citrulline and nitric oxide (*NO*) from L-arginine and oxygen (O_2). (*b*) The activation of NO synthase at suboptimal levels (*dashed line*) of (6R)-5,6,7,8-tetrahydrobiopterin (*H_4 Biopterin*) generates superoxide anion (O_2^-) followed by the production of hydrogen peroxide (H_2O_2) and/or peroxynitrite ($ONOO^-$) from the rapid reaction of O_2^- and NO.

Methods

Animals. The experiments were performed on aortas of male SHR and WKY rats (4-wk-old; IFFA CREDO, L'Arbresle, France). All procedures were in accordance with institutional guidelines. Blood pressure was measured in the left femoral artery with a Letica PRI 256/2 equipment (Letica S/A Instruments, Hospitalet, Spain). Mean blood pressure of SHR did not differ from that of age-matched WKY rats (69±8 and 68±5 mmHg; $n = 6$ for SHR and WKY rats, respectively). Animals were anesthetized with thiopental (50 mg/kg IP; Abbot Laboratories Diagnostic Division, Chicago, IL). The aorta was excised and immediately placed into cold (4°C) control solutions. Under a dissection microscope (Wild M3C; Wild AG, Heerbrugg, Switzerland) the isolated aorta was cleaned of adherent connective tissue. For measurements of NO, O_2^-, H_2O_2 concentrations and organ chamber experiments, the preparations were immersed in HBSS and modified Krebs-Ringer bicarbonate solution, respectively. For measurements of H_4B levels and determination of NOS activity, the aortas were frozen in liquid nitrogen and stored at −70°C until assay was performed.

Organ-chamber experiments. Aortas were cut into rings (3–4 mm long). In certain rings, the endothelium was mechanically removed. To inhibit cyclooxygenase, all experiments were performed in the presence of indomethacin (10^{-5} mol/liter). Each ring was connected to an isometric force transducer (SCAIME, Paris, France), suspended in an organ chamber filled with 25 ml of modified Krebs-Ringer bicarbonate solution of the following composition (mmol/liter): NaCl 118.6, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.1, calcium EDTA 0.026, glucose 10.1 (37°C; pH 7.4), and bubbled with 95% O_2–5% CO_2. Isometric tension was recorded continuously. After a 30-min equilibration period, rings were gradually stretched to the optimal point of their length–tension curve (2.5±0.2 g) as determined by the contraction to KCl (100 mmol/liter). The functional integrity of the endothelium was tested by the presence of relaxations to acetylcholine (10^{-6} mol/liter). Concentration–response curves were obtained in a cumulative fashion. Several rings cut from the same artery were studied in parallel; only one concentration-response curve was made per preparation. In quiescent preparations, indomethacin, N^G-nitro-L-arginine methyl ester (L-NAME), catalase, and SOD did not affect resting tension. Responses to calcium ionophore A23187 were obtained during submaximal contraction to norepinephrine (3×10^{-7} mol/liter). Incubation time was 30 min for indomethacin, 15 min for L-NAME, and 5 min for CAT and SOD. Relaxations were expressed as a percentage of maximal relaxations induced by papaverine (3×10^{-4} mol/liter).

Measurement of nitric oxide. NO was measured using a porphyrinic microsensor as described previously (17, 18). The porphyrinic microsensor has a response time of 0.1 ms at micromolar NO concentrations and 10 ms at the detection limit of 10^{-9} mol/liter.

Briefly, the NO microsensor was produced from a single carbon fiber (6 μm diameter, 12 ohm cm; Amoco Performance Products, Greenville, SC). The tip of the carbon fiber was made more sensitive to potential interferents by deposition of a highly conductive polymeric porphyrin from a solution of 2.5×10^{-4} mol/liter nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin subsequently covered with Nafion®. Linear calibration curves were constructed for each sensor (19). The porphyrinic NO sensor was routinely tested for possible interferences from all reagents and known secretable and readily oxidizable mammalian biochemicals at concentrations 10 times higher than the concentrations used in this study.

Amperometry was used for fast (resolution time 0.1–1 ms) and continuous measurement of the changes of NO concentration. A three-electrode system was used for these measurements. It consisted of a porphyrinic microsensor working electrode, a platinum counter electrode (diameter 0.5 mm), and a silver/silver chloride reference electrode (SSCE). Current was monitored using a Princeton Applied Research, PAR model 273 voltametric analyzer interfaced with an IBM 80486 computer with data acquisition and control software. Current-time curves (amperograms) were obtained at a constant potential (0.66 V), the peak potential for the oxidation of NO versus SSCE.

