Neuronal Nitric Oxide Synthase Is Expressed in Rat Vascular Smooth Muscle Cells
Activation by Angiotensin II in Hypertension

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Abstract—The nitric oxide synthase (NOS) inhibitor nitro-L-arginine augmented the contractions to angiotensin (Ang) II in carotid artery rings without endothelium from spontaneously hypertensive rats (SHR) but not normotensive Wistar-Kyoto rats, suggesting the possibility of nonendothelial NOS activity in SHR arteries. In SHR artery without endothelium, the potentiation of Ang II contraction by nitro-L-arginine was prevented by L-arginine, but not by D-arginine, and was observed also in the presence of oxyhemoglobin, monomethyl-L-arginine, and 7-nitroindazole, but not in the presence of aminoguanidine. In further support of NOS activation by Ang II in nonendothelial cells, Ang II but not acetylcholine stimulated cGMP levels by 2-fold in SHR arteries without endothelium; nitro-L-arginine decreased both basal and Ang II–stimulated cGMP levels. When NOS activity in SHR arteries was measured, no calcium-independent L-citrulline formation was detectable, while up to 47% of the total calcium-dependent NOS activity was present in nonendothelial cells. Expression of neuronal NOS was revealed in the media of SHR arteries by immunohistochemistry, Western blot, and reverse transcriptase–polymerase chain reaction. Expression of this NOS isoform was greater in SHR than in Wistar-Kyoto rat preparations. Finally, endothelial NOS was observed in the endothelium, but no detectable levels of inducible NOS were found in these tissues. These results demonstrate the expression of neuronal NOS in rat vascular smooth muscle cells and its activation on stimulation by Ang II in spontaneously hypertensive, but not normotensive, animals. (Circ Res. 1998;83:1271-1278.)

Key Words: cGMP • neuronal nitric oxide synthase • contraction • nitric oxide synthase activity • AT1 receptor

Under physiological conditions, nitric oxide (NO) is produced in the vessel wall by endothelial NO synthase (eNOS) localized in endothelial cells. The release of NO following activation of eNOS, together with the production of prostacyclin and endothelium-derived hyperpolarizing factor(s), modulates the response of vascular smooth muscle cells to contractile agents.1–4

The response of the vessel wall to the potent vasoconstrictor angiotensin (Ang) II depends on the endothelium, the presence of which can lead either to increased (in canine basilar and cerebral arteries)5–7 or to impaired contractions (such as in the rat aorta, the porcine femoral, or the bovine coronary arteries).8–9 In rat carotid arteries, Ang II activates endothelial receptors to release NO, which in turn impairs the contraction of vascular smooth muscle caused by the peptide.10–12

Since the contribution of endothelial NO to the regulation of vascular tone may be modified during hypertension, the response to Ang II was examined in carotid artery from spontaneously hypertensive rats (SHR). Preliminary experiments show markedly different responses to Ang II that suggest an additional nonendothelial source of NO in arteries from hypertensive rats.13 Further experiments were aimed at identifying the NO synthase (NOS) isoform activated by Ang II in nonendothelial cells of the SHR carotid artery.

Materials and Methods
All experiments were performed on the common carotid artery of SHR and normotensive male Wistar-Kyoto rats (WKY) (10 to 12 weeks old: 260 to 290 g; Harlan Sprague-Dawley, Indianapolis, IN, and Iffa-Credo, Domaine des Oncins, St Germain sur l’Arbresle, France). In addition, carotid arteries from prehypertensive SHR and age-matched WKY (4 weeks old: 90 to 100 g) were studied in reverse transcriptase–polymerase chain reaction (RT-PCR) experiments.

