Functional expression of NOS 1 in vascular smooth muscle

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INCREASES IN cGMP have been associated with Ca2+-dependent contractions of various smooth muscle preparations (1). Thus it was initially postulated in 1975 that increases in cGMP led to smooth muscle contraction (1). Thus it was initially postulated in 1975 that increases in cGMP led to smooth muscle contraction (1). Subsequently, it was determined that nitric oxide (NO) and NO donors led to vasorelaxation via activating soluble guanylyl cyclase, which led to increases in intracellular cGMP concentrations in vascular smooth muscle (VSM; see Refs. 20, 23, 34, 37). However, the specific mechanisms by which increases in [Ca2+]i lead to increases in intracellular cGMP in endothelium-denuded VSM preparations are not known.

The specific mechanisms by which increases in cGMP lead to relaxation of the VSM are also unknown. Although many investigators have suggested that contraction of smooth muscle is associated with increases in intracellular Ca2+ concentrations ([Ca2+]i) and relaxation with decreases in [Ca2+]i, there are substantial data that muscle preparations can contract without increases in [Ca2+]i or when the [Ca2+]i is fixed, and the same is true with respect to relaxation (14, 25). Another proposed mechanism of cyclic nucleotide-dependent relaxation is via activation of the cyclic nucleotide-dependent protein kinases, AMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG; see Ref. 30). One recently identified substrate protein of PKA and PKG is the small heat shock-related protein, HSP20 (4). Cyclic nucleotide-dependent vasorelaxation is associated with increases in the phosphorylation of HSP20 (4, 27). HSP20 is an actin-binding protein, and the association with actin depends on the phosphorylation state of HSP20 (13). In a smooth muscle preparation that is uniquely refractory to cyclic nucleotide-dependent vasorelaxation, umbilical artery smooth muscle, there is no increase in the phosphorylation of HSP20 (7, 12). The site on the HSP20 molecule that is phosphorylated during cyclic nucleotide-dependent relaxation has been identified (serine 16), and the introduction of peptide analogs as inhibitors in transiently permeabilized VSM inhibits and augments serotonin (5-HT)-induced contraction (5). Thus it is possible that HSP20 modulates relaxation by a direct interaction with the contractile apparatus.

Neuronal nitric oxide synthase isoform; guanosine 3',5'-cyclic monophosphate; heat shock protein 20; 7-nitroindazole; N\(^{6}\)-(1-imino-3-butenyl)-L-ornithine
specific antibodies with Western blotting and immunospecific NOS enzymes present in VSM using isoform-activation in VSM. We then sought to characterize the provide biochemical and physiological evidence for NOS activation in VSM. This would 5-HT-induced contraction of endothelium-denuded VSM strips leads to activation of NOS. This would provide biochemical and physiological evidence for NOS activation in VSM. We then sought to characterize the specific NOS enzymes present in VSM using isoform-specific antibodies with Western blotting and immuno-histochemical techniques.

**MATERIALS AND METHODS**

Materials. N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) and IBMX were obtained from Calbiochem (La Jolla, CA), 5-HT, forskolin, and N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NNAME) were from Sigma Chemical (St. Louis, MO), 7-Nitroindazole (7-NI) and anti-NOS specific antibodies were from BIOMOL (Plymouth Meeting, PA). N\textsuperscript{S}-(1-imino-3-butenyl)-L-ornithine (L-VNI) was purchased from Alexis Biochemicals (San Diego, CA). \textsuperscript{125}I-labeled protein A, [\textsuperscript{3H}]arginine, and \textsuperscript{125}I-labeled cGMP were obtained from Amersham (Arlington Heights, IL). Amphotiles and electrophoresis reagents were purchased from Pharmacia/LKB (Uppsala, Sweden), and Immobilon from Millipore (Bedford, MA).

Preparation of muscle strips. Bovine carotid arteries were obtained from near-term calves at a local abattoir immediately after death (Shapiro’s, Augusta, GA). Human renal arterial tissue was obtained from organ donors. The arteries were transported in ice-cold HEPES-buffered salt solution (in mM: 140 NaCl, 4.7 KCl, 1.0 MgSO\textsubscript{4}, 1.0 NaH\textsubscript{2}PO\textsubscript{4}, 1.0 CaCl\textsubscript{2}, 10 glucose, and 10 HEPES (pH 7.4)). Smooth muscle strips were prepared by gently removing the connective tissue. The arteries were opened longitudinally, the adventitia was removed, and the endothelium was denuded by gently rubbing the intima with a cotton-tipped applicator. Complete removal of the endothelium with this technique has been previously confirmed with scanning electron microscopy (43). Transverse muscle strips, 1 mm in width, were cut and equilibrated in bicarbonate buffer [in mM: 120 NaCl, 4.7 KCl, 1.0 MgSO\textsubscript{4}, 1.0 NaH\textsubscript{2}PO\textsubscript{4}, 10 glucose, 15 CaCl\textsubscript{2}, and 25 Na\textsubscript{2}HCO\textsubscript{3} (pH 7.4)], bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2} and maintained at 37°C. All experiments were performed on freshly prepared carotid arteries.