The sensor was lowered with a computer controlled micromanipulator until it reached the endothelial surface as described (17, 18). Immediately before NO measurement, an isolated open vascular ring was placed in an organ chamber with fresh, phenol red free HBSS, and the active tip of the microsensor was placed on the endothelium of the vascular ring, and a platinum counter electrode and a SSCE were placed on adjacent tissue. Then enough calcium ionophore A23187 was injected into the organ chamber to reach 8×10^{-6} mol/liter. The experiment was then repeated in the presence of SOD or membrane permeable PEG-SOD (100 U/ml; from bovine erythrocytes coupled to methoxy-polyethylene glycol); after incubation for 60 min with H_4B (10^{-4} mol/liter), in the presence or in the absence of SOD, and after incubation with N^G-monomethyl-L-arginine (L-NMMA) or L-NAME (2×10^{-5} mol/liter) in HBSS.

Measurement of hydrogen peroxide. A H_2O_2 electrode was prepared as described (20). Briefly, horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) was immobilized in epoxy hydrogel on a current collector (carbon fiber Amoco; diameter, 7 μm). The redox hydrogel was made of a poly (4 – vinyl pyridine) backbone complexed by osmium redox polyamine as described (21). Electrode coating was prepared by mixing the enzyme, (30% wt/wt) polymer and the cross-linker solution (1:2 molar ratio). The amperometric measurements were performed at −0.2 V (versus SSCE) using the same set up described above for NO measurements. The detection limit for H_2O_2 was 10^{-8} mol/liter.

Measurement of superoxide by lucigenin-mediated chemiluminescence. The superoxide (O_2^-) concentration in aortic tissue was determined by a chemiluminescence method (22). O_2^- produced chemilu-

minescence of lucigenin (bis-*N*-methylacridinium nitrate; Aldrich Chemical Co., Milwaukee, WI) was detected with a scintillation counter (Beckman 6000 LS, with a single photon monitor; Beckman Instruments, Inc., Fullerton, CA). Each (0.8–1.5 mg) tissue sample was placed in 2 ml of HBSS adjusted to pH 7.4, then enough lucigenin was added to make its concentration 2.5×10^{-4} mol/liter. Basal O_2^- concentration produced by the tissue was measured after a 2-min incubation in HBSS. The total of the O_2^- produced was measured in a similar manner: the 2-min incubation period was followed by injection of 10 μl of 1.2×10^{-3} mol/liter A23187. Photons were counted during the first 20 s after addition of A23187 and were calibrated as O_2^- concentration by constructing standard curves based on photons emitted by O_2^- stoichiometrically generated by reaction of xanthine and xanthine oxidase. An absolute colorimetric method of standardization based on the integration of electrical charge transferred during the reaction of O_2^- with an electrode was used to correlate photon counts measured by chemiluminescence to the actual concentrations of O_2^- (19).

Preparation of tissue extracts. Tissue was homogenized in a glass pistill in distilled water containing 0.1 mg/ml pefabloc SC (Merck, Darmstadt, Germany) and 5×10^{-3} mol/liter dithioerythrol, then centrifuged at 4°C and 13.000 *g* for 15 min. In some experiments, the pellet of the first centrifugation was resuspended in the same solution containing 20 mmol/liter 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate (CHAPS; Sigma Chemical Co.) and centrifuged again at the same conditions.

Determination of tetrahydrobiopterin concentrations by high performance liquid chromatography (HPLC). Determination of H_4B concentrations by HPLC was performed as described (23). Briefly, for the oxidation of the reduced nonfluorescent di- and tetrahydrobiopterin to a fluorescent biopterin, 100 μl of tissue extract was mixed with 10 μl 1 N HCl and 10 μl 0.1 M I_2 (dissolved in 0.25 M KI) and incubated for 60 min in the dark. After centrifugation (2 min, 13.000 *g*), 10 μl 0.1 M ascorbic acid was added to the supernatant. 30 μl was then injected onto a 250 mm long, 4 mm inner diameter RP-18 column (Lichrospher, 5 μm particle size; Merck), isocritically eluted with 15 mmol/liter potassium phosphate buffer, pH 6.0, at a flow rate of 0.8 μl/min. H_4B was detected by fluorescence at 350 nm (excitation) and 440 nm (emission).

Determination of cNOS activity. Determination of cNOS activity was carried out as described (24). Briefly, tissue extracts were freed from low molecular weight compounds with NAP-5 columns (Pharmacia Fine Chemicals, Uppsala, Sweden). Protein fraction was eluted with 40 mmol/liter Tris-HCl buffer, containing 0.1 mg/ml Pefabloc SC. Standard reaction mixtures contained 100 mmol/liter L-arginine, 25 μmol/liter FAD, 25 μmol/liter FMN, 2 mmol/liter NADPH, 0.15 mmol/liter EGTA, 0.9 mmol/liter EDTA, 1.78 mmol/liter $MgCl_2$, 0.27 mmol/liter $CaCl_2$, 5 μmol/liter (6R)-5,6,7,8-tetrahydrobiopterin and 60,000–80,000 cpm of purified L-[2,3,4,5 3*H*] arginine (Amersham Life Sciences, Arlington Heights, IL), and 100 μl of tissue extract in a final volume of 200 μl. After incubation at 37°C for 30 min, the reaction was stopped by addition of 800 μl 200 mmol/liter sodium acetate, pH 5.0, containing 200 μmol/liter EDTA and 1 mmol/liter L-citrulline. [^{3}H]Citrulline was quantified after separation from [3H]arginine by cation exchange on Dowex 50W.