All procedures were performed in accordance with the guidelines of the Animal Protocol Review Committee of Baylor College of Medicine and those from the European Community standards for animal care and euthanasia (French Ministère de l’Agriculture; authorization number 07430). Systolic arterial blood pressure was measured by the tail cuff method and averaged 140±5 (n=14) and
Organ Chamber Experiments
The common left and right carotid arteries of WKY and SHR (10 to 12 weeks old) were dissected free, excised, and placed in ice-cold modified Krebs-Ringer bicarbonate solution of the following composition (in mmol/L): NaCl 118.3, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25.0, EDTA 0.026 and glucose 11.1 (control solution). The blood vessels were cleaned of adherent connective tissue and cut into rings (5 mm long). Unless specified, the endothelium was removed by gently rubbing the intimal surface with the tip of a pair of small forceps. Rings were suspended horizontally between 2 stainless steel wires in organ chambers that contained 25 mL of control solution (37°C) aerated with 95% O2 and 5% CO2 and the tip of a pair of small forceps. Carotid rings were incubated in control solution (37°C, 60 minutes) and cut into rings (5 mm long). Unless specified, the endothelium was confirmed by the lack of effect of acetylcholine on the rings without endothelium. In preparations without endothelium, the absence of endothelium was confirmed by the lack of effect of acetylcholine on the preparations 30 minutes before the dose-response curve to Ang II and did not affect the basal tension of the preparations as compared with control conditions. In SHR preparations without endothelium, the absence of endothelium was confirmed by the lack of effect of acetylcholine on the basal level of cGMP (control, 64.8±6.0 fmol cGMP/10 mg tissue; n=6). In SHR preparations with endothelium, a comparable exposure to acetylcholine caused a 4-fold increase in intracellular cGMP levels (from 88.3±14.3 to 352.7±38.5 fmol cGMP/10 mg tissue; n=4).

NOS Activity Measurement
NOS activity was determined by measuring the conversion of L-[^3H]arginine to L-[^3H]citrulline.11 Experiments were performed in parallel using homogenates from SHR carotid arteries with or without endothelium (10 to 12 weeks old). The endothelium of some SHR carotid arteries was removed by opening the preparation longitudinally in cold Krebs-Ringer solution, pinning it down (lumen facing up) and removing mechanically the endothelium with a pair of forceps and a wetted-cotton tip. Carotid arteries with or without endothelium were homogenized at 4°C in 500 μL of homogenization buffer (composition: Tris-HCl 50 mMol/L; pH 7.4; CHAPS 10 mMol/L, EDTA 2.0 mMol/L, DTT 1.0 mMol/L, phenylmethylsulfonyl fluoride 1.0 mMol/L, pepstatin A 1.0 mMol/L, and leupeptin 2.0 mMol/L). Homogenates were centrifuged at 10,000g for 20 minutes. Then, aliquots of supernatant (about 150 μg of protein) were incubated in Tris-HCl buffer (50 mMol/L, pH 7.4) containing NAPDH (1 mMol/L), flavin adenine dinucleotide (4.0 mMol/L), flavin mononucleotide (4.0 mMol/L), tetrahydrobiopterin (10.0 mMol/L), calmodulin (1.0 mg/L), and L-[^3H]arginine (1.0 μCi/L; 23 μmol/mL; Amersham, Les Ulis, France). Reactions were conducted at 37°C for 60 minutes in a final volume of 450 μL, either in the presence of CaCl2 (25 mMol/L) or in that of EDTA (20 mMol/L); they were terminated by adding 1 mL ice-cold stopping buffer (composition: HEPES 30 mMol/L, pH 5.5, and EDTA 3.0 mMol/L). The L-[^3H]citrulline was separated from the L-[^3H]arginine by cationic exchange chromatography (Dowex AG50W-X8; Na+ form; Bio-Rad Laboratories). Concentration of the specifically eluted L-[^3H]citrulline was determined by liquid scintillation counting. The calcium-dependent NOS activity was evaluated by the difference in activity between samples assayed in the presence of CaCl2 and those assayed in the presence of EDTA.