Measurement of intracellular cGMP content. Strips of bovine carotid artery smooth muscle were equilibrated in bicarbonate buffer, and agonists and/or inhibitors were added to the buffer. cGMP was extracted from the tissue according to the protocol of Bansinath et al. (3). The strips (0.15 g of tissue) were snap-frozen in acetone-dry ice and were homogenized in 1 ml of ice-cold 6% TCA using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) at a speed setting of 7 for 30 s. The homogenate was centrifuged 15,000 g for 15 min at 4°C. The supernatant was washed four times with 5 vol of water-saturated diethyl ether, and the upper ether phase was discarded with each wash. The aqueous extract was dried using a speed evaporator (Speed Vac Plus, Savant Instruments, Holbrook, NY). The dried extract was reconstituted in 1 ml of acetate buffer (50 mM, pH 6.2), and cGMP was quantitated by RIA (36). The pellet was resuspended in 500 µl of 1 N NaOH, and protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL).

Measurement of NOS activity by conversion of [\textsuperscript{3H}]arginine to [\textsuperscript{3H}]citrulline. Endothelium-denuded bovine carotid vessels were weighed and snap-frozen in liquid nitrogen. The frozen vessels were pulverized, placed in homogenization buffer at a 20 times vol/wt ratio (25 mM Tris·HCl, pH 7.4, 1 mM EDTA, and 1 mM EGTA) in the presence of protease inhibitors (1 mM PMSF, 1 µM pepstatin A, 2 µM leupeptin, and 0.1% aprotinin), and homogenized with a glass-glass homogenizer for 10 strokes on ice. Glyceral was added for a final 10% solution, and the homogenate was snap-frozen in aliquots. Protein concentration was determined by standard Bradford assay (Bio-Rad) using BSA as the standard. Aliquots of the bovine vessel homogenate or rat brain preparation were incubated with [\textsuperscript{3H}]arginine (10 µM final arginine, 71 Ci/mmol) in the presence of 1 mM NADPH, 30 mM cysteine, 3 µM tetrahydrobiopterin, 2 mM Ca\textsubscript{2+}, 1 µM FAD, and 1 µM FMN in a total volume of 50 µl (36). Additional aliquots were incubated with 1 mM L-NNAME or 1 µM L-VNIO. After a 30-min incubation at 37°C, the reaction was terminated by the addition of 1 ml 50 mM HEPE, pH 5.5, containing 2 mM EDTA and 2 mM EGTA. The reactions were applied to 1 ml Dowex AG 50WX-8 columns (Na form; Bio-Rad) and the [\textsuperscript{3H}]citrulline eluted with 2 vol of 0.75 ml water. The eluted radioactivity was quantitated by liquid scintillation counting (Beckman 6500; Beckman-Coulter Instruments).