Drugs. Acetylcholine chloride, calcium ionophore A23187, catalase, indomethacin, L-NAME, L-NMMA, norepinephrine, papaverine hydrochloride, sodium nitroprusside, superoxide dismutase (from bovine erythrocytes, 4,400 U/mg protein) and chemical components of the physiological salt solutions (HBSS and Krebs-Ringer) were obtained from Sigma Chemical Co. (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride was obtained from Schircks Laboratories (Jona, Switzerland). Stock solutions of the drugs were freshly prepared every day. Drugs were dissolved in distilled water such that volumes of less than 0.2 ml were added to the organ chambers. A stock solution of 10^{-5} mol/liter indomethacin was prepared in equal molar concentrations of Na_2CO_3. A stock solution of 1.2×10^{-3} mol/liter of A23187 was prepared in dimethyl sulfoxide. All concentrations are expressed as final molar (mol/liter) concentration in the bath solutions.

Statistical analysis. All experiments were performed in parallel on preparations from SHR and WKY rats. In each set of experiments, *n* equals the number of experiments. Results are expressed as mean±SEM. Statistical evaluation of the data was performed by using Student's *t* test for unpaired observations. A value of $P < 0.05$ was considered statistically significant.

Results

Nitric oxide release. After stimulation with calcium ionophore A23187 (8×10^{-6} mol/liter), NO concentration bursts from the aortas of 4-wk-old SHR and WKY rats, were measured using a porphyrinic sensor placed near the endothelial surface (5±2 μm). Amperometric curves showing the change of NO concentration with time, recorded in the absence and presence of SOD (100 U/ml) or H_4B (10^{-4} mol/liter), are depicted in Fig. 2. After the addition of A23187, a rapid increase of NO concentration was observed; peak NO concentrations were higher for WKY rats than for SHR (200±5 and 120±5 nmol/liter, respectively, $n = 7$, $P < 0.005$).

Since SOD catalyzes O_2^- dismutation, we used the difference in NO concentration recorded with and without SOD as

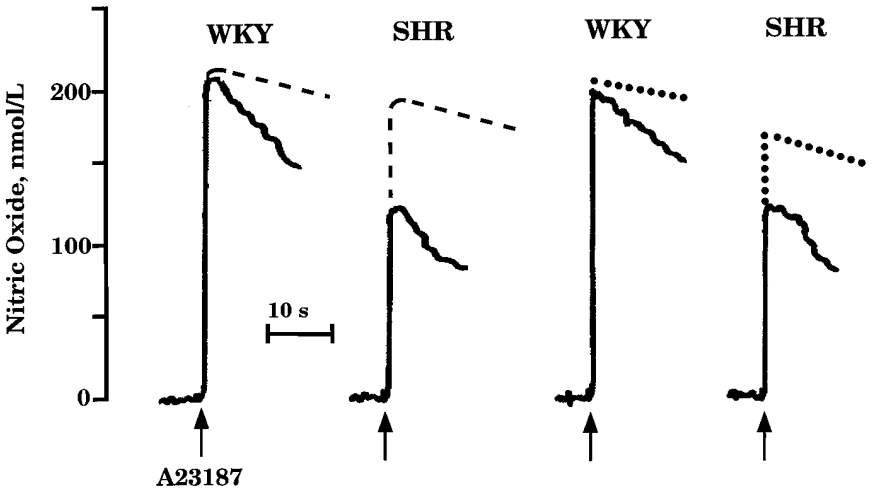

Figure 2. Typical amperograms showing changes of nitric oxide concentrations near the surface of endothelial cells from the aorta of 4-wk-old Wistar-Kyoto (*WKY*) and spontaneously hypertensive rats (*SHR*). NO release was stimulated with calcium ionophore A23187 (8×10^{-6} mol/liter, *solid line*). Amperograms obtained during stimulation with A23187 in the presence of SOD (100 U/ml, *dashed line*), or after incubation with H_4B (10^{-4} mol/liter, *dotted line*) are shown.

an indirect approach to estimate production of O_2^- at the time of NO release. In the presence of SOD a small 8% increase of peak NO concentration was observed for WKY rats and a significant 54% increase for SHR (216±6 and 185±5 nmol/liter, respectively). A small difference in NO signal (10 and 62% increase of peak NO concentration in WKY and SHR, respectively) was observed with membrane permeable PEG–SOD (data not shown). Similar increases in peak NO production were observed after incubation of aortas with H_4B; 5% for WKY and about 50% for SHR (210±5 and 178 nmol/liter, respectively, $n = 7$, $P < 0.005$). After incubation of aortas with H_4B we did not detect a further increase of NO signal in the presence of either SOD or PEG–SOD (data not shown).