Immunocytochemistry
Tissue Preparation
Carotid arteries of 10- to 12-week-old SHR were sectioned into 3- to 4-mm segments and either fixed in 10% neutral buffered formalin supplemented with zinc chloride (Antech, Ltd) or flash frozen in a freezing medium (TBS) for frozen section microtomy. Tissue to be used for paraffin sections remained in formalin for 18 to 24 hours and were then transferred to 70% ethyl alcohol until processing. Standard histological methods of dehydration were used in ascending grades of ethyl alcohol, clearing in xylene, and paraffin infiltration; tissues were transferred in paraffin wax blocks to present a luminal cross section of the vessel. The paraffin blocks were sectioned on a standard rotary microtome (Leitz 1512) at 3 μm; the sections were floated on a water bath and then retrieved on BioTek capillary gap slides (Ventana Medical Systems, Inc). The sections were allowed to dry for 1 hour in a 60°C oven. Slides were then rinsed well in standard PBS, pH 7.3.

Immunohistochemistry
Antigen retrieval was performed on the paraffin sections before immunohistochemistry. The sections were placed in a working solution of epitope recovery buffer (10× heat-induced enhanced retrieval [HIER]; HIER101; Ventana Medical Systems, Inc) and microwaved at high power for 5 minutes, distilled water was added to replenish the evaporated solvent, and slides were microwaved for an additional 5 minutes at the same power setting and then allowed to cool for a minimum of 15 minutes. The slides were then rinsed in distilled water and placed in PBS to which had been added 0.2% Tween 20 (Sigma Chemical Co). Immunohistochemistry of the paraffin and frozen sections was performed on a TekMate500 (Ventana Medical Systems, Inc) automated slide stainer. All reagents were applied to the sections according to the manufacturer's instructions.
used in the immunohistochemical procedure were constituted in a
radiolabelled with 35S (Amersham). Briefly, sections were incubated with protein-blocking
serum to minimize spurious background staining. The sections were then incubated overnight at room temperature with
the primary antibody (polyclonal anti-neuronal NOS [nNOS], monoclonal anti-eNOS, or monoclonal anti-iNOS [iNOS], all at 1
µg/mL, from Transduction Laboratories), a nonimmune IgG control
(rabbit or mouse IgG, 1 µg/mL, from Cappel), or diluent. Following washes in the PBS buffer, the slides were incubated with a biotinylated, species-specific secondary antibody (anti-mouse IgG or
anti-rabbit IgG; Vector Laboratories) at a concentration of 2.25
µg/mL for 45 minutes at room temperature. Endogenous peroxidase
activity was quenched by incubating sections in 0.3% H2O2 in
methanol for 15 minutes at room temperature. Sections were incubated in a peroxidase-conjugated avidin-biotin complex (Vector Laboratories) for 45 minutes at room temperature, and antigenic sites were visualized using diaminobenzidine as the chromagen (Sigma Chemical Co); buffer washes were carried out between each of the above steps. Sections were counterstained with hematoxylin, rinsed in buffer for bluing, and then processed through ascending grades of ethyl alcohol and finally into xylene. The sections were coverslipped using a synthetic mountant.

Western Blot Analysis

Tissue Preparation

Cardiot arteries used for Western blot and total RNA extraction were excised aseptically from WKY and SHR (4 and 10 to 12 weeks old), gently flushed with cold, sterile Hanks’ balanced solution (Gibco; Life Technologies, Inc, Paisley, Scotland, UK) and incubated for 30
to 40 minutes at 37°C in 175 U of collagenase (Worthington) in 2 mL of Hanks’ solution. At the end of the incubation, the adventitial layer was quickly removed with a pair of forceps. Then the endothelium was mechanically scraped away after the arteries were longitudinally opened. The resultant medial layer obtained was frozen in liquid nitrogen until assayed.