HSP20 phosphorylation. Phosphorylation of HSP20 is associated with a shift of the protein from a more basic nonphosphorylated isofrom to multiple phosphorylated isoforms. Using this property, and a highly specific affinity-purified polyclonal antibody against HSP20 (28), isoelectric focusing immunoblots were performed to determine HSP20 phosphorylation. The strips were equilibrated in buffer, and agonists and/or inhibitors were added directly to the buffer. After the appropriate time points, the vessels were then snap-frozen in acetone/dry ice and ground to a fine powder under liquid nitrogen. The powder was placed in acetone, TCA (10%), and dithiothreitol (DTT, 100 mM) solution, frozen in liquid nitrogen again, and allowed to come to room temperature. The TCA was removed by washing three times in ac.etone/DTT, and the samples were speed evaporated. The powder was reconstituted in urea (9 M), 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (2%), and DTT (100 mM), and protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce). Protein (30 µg) was loaded in each well of an IEF slab gel containing Ampholines (pH 4–6, 6–8, 1:1). The proteins were focused for 1 h at 100 V, 1 h at 200 V, and 75 min at 500 V. The proteins were then transferred to Immobilon for 210 volt hours. The blot was air-dried and subsequently blocked with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.4)/5% milk for 1 h. The blots were then incubated with anti-HSP20 antibodies (1: 1,000) in TBS/mlk for 1 h at room temperature. The blots were washed three times (5 min each) in TBS/Tween 20 (0.5%). Immunoreactive protein was determined using \textsuperscript{125}I-protein A (1:1,000 dilution). The blots were again washed six times (5 min each) in TBS/Tween-20. The blots were then exposed on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) screen for 18 h.
Immunohistochemistry. Strips of tissue were fixed in 4% buffered paraformaldehyde and paraffin-embedded, and 8-µm sections were cut and placed on poly-L-lysine slides. The tissue sections were stained by the avidin-biotin-peroxidase complex (ABC) method. Endogenous peroxidase and biotin activity was blocked by immersing slides in 0.03% hydrogen peroxide in methanol for 30 min followed by washing in PBS (3 × 10 min) and a biotin blocking kit (Vector). After blocking (nonspecific binding by incubation in 3% normal horse serum in PBS for 20 min), sections are incubated overnight with primary antibody (1:100) or control solution in PBS containing 0.05% BSA and 0.1% sodium azide. After being washed in PBS (3 × 10 min), sections were incubated successively with biotinylated horse anti-mouse IgG (1:100) and freshly prepared ABC (Vectastain; Vector) for 30 and 60 min, respectively. Peroxidase activity is detected using glucose oxidase-3,3'-diaminobenzidine (DAB) with the nickel enhancement method. Hematoxylin was used as a counterstain. The slides were viewed with a Zeiss Axioshot 2 microscope interfaced with a Spot camera (Diagnostic Instruments, Sterling Heights, MI).

Western blotting of NOS 1. Tissue homogenates were prepared for Western blotting from the endothelium-denuded bovine carotid vessels as outlined above for NOS activity. Briefly, the proteins were separated on 7.5% SDS-PAGE and transferred to nitrocellulose with a Trans-blot (Bio-Rad) for 45 min. The blots are allowed to air-dry for 30 min and were blocked with 5% nonfat dry milk diluted in TBS (blocking buffer) for 1 h at room temperature. The blots were incubated with the primary antibody (polyclonal anti-NOS 1; 1:1,000) diluted in blocking buffer overnight at 4°C followed by two washes with blocking buffer at room temperature. The blots are then incubated with the secondary antibody (horse radish peroxidase-conjugated goat anti-rabbit antibody; Amersham) for 1 h at room temperature followed by four washes with TBS. The specific bands are detected using the enhanced chemiluminescence system (Amersham). The immunogen that was used to generate the rabbit polyclonal antibodies was human NOS 1 synthetic peptide (1414–1434) conjugated to Keyhole limpet hemocyanin.

Physiological contractile responses. Transverse strips, 1.0 mM in diameter, were cut, and a loop of 3–0 silk was tied to each end. The strips were suspended in a muscle bath containing bicarbonate buffer (in mM: 120 NaCl, 4.7 KCl, 1.0 MgSO4, 1.0 NaH2PO4, 10 glucose, 1.5 CaCl2, and 25 Na2HCO3, pH 7.4) and were equilibrated with 95% O2-5% CO2 at 37°C for at least 1 h. Changes in isometric force were registered by tension transducers (Grass Instrument, Quincy, MA) and were recorded on a chart recorder (Gould Instrument, Norcross, GA). The strips were progressively stretched, the isometric forces generated in response to 110 mM KCl (made with equimolar replacement of NaCl in bicarbonate buffer) were determined until an optimal tension was identified for the given length of the muscle strip, and reproducible contractile responses were obtained with repeated additions of KCl. Agonists and inhibitors were diluted in bicarbonate buffer and were added directly to the muscle bath. The mean resting tension was 1 g. The muscles were incubated in the presence of 7-NI for 15 s and were treated with high KCl (110 mM). Next, the maximal tension was determined after 10 min. The strips were washed three times with bicarbonate buffer and allowed to equilibrate for 30 s after each contraction. After each experiment, the strips were blotted, and wet weight was measured. Stress (10² N/m²) was calculated as force (g) × 0.0987/area, where area = wet weight (mg)/length (mm at maximal length)/1.055.