Superoxide anion and hydrogen peroxide release. The difference in peak NO concentrations in the presence and absence of SOD suggests that A23187 not only stimulates NO release, but also causes the simultaneous release of O_2^-. This possibility was confirmed by the use of a chemiluminescence method to make direct in vitro measurement of O_2^- in aortic tissue. Also, one of the O_2^- dismutase products, H_2O_2, was measured with a H_2O_2 selective electrode.

O_2^- and H_2O_2 concentrations were measured in the aorta of SHR and WKY rats before and after stimulation with A23187 (Fig. 3). In WKY, the concentration of O_2^- increased from 10±2 to 16±3 nmol/mg tissue, respectively (Fig. 3, *top*); the corresponding increase in H_2O_2 production was insignificant. After incubation with SOD or H_4B, O_2^- production stimulated by A23187 decreased to its basal level. Furthermore, it is interesting to note that in WKY, after SOD treatment H_2O_2 production increased to 10±2 nmol/liter. By contrast, in SHR the basal production of O_2^- and H_2O_2 was higher than that observed in WKY rats (Fig. 3, *bottom*); the O_2^- concentration increased significantly after stimulation with A23187 (18±3 and 65±4 nmol/mg tissue, respectively). In the presence of SOD the O_2^- concentration decreased to basal level and H_2O_2 increased three times in comparison to the control (59±4 nmol/liter). This clearly indicates that O_2^- is entirely dismutated by SOD in this system. The presence of membrane permeable PEG–SOD caused additional 15% decrease/increase in O_2^- and H_2O_2 production, respectively, as compared with SOD treatment (data not shown).

As a negative control, 2×10^{-5} mol/liter doses of known inhibitors of cNOS, after 10 min incubation, were found to reduce O_2^- from both SHR and WKY rats after stimulation with