Western Blot

Arteries from 10- to 12-week-old WKY and SHR were homogenized in 500 µL of buffer (200 mmol/L sucrose and 20 mmol/L HEPES, pH 7.4) containing protease inhibitors (1.1 µmol/L leupeptin, 0.7 µmol/L aprotinin, 120 µmol/L phenylmethylsulfonyl fluoride, 1 mmol/L iodoacetamide, 0.7 µmol/L pepstatin, and 1 mmol/L diisopropylfluorophosphate). The homogenate was centrifuged (14,000 rpm) for 15 minutes. Protein content in the supernatant was determined by the method of Bradford. Equal amounts of proteins (50 µg per lane) were denatured and separated in denaturing SDS/7% polyacrylamide gels. Proteins were then transferred onto a nitrocellulose membrane (Hybond enhanced chemiluminescence [ECL]; Amersham, Les Ulis, France). Blots were blocked for 2 hours at room temperature with 5% nonfat dry milk in TBS-T (20 mL of TBS- Tris-HCl, 137 mmol/L NaCl, and 0.1% Tween 20) before incubation with rabbit polyclonal anti-nNOS (dilution 1:500; Transduction Laboratories) for 2 hours at room temperature. The membrane was washed and incubated with the anti-rabbit IgG conjugated to horse-radish peroxidase (1:5000 dilution for 2 hours at room temperature). After successive washes with TBS-T, the immunocomplexes were detected by chemiluminescent reaction (ECL+ kit; Amersham, Les Ulis, France) followed by exposition of the membranes to Hyperfilm ECL (15 minutes).

RT-PCR

For total RNA extraction and RT-PCR, carotid arteries without endo-
thelium and without adventitia from WKY and SHR (4 and 10 to 12 weeks old) were obtained as described above for Western blot analysis. Total RNA was extracted according to Trizol reagent protocol (Life Technologies, Inc, Cergy Pontoise, France). Purified RNA was dis-
solved in water and the concentration measured by absorbance at 260
nm. The quality of RNA was confirmed by ethidium bromide staining in 1% agarose gel. RT-PCR was then performed for nNOS and GAPDH
gene expression. The primer chosen for rat nNOS was, for sense, 5'-CTGGCTCAACAGAATACGGCT-3' and, for antisense, 5'-ACAGTTGACAGCTCCTGGAAGA-3', thus amplifying a 293-bp fragment. For rat GAPDH, the following primers were used: 5'-TGAAGTGGCTGTGCAACGGATTTTGTC-3' (sense) and 5'-CAGTGGCATAGGTCCTACAC-3' (antisense), resulting in a 982-bp band. First-strand cDNA was performed on 100 ng of total RNA. The single-strand cDNA synthesis was carried out in 20 µL of reaction buffer, consisting of 20 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.6 mmol/L MgCl2, 1 mmol/L dNTP, 10 mmol/L DTT, 0.2 µmol/L oligo(dT). The reaction mixture was incubated for 10 minutes at 25°C and then 60 minutes at 37°C. Then, nNOS and GAPDH were coamplified using 2.5 U of Taq DNA polymerase (Boehringer) and 0.5 µmol/L of the sense and antisense primers of each set in 50 µL of 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 4.5 mmol/L MgCl2, 1 mmol/L dNTP, and 0.01% gelatin. Preliminary studies showed that the amplification reaction reached a plateau phase after 25 and 32 cycles for GAPDH and nNOS, respectively. Therefore, the GAPDH primers were added to the nNOS amplification tube 7 cycles after the beginning of the reaction. In these conditions, the reaction is linearly related to the initial cDNA concentration, and the nNOS/GAPDH ratio is not depen-
dent on the amount of cDNA used for the coamplification reaction. Semiquantitative RT-PCR was then performed by coamplifying nNOS and GAPDH. To quantify nNOS and GAPDH mRNA levels, a trace amount of [3H]dCTP was included in the PCR reaction. The PCR products were then separated on a 5% polyacrylamide gel, and radio-
active signals were analyzed using a computer-based imaging system (Fuji Bas 1000; Fuji Medical Systems).

