Smooth Muscle Cell Responsiveness to Nitrovasodilators in Hypertensive and Normotensive Rats

Andreas Papapetropoulos, Nándor Marczin, Mary D. Snead, Charles Cheng, Antonio Milici, John D. Catravas

Abstract Endothelium-derived relaxing factor and exogenous nitrovasodilators are thought to produce smooth muscle relaxation by activation of soluble guanylate cyclase. To investigate whether diminished cyclic GMP (cGMP) accumulation underlies the differences in vascular reactivity to nitrovasodilators between Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), we determined cGMP formation in aortic smooth muscle cells from the two strains. Both cultured cells and aortic rings from 12- to 14-week-old SHR accumulated greater amounts of cGMP on stimulation with exogenous nitrovasodilators (ie, sodium nitroprusside) than those from WKY rats, whereas there was no difference observed in cells from prehypertensive animals (5- to 6-week old) between the two strains. Responsiveness of smooth muscle cells to endothelium-derived relaxing factor was investigated in cocultures of bovine aortic endothelial cells (BAE) and smooth muscle cells from SHR and WKY rats. cGMP accumulation elicited by endothelium-derived relaxing factor released either basally or in response to bradykinin and the calcium ionophore A23187 was greater in smooth muscle from 12- to 14-week-old SHR than from age-matched WKY rats (80±17 versus 11±2 for basal; 152±12 versus 80±26 for A23187; 163±21 versus 40±12 pmol/mg protein per 15 minutes for bradykinin) in SHR/BAE and WKY/BAE cocultures, respectively. Northern blot analysis of steady-state messenger RNA levels for the β1 subunit of soluble guanylate cyclase revealed higher levels of the message in SHR. These results indicate that smooth muscle cells from SHR accumulate greater amounts of cGMP than WKY cells in response to endogenous or exogenous nitric oxide, possibly because of increased levels of soluble guanylate cyclase. (Hypertension. 1994;23:476-484.)

Key Words • rats, inbred SHR • guanosine cyclic monophosphate • nitroglycerin • endothelium-derived relaxing factor • guanyl cyclase • nitroglycerin

Nitrovasodilators are thought to produce vascular smooth muscle relaxation through activation of the soluble isoenform of guanylate cyclase, increasing cellular cyclic GMP (cGMP). Although the mechanism of action of cGMP is not fully understood, it has been shown that cGMP lowers intracellular Ca2+ levels and alters the phosphorylation pattern of cellular proteins involved in contraction. It has been suggested that some of the actions of cGMP are mediated by activation of cGMP-dependent protein kinase. Vascular endothelium has been shown to release, both under basal conditions and after agonist stimulation (ie, bradykinin, acetylcholine, calcium ionophore A23187), an endogenous nitrovasodilator, known as endothelium-derived relaxing factor (EDRF), which is nitric oxide or a nitric oxide-containing molecule, synthesized from l-arginine. EDRF release produces vascular relaxation through elevation of cGMP levels in the smooth muscle, Basal release of EDRF in vivo regulates vascular tone because l-arginine analogues, which inhibit nitric oxide synthase, elevate blood pressure. Moreover, vascular endothelial cells in culture release EDRF, which leads to increased accumulation of cGMP in smooth muscle—endothelium cocultures.

In several models of hypertension, both endothelium-dependent and -independent responses are altered. Increased total peripheral resistance has been hypothesized to be partly due to decreased EDRF action and/or increased release of endothelium-derived constricting factors, which in the spontaneously hypertensive rat (SHR), the most commonly used model of genetic hypertension, are mediated by a cyclooxygenase pathway product. Studies on both endothelium-dependent and -independent relaxations in SHR have yielded conflicting results. Whereas many investigators report decreased relaxation to sodium nitroprusside (SNP) or glyceryl trinitrate (GTN) in the aortas and carotid arteries of SHR, others have shown unchanged or even enhanced relaxation in response to nitrovasodilators in the aorta and mesenteric resistance arteries from SHR or stroke-prone SHR compared with Wistar-Kyoto (WKY) rats. The use of different agents to constrict the vessel rings as well as the presence or absence of endothelium may explain some of these discrepancies. When norepinephrine was used to preconstrict mesenteric arteries from SHR and WKY rats, vasodilation in response to acetylcholine, an endothelium-dependent vasodilator, was smaller in SHR; however, relaxation to acetylcholine was comparable when the arteries were preconstricted with arginine or lysine vasopressin. Furthermore, SNP- and sodium nitrate–induced relaxations were lower in SHR than...
WKY rats in endothelium-intact but not endothelium-denuded aortas.27