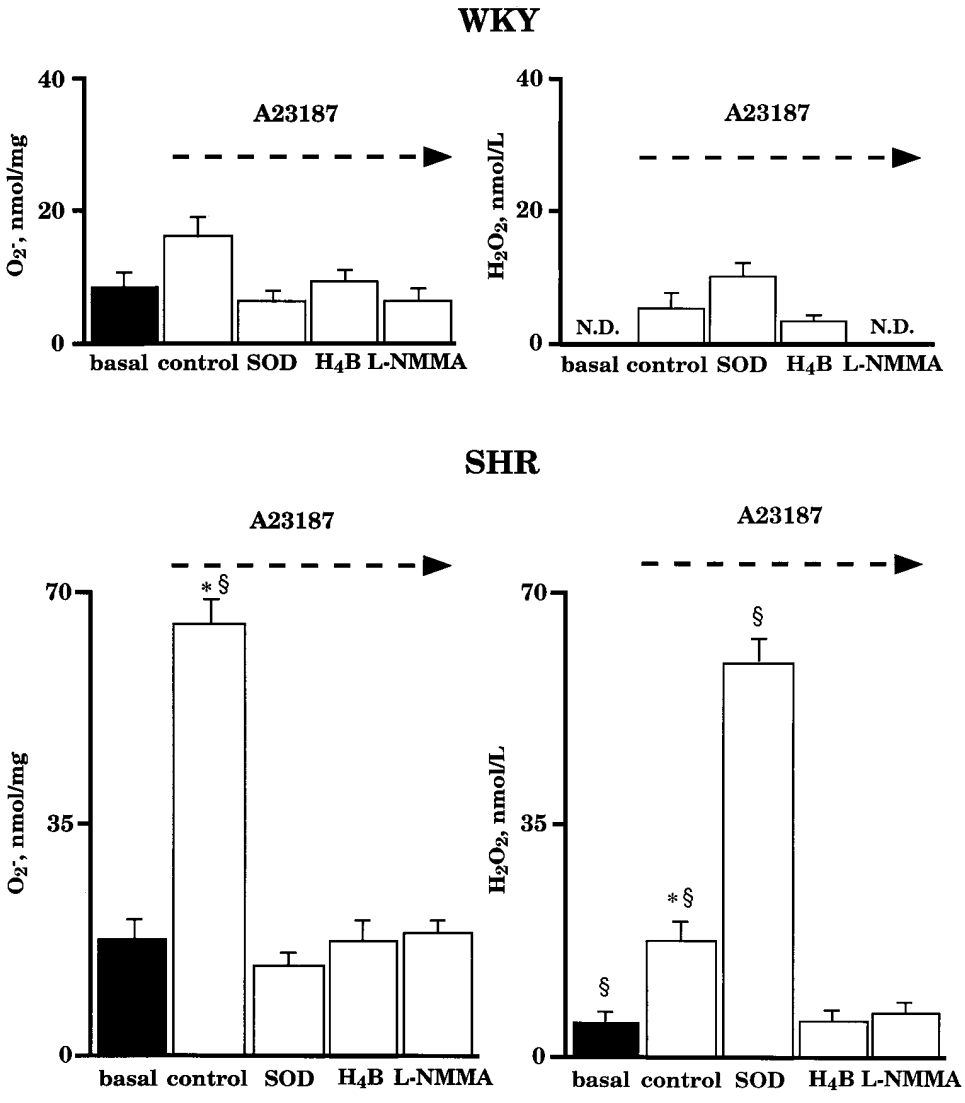

Figure 3. Bar graphs showing the concentration of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in aortic tissue from 4-wk-old Wistar-Kyoto rats (*WKY, top*) and spontaneously hypertensive rats (*SHR, bottom*). Basal concentrations were measured without stimulation of cNOS by calcium ionophore A23187 (8×10^{-6} mol/liter). Total concentrations (*control*) were measured after stimulation with A23187; also, in the presence of SOD (100 U/ml), or after incubation with (6R)-5,6,7,8-tetrahydrobiopterin (H_4B, 10^{-4} mol/liter), and in the presence of N^G-monomethyl-L-arginine (*L-NMMA*, 2×10^{-5} mol/liter). Values are means±SEM ($n = 7$). *$P < .05$ compared with corresponding basal values; §$P < .05$ compared with values in WKY rats.

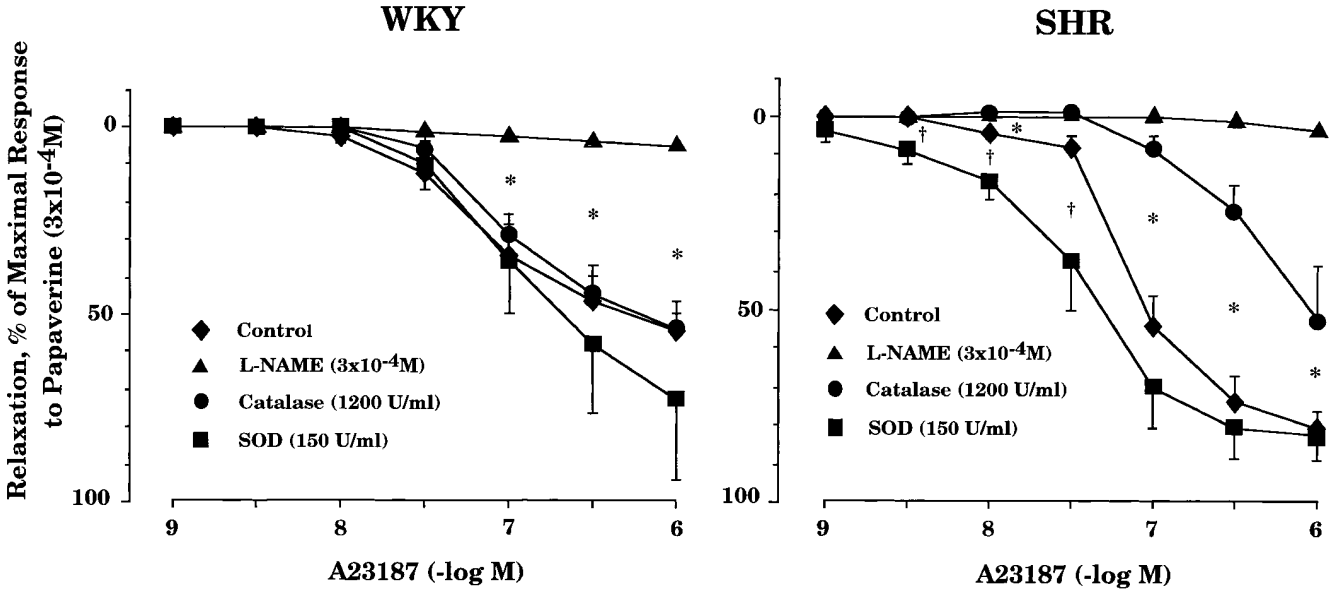

Figure 4. Line graphs showing concentration-response curves to calcium ionophore A23187 in aortas with endothelium of Wistar-Kyoto (*WKY*, *left*) and spontaneously hypertensive rats (*SHR*, *right*). Relaxations were obtained during contractions to norepinephrine (3×10^{-7} mol/liter) in control rings and rings treated with L-NAME, catalase, or SOD. Data are means±SEM (n = 5–6 for each group). *Significant difference between control and L-NAME–treated rings or control and catalase-treated rings ($P < .05$). †Significant difference between control and SOD-treated rings ($P < 0.05$).

A23187. L-NAME and L-NMMA inhibited A23187-induced production of O_2^- by 25% (data not shown) and 70% (Fig. 3). The relative inhibition of O_2^- production by L-NAME and L-NMMA was similar to the inhibitory effect of the two L-arginine analogs on A23187-induced NO release (28% and 68%, respectively).