Statistical Analysis

Results are given as mean±SEM. For organ chamber experiments, data are expressed as percentage of the contraction evoked by phenylephrine (30 µmol/L). Concentration-response curves were fitted to the equation:

yp=yo+UpperLimit*(concentration/LowerLimit)-1

using non-linear regression (GraphPad, San Diego, CA). The 50% of the maximal response (EC50) values represent the concentration of Ang II (in mmol/L) that elicits 50% of the maximal response. Experiments with Ang II antagonists (losartan and PD-123319) were performed on rings from the same rat studied in parallel. The pA2 value (estimates of the equilibrium dissociation constant) for losartan was determined from the graph of log concentration ratios minus 1 versus log concentra-
tion of Ang II that elicits the same degree of response in the absence of the antagonist. The pA2 value was calculated only if the slope of the plot was not different from unity. 10,16

Statistical Analysis

Results are given as mean±SEM. For organ chamber experiments, data are expressed as percentage of the contraction evoked by phenylephrine (30 µmol/L). Statistical analysis was performed using the GraphPad InStat software (GraphPad, San Diego, CA). The 50% of the maximal response (EC50) values represent the concentration of Ang II (in mmol/L) that elicits 50% of the maximal response. Experiments with Ang II antagonists (losartan and PD-123319) were performed on rings from the same rat studied in parallel. The pA2 value (estimates of the equilibrium dissociation constant) for losartan was determined from the graph of log concentration ratios minus 1 versus log concentra-
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Results

The response to Ang II was evaluated in WKY and SHR carotid arteries. In both strains, the contractions to the peptide were augmented following removal of the endothelium (Figure 1). However, the contribution of the endothelium was different between WKY and SHR preparations. Indeed, the endothelial inhibitory component of the response to Ang II (defined as the difference in areas under the concentration-dependent curves obtained in preparations with and without endothelium) was greater in WKY than in SHR (493±145 versus 132±46 arbitrary units, respectively; n=6; P<0.05).

In the normotensive rats, L-NA (0.1 mmol/L) significantly augmented the contractions evoked by Ang II in preparations with endothelium to reach a comparable response to that of rings without endothelium (Figure 1A). L-NA did not affect the contractions to Ang II in preparations without endothelium.
Contractions to Ang II of carotid arteries from normotensive WKY (A) and SHR (B). Experiments were performed on preparations with (+E; closed symbols) and without endothelium (−E; open symbols). Data are given as mean±SEM and are expressed as a percentage of the response of each preparation to phenylephrine (30 μmol/L). A, The response to the peptide was examined in control conditions in WKY rings with (●) and without (○) endothelium; contractions to Ang II were also examined in parallel in the presence of L-NA (0.1 mmol/L) in preparations with (■) and without endothelium (□). The contractions evoked by phenylephrine were not significantly different between the 4 groups of rings (control without endothelium, 1.9±0.1 g; control with endothelium, 2.0±0.2 g; with endothelium in the presence of L-NA, 1.9±0.2 g; without endothelium in the presence of L-NA, 1.7±0.2 g; n=6; P>0.05). B, The response to the peptide was examined in control conditions in SHR rings with (●) and without (○) endothelium; contractions to Ang II were also examined in parallel in the presence of L-NA (0.1 mmol/L) in preparations with (■) and without endothelium (□). The contractions evoked by phenylephrine were not significantly different between the 4 groups of rings (control without endothelium, 1.8±0.2 g; control with endothelium, 2.0±0.2 g; with endothelium in the presence of L-NA, 1.8±0.2 g; without endothelium in the presence of L-NA, 1.7±0.2 g; n=6; P>0.05). *Significant difference of endothelium removal or L-NA. n.s. indicates not significant.
Effect of the Ang II Receptor Antagonists Losartan and PD-123319 on the Contractions Evoked by Ang II in Rings Without Endothelium From SHR Carotid Arteries (n=6)

<table>
<thead>
<tr>
<th></th>
<th>Ang II EC50, nmol/L</th>
<th>Ang II Maximal Response, mg</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.45±0.10</td>
<td>1322±151</td>
</tr>
<tr>
<td>Losartan (10 nmol/L)</td>
<td>18.20±3.01*</td>
<td>1248±132</td>
</tr>
<tr>
<td>Losartan (100 nmol/L)</td>
<td>252.76±24.16*</td>
<td>1476±147</td>
</tr>
<tr>
<td>Losartan (300 nmol/L)</td>
<td>598.75±107.07*</td>
<td>1310±70</td>
</tr>
<tr>
<td>PD-123319 (1 µmol/L)</td>
<td>0.62±0.12</td>
<td>1267±189</td>
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*Antagonist has significant effect when compared with control.