Statistical analysis. The results are reported as means ± SE. Comparisons were made by one-way ANOVA using the multiple-comparison Tukey's Test on Sigma Stat software (Jandel Scientific, San Rafael, CA), with P < 0.05 considered significant. Densitometric analysis was performed with a PhosphorImager using Imagequant software (Molecular Dynamics).

RESULTS

cGMP content and NOS activity. To determine if stimulation with the contractile agonist 5-HT leads to increases in intracellular cGMP content, strips of bovine carotid artery smooth muscle were equilibrated in bicarbonate buffer and treated with 5-HT (10 µM, 10 min). Some strips were preincubated with the NOS inhibitor L-NMMA (1 mM) for 15 min and then were treated with 5-HT (10 µM, 10 min). The addition of the guanylyl cyclase activator sodium nitroprusside (SNP, 10 µM) was used as a positive control. All experiments were performed in the presence of the phosphodiesterase inhibitor IBMX (1 mM) to prevent breakdown of cGMP. Stimulation with 5-HT led to significant increases in cGMP (Fig. 1). Pretreatment with L-NMMA inhibited the increase in cGMP content. Treatment with SNP (positive control) also led to significant increases in cGMP.

NOS activity in the endothelium-denuded bovine vessel was assessed by the conversion of radiolabeled L-arginine to L-citrulline. Three separate preparations of the bovine vessels revealed a consistent amount of NOS activity at a rate of 0.71 ± 0.23 pmol citrulline/mg protein. Inhibition by L-NAME (1 mM), a nonspecific NOS inhibitor, decreased the activity by 98%. L-VNIO (1 µM), a newly described NOS 1-specific inhibitor (2), reduced the bovine VSM NOS activity by 99%. According to Babu and Griffith (2), the inhibitory constant for L-VNIO for NOS 1 is 0.1 µM; that for NOS 2 and NOS 3 isoforms are 60 and 12 µM, respectively. For compari-

![Fig. 1. cGMP content. Strips of bovine carotid artery smooth muscle were incubated in the presence of buffer alone (control) or serotonin (5-HT, 10 µM, 10 min), preincubated with N-monomethyl-L-arginine (L-NMMA; 1 mM, 15 min) followed by 5-HT (10 µM, 10 min), or 5-HT (10 µM, 10 min) followed by sodium nitroprusside (SNP, 10 µM, 10 min). Stimulation with 5-HT or SNP led to significant increases in cGMP content (*P < 0.05, ANOVA, n = 4 separate muscle preparations). However, when the strips were preincubated with L-NMMA, there was no increase in cGMP content. The results are represented as %increase over control within each experiment.](image-url)
son purposes, we evaluated the inhibition of NOS 1 derived from rat brain with L-NAME and L-VNIO. L-NAME (1 mM) and L-VNIO (1 µM) decreased rat brain NOS activity by 96 and 80%, respectively.

Phosphorylation of HSP20 in VSM. To determine if activation of the guanylyl cyclase/cGMP pathway led to increases in the phosphorylation of the PKG substrate HSP20, strips of carotid artery smooth muscle were treated with 5-HT (10 µM) with and without preincubation with L-NMMA (1 mM). Stimulation of carotid artery smooth muscle with 5-HT led to significant increases in the phosphorylated isoform of HSP20 (Fig. 2). To determine if the 5-HT-induced increase in the phosphorylation of HSP20 was due to activation of NOS, strips of carotid arteries were preincubated with L-NMMA and then were treated with 5-HT. Preincubation with L-NMMA inhibited the increase in phosphorylation of HSP20 associated with 5-HT stimulation.

Immunohistochemistry and Western blotting. Immunohistochemistry revealed the presence of NOS 3 in the endothelium of the bovine carotid artery (Fig. 3A) and human renal artery (data not shown) and expression of NOS 1 in the media of bovine carotid artery (Figs. 3C and 4B). The specificity of the anti-NOS 1 antibody was confirmed immunohistochemically by a positive reaction in neurons of the rat brain (data not shown). There was no immunoreactive NOS 2 in bovine carotid artery (Figs. 3B and 4A). There was no immunoreactive product when samples were prepared without the primary antibody (data not shown). Western blotting confirmed the presence of NOS 1 in tissue homogenates of endothelium-denuded carotid artery with specific anti-NOS 1 antibodies (Fig. 5).