Endothelium-dependent relaxations to A23187. During contractions to norepinephrine (3×10^{-7} mol/liter) calcium ionophore A23187 (10^{-9}–10^{-6} mol/liter) caused endothelium-dependent relaxations both in WKY and SHR (Fig. 4). A23187-induced relaxations were greater in SHR. Inhibition of cNOS with L-NAME (3×10^{-4} mol/liter) abolished relaxations to A23187 in both strains (Fig. 4). Accordingly, A23187-induced relaxations were abolished by a different cNOS inhibitor diphenylene iodonium (10^{-5} mol/liter; data not shown). Only in SHR, the relaxations were significantly inhibited by catalase (1,200 U/ml) and enhanced by SOD (150 U/ml; Fig. 4). By contrast, SOD plus catalase did not exert any effect on the response to A23187 (Table I). Also, inhibition of xanthine oxidase with oxypurinol (10^{-5} mol/liter) did not affect these relaxations (Table I). Furthermore, the effects of catalase or SOD on A23187-induced relaxations were abolished in the presence of the natural cofactor for cNOS, H_4B (10^{-4} mol/liter; Fig. 5), but not affected by exogenous L-arginine (10^{-3} mol/liter; data not shown). The absence or the presence of cyclooxygenase inhibitor indomethacin (10^{-5} mol/liter) did not affect the response to A23187 in SHR aortas (-Log EC_{50}: 7.2±0.04 and 7.2±0.06; n = 8; with and without indomethacin, respectively). Similar findings were obtained with acetylcholine (10^{-9}–10^{-4} mol/liter; data not shown).

Endothelium-independent relaxations to sodium nitroprusside. During contractions induced with norepinephrine, the NO donor sodium nitroprusside (10^{-10}–10^{-5} mol/liter) caused concentration-dependent relaxations in SHR aorta without endothelium. Catalase, SOD, and L-NAME did not affect the relaxations to sodium nitroprusside (Table I).

Levels of tetrahydrobiopterin and measurements of cNOS activity. The levels of H_4B, assessed by HPLC, were not significantly different in SHR and in WKY aortas (6.4±0.5 and 7.5±0.7 pmol/mg, respectively; n = 15). By contrast, cNOS activity was significantly higher in WKY than in SHR aortas after H_4B supplementation (Fig. 6).

Table I. EC_{50} and Maximal Relaxations in SHR Aortas With (A) and Without Endothelium (B) in Response to A23187 and Sodium Nitroprusside, Respectively. Effect of SOD plus Catalase, Oxypurinol, SOD, and Catalase alone, N^G-nitro-L-arginine methyl ester

	-Log EC_{50}	Maximal relaxation (%)	n
A			
Control	7.2±0.1	74±9	4
SOD (150 U/ml) + Catalase (1200 U/ml)	7.3±0.1	79±5	4
Oxypurinol (10^{-5} M)	7.0±0.1	71±9	6
B			
Control	7.8±0.1	100	4
SOD (150 U/ml)	8.0±0.1	100	4
Catalase (1200 U/ml)	7.9±0.1	100	4
L-NAME (3×10^{-4} M)	7.8±0.1	100	4

EC_{50} indicates half-maximal effective concentration; n, number of rats. Values are mean±SEM. Relaxations are expressed as percent of maximal relaxation to papaverine (3×10^{-4} mol/l). Relaxations were obtained during contractions to norepinephrine (3×10^{-7} mol/l).

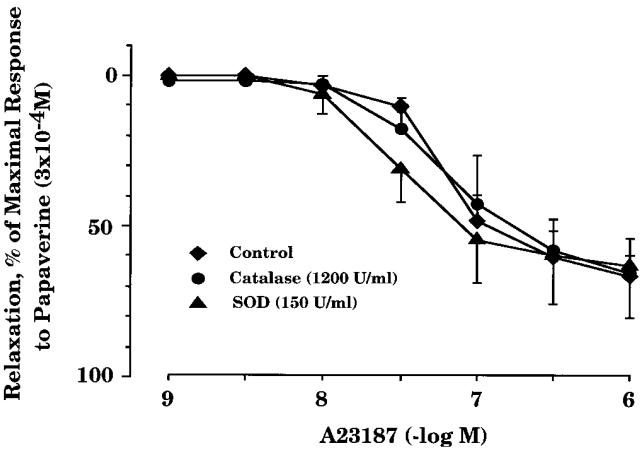

Figure 5. Line graph showing concentration–response curves to calcium ionophore A23187 in aortas with endothelium of spontaneously hypertensive rats incubated in the presence of (6R)-5,6,7,8-tetrahydrobiopterin (10^{-4} mol/liter). Relaxations were obtained during contractions to norepinephrine (3×10^{-7} mol/liter) in control rings and rings treated with catalase or SOD. Data are means±SEM ($n = 4$ for each group).