Discussion

The present experiments show that nNOS is expressed in smooth muscle cells of rat carotid artery. Ang II stimulates the release of NO from this isoform only in arteries from SHR, leading to an impairment of the direct contractile effect of the peptide.

The first sign of the presence of a NOS activated by Ang II in nonendothelial cells of the SHR carotid artery came from in vitro experiments showing the augmentation of the contraction to the peptide by L-NA in preparations without endothelium. It is unlikely that this potentiating effect of L-NA is caused by a nonspecific effect of the compound. Indeed, it could be mimicked by another nonpreferential inhibitor of NOS, L-NMMA, and with oxyhemoglobin, a scavenger of NO. Further, the reversal by L-arginine, but not D-arginine, of the potentiating effect of L-NA confirmed the

limum (Figure 3). In addition, the contraction to Ang II was not augmented after exposure to dexamethasone (0.1 µmol/L) for a 3.5-hour incubation (control, 1.3±0.1 g; with dexamethasone, 1.2±0.1 g; n=8; NS).26

In SHR rings without endothelium, losartan (10 to 300 nmol/L; a preferential AT1 receptor antagonist) caused a parallel rightward displacement of the concentration-response curve to Ang II without affecting the maximal response to the peptide (Table). The slope of the Arunlakshana-Schild plot was not different from unity (slope=1.03±0.05; n=6; P=0.55), and the pA2 value was estimated to be 9.6±0.1 (n=6). PD-123319 (1 µmol/L; a preferential AT1 receptor antagonist) did not significantly affect the response to Ang II in SHR carotid arteries without endothelium (Table).

In SHR carotid arteries without endothelium, the basal content in cGMP was significantly augmented after exposure to Ang II (100 nmol/L; 1 minute), (Figure 4). However, the peptide (100 nmol/L; 1 minute) did not significantly affect cGMP levels in WKY carotid arteries without endothelium (basal, 75±15; Ang II, 81±9 fmol/10 mg tissue; n=4; NS). In SHR preparations without endothelium, L-NA (0.1 mmol/L) significantly decreased the level of cGMP, both under basal conditions and following exposure to Ang II (Figure 4).

The endogenous NOS activity was estimated by the conversion of l-arginine into l-citrulline in SHR carotid arteries with and without endothelium. In arteries without endothelium, there was a detectable calcium-dependent NOS activity that was significantly less than that of intact carotid arteries with endothelium (with endothelium, 1.35±0.1; without endothelium, 0.64±0.06 nmol/mg protein/min; n=6; P<0.05). There was no detectable formation of l-citrulline either in arteries with or without endothelium when assays were performed in the absence of calcium (n=6).

The immunohistochemistry studies (n=3) using specific antibodies against different isoforms of NOSs demonstrated that eNOS was located exclusively in the endothelial cells of the SHR carotid artery (Figure 5A). The presence of the inducible isoform (iNOS) was detected only following prolonged (24-hour) in vitro exposure of the preparations to interleukin-1β (10 ng/mL); under these conditions, staining was observed in the intimal, the medial, and the adventitial layers of the artery (Figure 5C and 5D). The specific antibody against the third isoform (nNOS) revealed the presence of a nNOS mainly in the tunica media and in a few cells of the adventitia (Figure 5B). A similar conclusion was obtained for WKY carotid arteries: eNOS and nNOS were expressed in endothelial and smooth muscle cells, respectively, while iNOS expression was undetectable under control conditions (data not shown). Western blot analysis using the nNOS antibody revealed the presence of a 155-kDa protein in smooth muscle cells of both WKY and SHR carotid arteries; nNOS expression was greater in SHR than in WKY (Figure 6).