Physiological contractile responses. We have previously demonstrated that pretreatment of bovine carotid artery smooth muscle with L-NMMA increases the magnitude of the contractile response to 5-HT and high extracellular KCl (43). To determine if other NOS inhibitors also increase contractile responses to agents that increase intracellular Ca²⁺, strips of bovine carotid artery smooth muscle were stimulated with high extracellular KCl (110 mM). The muscles were then treated with 7-NI, a more specific inhibitor of NOS 1, and again were stimulated with KCl. Pretreatment with increasing doses of 7-NI produced a dose-dependent increase in the magnitude of the KCl-induced contraction (Fig. 6). There was no increase in the magnitude of repeated KCl-induced contractions without pretreatment with 7-NI (Fig. 6).

DISCUSSION

In this investigation, we confirmed that stimulation of VSM with an agonist that is known to increase [Ca²⁺], 5-HT, leads to increases in intracellular cGMP content. These increases in cGMP content do not occur when the muscle strips are pretreated with the NOS inhibitor L-NMMA. A more direct assessment of NOS
activity, conversion of radiolabeled L-arginine to L-citrulline, determined that a small but consistent amount of NOS is present in the VSM homogenate. Additionally, a newly described NOS 1-specific inhibitor, L-VNIO, abolished the activity. L-VNIO is \( \approx \) 100- and 600-fold more potent as an inhibitor of NOS 1 over NOS 3 and NOS 2, respectively. We have recently determined that a major substrate protein for PKG in VSM is the small heat shock-related protein HSP20 (4).

Stimulation of carotid artery smooth muscle preparations with 5-HT resulted in increases in the phosphorylation of HSP20. This increase in phosphorylation was inhibited by preincubation of the muscle strips with L-NMMA.

Using NOS isoform-specific antibodies in immunohistochemical and Western blotting analysis, we determined that NOS 1 is expressed in VSM. Specific anti-NOS 3 antibodies did confirm expression of NOS 3 in the endothelium; however, no positive reaction was demonstrated in the media of the vessel wall. There was also no immunoreactive NOS 2 in the media of bovine carotid or human renal arteries. Western blotting analysis with anti-NOS 1 specific antibodies demonstrated that the specific band detected was at 160 kDa, thus indicating that the antibody does not cross-react with NOS 3 or NOS 2.

Finally, we determined that 7-NI, a more specific NOS 1 inhibitor, augmented high KCl-induced contractions in a dose-dependent manner. 7-NI has been suggested to be an NOS 1 inhibitor based on its inhibitory profile with in vivo studies (32, 40, 44). However, enzymatic in vitro assays have not shown 7-NI to be specific for the NOS 1 isoform (31). KCl induces VSM contractions by depolarizing the membrane and allowing extracellular Ca\(^{2+}\) to enter the cells. We have previously reported that pretreatment of bovine carotid artery smooth muscle with the nonspecific NOS inhibitor L-NMMA increases the magnitude of the contractile response to 5-HT stimulation (43). However, contractions induced by the phorbol ester, phorbol 12,13-dibutyrate, are not augmented by L-NMMA when Ca\(^{2+}\) is chelated. Taken together, these data suggest that a Ca\(^{2+}\)-dependent NOS is present in VSM and can be activated by agents that increase intracellular Ca\(^{2+}\).

Our previous reports (26, 43) have indicated that there is pharmacological and physiological evidence for NOS activation in VSM. Bovine carotid smooth muscle
contractions induced by 5-HT or norepinephrine were shown to be attenuated by preincubation with L-NMMA. Other reports have demonstrated evidence for NOS activation in smooth muscle as well (6, 8, 25), although these reports did not discern between constitutive and inducible NOS. Increased NO production has been measured in endothelium-denuded bovine intrapulmonary arteries (45). Wood and colleagues (42) have found that the VSM is capable of producing a relaxing factor pharmacologically and chemically similar to NO. Cumulative addition of the NOS inhibitor L-NMMA to muscle bathings containing rings of rat aortic smooth muscle causes contractions in these preparations (16). Endothelium-denuded canine saphenous vein smooth muscle rings revealed an NOS activity only when cyclooxygenase activity was inhibited (24). Papadaki et al. (35) have recently reported that cultured human aortic smooth muscle cells produce NO in response to fluid flow, and Western blotting with a monoclonal anti-NOS 1 demonstrated a specific product. Finally, Boulanger et al. (10) have shown that nitro-L-arginine augments contractions to ANG II in VSM (10).

These data provide physiological, biochemical, and morphological evidence for the presence of NOS 1 in VSM from intact vessels. Ca2+-dependent activation of NOS 1 may function as an autocrine feedback mechanism to inhibit VSM contraction. Thus smooth muscle NOS 1 may be important in the regulation of vasomotor tone.

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