Discussion

This study demonstrates that cNOS-dependent generation of O_2^- occurs in prehypertensive SHR aorta. This conclusion is supported by several lines of evidence. First, the peak endothelial NO concentrations, after A23187 stimulation, were significantly smaller in the aortas of SHR than in WKY rats. Second, H_4B supplementation in SHR diminished the cNOS-dependent generation of O_2^- and its dismutase product H_2O_2, while it increased the net NO production. Finally, only in SHR were the endothelium-dependent relaxation in response to A23187 significantly enhanced by SOD and inhibited by catalase.

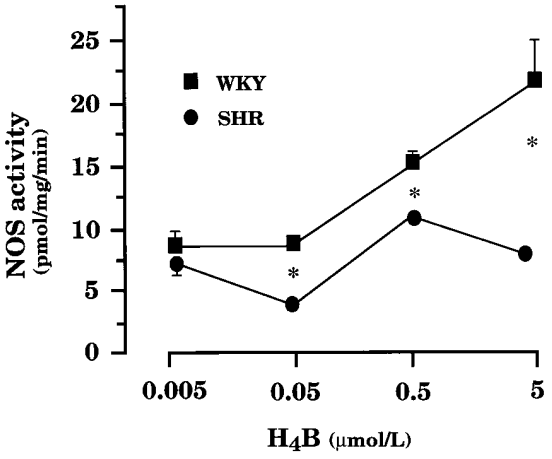

Figure 6. Line graph showing the effect of various doses of (6R)-5,6,7,8-tetrahydrobiopterin (expressed as micromoles per liter) on constitutive nitric oxide synthase activity (expressed as picomoles of L-citrulline generated per milligram of aortic tissue per minute) in aortas with endothelium of Wistar-Kyoto (*WKY*) and spontaneously hypertensive rats (*SHR*). Data are means±SEM ($n = 3$). *Significant difference between WKY and SHR ($P < 0.05$).

There are many sources of O_2^- in the endothelium including cyclooxygenase, xanthine oxidase, and NADH oxidoreductase (15, 25, 26). However, it has also been demonstrated that isolated neuronal cNOS may produce O_2^- in H_4B or L-arginine starved and SOD-free environments (10). In this work, confirmation that cNOS rather than other sources produced most of the O_2^- in prehypertensive SHR came from experiments showing O_2^- release after treatment with a NO agonist, as well as its inhibition after incubation with known cNOS inhibitors. The rapid accumulation of O_2^- concentration in the presence of A23187 suggests that production of O_2^- is calcium-dependent like the production of NO by cNOS. Furthermore, as with NO production, O_2^- production can be inhibited by L-arginine analogs, such as L-NMMA and L-NAME. We observed that the sum of the O_2^- produced by all non-cNOS sources, without A23187 stimulation (basal release), accounts for only 25–30% of the total O_2^- produced after stimulation by A23187. Therefore, it can be assumed that the remaining 70–75% is associated with calcium-dependent cNOS. Interestingly, the release of this particular fraction of O_2^- was almost entirely inhibited by L-NMMA.

The organ chamber experiments in this study were performed in the presence of indomethacin to rule out the possibility that free radical production was initiated by activation of arachidonic acid metabolism via the cyclooxygenase pathway. Endothelium-dependent relaxations in response to A23187 were present in aortas of both SHR and WKY rats. L-NAME abolished these relaxations, demonstrating that the effect of A23187 is mediated by activation of cNOS. Further analysis revealed that catalase inhibited, and SOD enhanced, the endothelium-dependent relaxations to A23187 only in SHR aortas, suggesting that H_2O_2, a product of O_2^- dismutation formed via cNOS, becomes a mediator of these relaxations. The lack of this SOD effect in the presence of catalase support this conclusion. Indeed, previous studies (14, 27, 28) provided evidence that H_2O_2 is a potent vasodilator through direct activation of soluble guanylyl cyclase in smooth muscle cells and increase of cyclic GMP. Also, we ruled out the possibility that xanthine oxidase could be a source of reduced oxygen species. Oxypurinol a selective inhibitor of this enzyme did not exert any effect on A23187-induced relaxations. Targeting of other oxidative enzymes by pharmacological inhibitors may be difficult. Diphenylene iodonium, a selective inhibitor of NADPH oxidoreductase, also inhibits NOS (29, 30). Accordingly, A23187-induced relaxations were abolished by this agent. Its inhibitory effect on both enzymes may further confuse the issue of which and how much each source contributes to increase O_2^- generation. On the basis of the specificity of L-arginine analogs (L-NMMA, L-NAME) for NOS and the fact that reciprocal changes in O_2^- release are observed, it is unlikely the increase of O_2^- is derived from non-NOS. However, we are aware that our data do not completely exclude such a possibility.

Furthermore, SOD, catalase, and L-NAME did not affect relaxations to a NO donor sodium nitroprusside in preparations without endothelium, which strongly suggests that O_2^- is not produced by smooth muscle cells in SHR aortas.