Expression of nNOS mRNA was observed by RT-PCR in the medial layer of SHR and WKY carotid arteries (Figure 7). The ratio of nNOS to GAPDH coamplified cDNAs was compared in 4-week-old (prehypertensive) and 10- to 12-week-old WKY and SHR, respectively. The expression of nNOS was significantly greater in 10- to 12-week-old SHR arteries as compared with age-matched WKY. However, there was no significant difference in nNOS expression between prehypertensive 4-week-old SHR and age-matched WKY (Figure 7).

The first sign of the presence of a NOS activated by Ang II in SHR carotid arteries without endothelium (n=6). All experiments were performed in the presence of isobutylmethylxanthine (0.1 mmol/L; 45 minutes) to prevent activation of phosphodiesterases. This was done under control conditions (open bars) or following incubation with L-NA (0.1 mmol/L; 30 minutes; gray bars) Data are given as mean±SEM and expressed as fmol of cGMP/10 mg wet weight. There was no weight difference between the 4 groups of SHR preparations without endothelium (basal, 4.8±0.5 mg; basal+L-NA, 5.5±0.5 mg; Ang II, 4.1±0.9 mg; Ang II+L-NA, 4.6±0.7 mg; n=6). *Significant effect of Ang II. **Significant effect of L-NA.
stereospecificity of the interaction of L-NA with a NOS in nonendothelial cells of the SHR carotid artery.

The presence of an iNOS could explain the augmenting effect of the NOS inhibitor on the response to Ang II. Indeed, this isoform is induced following cytokine or lipopolysaccharide exposure of cultured smooth muscle cells or isolated blood vessels. However, this hypothesis appears unlikely. First, aminoguanidine, a preferential inhibitor of iNOS, did not affect the contraction evoked by Ang II in denuded carotid arteries. In addition, dexamethasone, which prevents the induction of NOS in the vessel wall, did not augment the contractions to Ang II under the present experimental conditions. The lack of pharmacological evidence for the presence of an inducible form of NOS in SHR carotid arteries without endothelium was reinforced by the fact that no calcium-independent NOS activity could be detected in this blood vessel. Furthermore, the presence of this isoform was not observed by immunohistochemistry, except after prolonged exposure to cytokines, as evidenced earlier. These results also demonstrate that iNOS is not constitutively expressed in the SHR carotid artery, as observed earlier in the kidney.

On the other hand, several observations support the existence of a NOS isoform other than the inducible one in carotid arteries without endothelium. First of all, a significant calcium-dependent NOS activity could be found in denuded arteries, suggesting the presence of at least 1 of the so-called constitutive isoforms in the media and/or the adventitia. This was supported by RT-PCR, which demonstrated the presence of the nNOS isoform message in SHR carotid arteries without endothelium and without adventitia. Immunohistochemistry studies and Western blot analysis revealed the expression of nNOS protein in the smooth muscle cells while the staining for eNOS was restricted to the

Figure 5. Immunostaining of SHR carotid arteries with endothelium with the anti-eNOS (A), anti-nNOS (B), and anti-iNOS (C and D) antibodies. Experiments were performed under control conditions (A through C) or following exposure of the tissue to interleukin-1β (20 μg/mL) for 24 hours as a positive control for the anti-iNOS antibody (D).

Figure 6. Representative Western blot of nNOS protein expression in carotid arteries from 10- to 12-week-old WKY and SHR.
Activation of this calcium-dependent nNOS by Ang II likely contributes to the L-NA–sensitive increase in intracellular cGMP levels in preparations without endothelium. A putative endothelium-dependent increase in cGMP by Ang II can be ruled out under these experimental conditions, because acetylcholine was no longer able to stimulate soluble guanylate cyclase following activation of the eNOS. The potentiation of Ang II contractions by 7-NI, at a concentration reported to preferentially inhibit the nNOS, is consistent with the presence of the neuronal isoform protein and mRNA in the media, as evidenced by immunohistochemistry, Western blot, and RT-PCR.