Interestingly, incubation of SHR aortas with supplementary H_4B, abolished the effect of catalase and SOD on A23187-induced relaxations. Furthermore, after treatment with elevated H_4B concentration, any significant difference in the concentrations of NO, O_2^- and H_2O_2 between the two strains disap-

peared. By contrast, L-arginine only slightly attenuated the inhibitory effect of catalase on the response to A23187. The effect of exogenous supplementation of H_4B is consistent with the concept of an altered H_4B-cNOS interaction which may be related to an impaired synthesis (13) or decreased affinity of the enzyme for the cofactor (31). To more precisely characterize the mechanisms leading to such cNOS-catalyzed production of O_2^-, we measured the levels of H_4B in the vessel wall as well as the activity of NOS in response to exogenous H_4B. Although H_4B concentrations were lower in SHR than in WKY aortas, the difference was not statistically significant. In contrast, cNOS activity (measured as picomol of L-citrulline produced per milligram of aortic tissue per minute) was significantly higher in WKY when compared with SHR aortas. These results would suggest that an increased requirement for H_4B may trigger an uncoupling of the oxidative and reductive domain of the enzyme resulting in dysfunctional cNOS activity in prehypertensive SHR. However, the possibility that H_4B has no allosteric effect on cNOS cannot be excluded based on our current data. It is possible that when the cNOS–H_4B interaction decreases below some critical level, no conformation changes occur between the two domains of the enzyme; cNOS simply starts reducing its other substrate O_2 into O_2^-. Neither dynamic nor static model of cNOS–H_4B interaction can be proven without tertiary crystallographic structures of the enzyme in the presence and in the absence of H_4B. Therefore, at this time, it is safe to assert, based on direct measurements of O_2^- by a chemiluminescence method, that O_2^- is produced in relatively high concentration in endothelium of prehypertensive SHR and this production is inversely related to NO accumulation.

Even though the total NO production in SHR (in the presence of SOD) is comparable with WKY, the concomitant increased production of O_2^- may rapidly react with it ($k = 3.8 \times 10^9$ L mol^{-1}s^{-1}) to produce the stable product peroxynitrite ($OONO^-$; 32). This reaction is even faster than the one of O_2^- with SOD ($k = 2 \times 10^9$ L mol^{-1}s^{-1}) to form H_2O_2 and O_2. Any $OONO^-$ produced when protonated ($pK_a = 6.8$) forms peroxynitrous acid (HOONO) which usually undergoes isomerization ($t_{1/2} < 1$ s) to form hydrogen cation and nitrate anion (33). However, if for any reason the HOONO concentration increases, as maximal O_2^- accumulations react with freshly synthesized NO, local HOONO concentration may become sufficient to assure its efficient transport to reactive sites as far as several cell diameters away (33). In the vicinity of certain reactive centers, HOONO may undergo homolytic cleavage to a hydroxyl free radical (OH) and nitrogen dioxide free radical (NO_2), or heterolytic cleavage to a nitronium cation (NO_2^+) and hydroxide anion (OH^-; 33). Three of these cleavage products (OH, NO_2 radicals, and NO_2^+), are among the most reactive and damaging species in biological systems (33).

The most intriguing and clinically useful finding of this paper is that the high concentration of the SOD product H_2O_2 observed in SHR may have some short term beneficial effect by virtue of its profound vasodilation properties. However, long term exposure of biological systems to elevated H_2O_2 is not advised, since any free ferrous ion (Fe^{2+}) that escapes the iron transporter ferritin will rapidly convert H_2O_2 into OH radical (Fenton reaction), the most destructive oxygen radical species (34).

The enhanced production of cNOS derived O_2^- in prehypertensive SHR may contribute to the development of hypertension, especially because this defect precedes the increase in blood pressure in this genetic model of hypertension. Endothelium-dependent contractions have been described in young SHR before the development of manifest hypertension (35). Interestingly, these contractions could be abolished with an inhibitor of O_2^- production. Intravenous injections of SOD reduced arterial blood pressure in SHR but not in WKY normotensive rats (36). Furthermore, a correlation between the expression of endothelium-dependent contractions and increase in arterial blood pressure has been reported in SHR (37). These findings suggest that an increased production of O_2^- may play a role in the development of high arterial tone in hypertension.

Whether O_2^- formed via cNOS in prehypertensive SHR serves this function remains to be determined. However, this study strongly supports the concept of a dysfunctional cNOS as a new source of reactive oxygen metabolites (14, 31). This cNOS-catalyzed formation of O_2^- and its subsequent transformation into HOONO cleavage products, or its dismutation into H_2O_2 and Fenton reaction product OH, may play a central role in the endothelial dysfunction and oxidative vascular injury described in a number of vascular diseases (38–42). Supplementation with H_4B, perhaps in combination with L-arginine (43) and free radical scavenger treatment (36) may find a future role in preventing essential hypertension and its complications.

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