Experiments with Ang II receptor antagonists suggest that the functional effects of the peptide (contraction and nNOS stimulation) are mediated solely by activation of the Ang AT1 receptor in the smooth muscle cells of the SHR carotid artery. Indeed, a large concentration of the preferential AT2 receptor antagonist PD-123319 does not affect the response to the peptide, while losartan, the preferential AT1 receptor antagonist, exerts a competitive antagonism on the response to Ang II in SHR arteries without endothelium (this study and Reference 10).

Expression of nNOS is not restricted to neurons. Indeed, this isoform has been found also in several cell types, including gastric smooth muscle and skeletal muscle. Expression of a constitutive NOS in vascular smooth muscle has been suggested also from experiments measuring NO release from cultured human vascular smooth muscle cells without deliberate exposure to cytokines. In addition, vascular smooth muscle cells from cerebral arteries display an NADPH-diaphorase activity that colocalizes, at least in part, with NOS activity. Furthermore, canine veins without endothelium exhibit a NO-like activity under experimental circumstances in which the inducible isoform is unlikely to be expressed. Finally, nNOS immunoreactivity has been observed in the media of coronary and pulmonary arteries from newborn rats.

Although Ang II has multiple effects on the release of endothelial vasoactive factors, several pieces of evidence show that the peptide activates the eNOS isoform in rat endothelial cells. This contributes to the impairing of the direct contractile effect of the peptide on vascular smooth muscle and may favor local blood flow when plasma or tissue levels of Ang II are increased. The present experiments show that the endothelial inhibitory component of the response to the Ang II is impaired in carotid arteries from SHR. Activation of nNOS in nonendothelial cells of the hypertensive wall could compensate for the endothelial dysfunction in response to Ang II observed in SHR carotid arteries. The expression of nNOS, or its activation by Ang II, in smooth muscle cells from SHR may be restricted to large vessels. Indeed, the response to Ang II of SHR mesenteric resistance arteries without endothelium appears to be insensitive to an inhibitor of NOS. However, the present results are consistent with the observation that renal NO production by Ang II is mediated by at least 2 NOS isoforms, 1 of them being nNOS.

Although nNOS is expressed also in carotid arteries from normotensive rats, this isoform is not activated by Ang II, as evidenced by the lack of effect of a NOS inhibitor on response of the smooth muscle to Ang II. Further evidence against nNOS activation in normotensive animals comes from the lack of effect of Ang II on cGMP levels in denuded arteries, in agreement with previous studies. One possible explanation could be the lower level of expression observed in this strain as compared with that of arteries from SHR. Alternatively, presence of an endogenous NOS inhibitor in WKY preparations or differences in NOS cofactor(s), L-arginine bioavailability, nNOS subcellular localization, or Ang II signal transduction pathway could explain the lack of activation of this NOS isoform by Ang II in vascular smooth muscle cells from normotensive animals.

In conclusion, the present study demonstrates the expression of nNOS in rat vascular smooth muscle cells from a large artery. The present data also provide biochemical and functional evidence that the nNOS isoform is stimulated following Ang II AT1 receptor subtype in arteries from spontaneously hypertensive, but not normotensive, animals. The increased expression of nNOS in SHR smooth muscle cells

Figure 7. A, Representative RT-PCR analysis of nNOS and GAPDH mRNA levels in carotid arteries from 10- to 12-week-old WKY and SHR (without endothelium and without adventitia). Total RNA (100 ng) was assayed for nNOS and GAPDH. Amplified products are electrophoresed on a 5% polyacrylamide gel. MWM indicates molecular weight marker. B, Ratio of amplified nNOS vs GAPDH in carotid arteries without endothelium and without adventitia from WKY and SHR (4 and 10 to 12 weeks old) (n=8). *Significant difference between WKY and SHR.
does not result from the different genetic backgrounds of WKY and SHR, but is likely to be associated with the development of hypertension. Activation of the nNOS isoform may compensate for a weakened endothelial response to Ang II in SHR. The nNOS isoform may not only counter-regulate vasoconstrictor responses such as that to Ang II but could participate also in the regulation of other smooth muscle cell functions during hypertension.

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References
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