The aim of the present study was to investigate, in smooth muscle cells derived from SHR and WKY rats, cGMP accumulation in response to both exogenous nitrovasodilators and endogenously produced EDRF from bovine aortic endothelial cells (BAE).

Methods

Cell Culture

Rat aortic smooth muscle cells were isolated from 12- to 14- and 5- to 6-week-old male SHR (Harlan Sprague Dawley, Inc) or WKY rats using previously published procedures,28 with four to five rats per isolation. Animal handling and euthanasia were in accordance with guidelines from the Institutional Committee on Animal Use for Research and Education. Cells were positively identified as smooth muscle cells by indirect immunofluorescent staining for α-actin using mouse anti-α-actin antibody and anti-mouse IgG fluorescein isothiocyanate conjugate. Smooth muscle cells were grown in T-75 tissue culture flasks (Corning Glass Inc) in 50% F12 and 50% Dulbecco’s modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc), 0.2 g/L l-glutamine, 100 μM penicillin, and 0.1 mg/mL streptomycin. Cells exhibited a typical cobblestone morphology and contact inhibition and were additionally characterized by immunofluorescent staining for a-actin using mouse anti-α-actin antibody and mouse anti-α-actin antibody and FITC conjugate. Smooth muscle cells were grown in T-75 tissue culture flasks in M199 (GIBCO) supplemented with 10% fetal calf serum, 100 μM penicillin, and 0.1 mg/mL streptomycin. Cells exhibited a typical cobblestone morphology and contact inhibition and were additionally characterized by immunofluorescent staining for von Willebrand factor, accumulation and binding of succinyl tyrosine methyl ester (a marker of smooth muscle actin) using monoclonal antibody for α-actin (Sigma Chemical Co). For the present study, cells between passages 1 and 12 were used. BAE were harvested nonenzymatically from aortas obtained at the local abattoir using previously published procedures,29 grown in T-25 tissue culture flasks in M199 (GIBCO) supplemented with 10% fetal calf serum, 100 μM penicillin, and 0.1 mg/mL streptomycin. Cells exhibited a typical cobblestone morphology and contact inhibition and were additionally characterized by immunofluorescent staining for von Willebrand factor, accumulation and binding of succinyl tyrosine methyl ester (a marker of smooth muscle actin) using monoclonal antibody for α-actin (Sigma Chemical Co). For the present study, BAE between passages 1 and 3 were used at or about confluence.

Radioimmunoassay for cGMP

Monoclonal antibody for cGMP was a generous gift from Dr Ferid Murad (Molecular Geriatrics). The radioligand ([3H]sucinyl cGMP-tyroso methyl ester) was prepared in our laboratory. Stock solutions of the succinyl tyrosine methyl ester of cGMP (Sigma) were made up in 50 mmol/L sodium acetate buffer, pH 4.75, and iodinated by the method of Hunter and Greenwood30 using carrier-free [125I] (Du Pont-NEN). The iodination reaction products were separated by reverse-phase high-performance liquid chromatography.32 Radioimmunoassay was performed using the Gammaflow automated radioimmunoassay system.33 Standard stock solutions of cGMP (20 μmol/L) were prepared in 0.1N HCl, and the absorbance of the solution was routinely monitored spectrophoto metrically (UV 160U, Shimadzu Scientific Instruments). Standard dilutions (0.63 to 80 pmol/L) were made from the stock solution. The HCl extract containing cGMP was used for radioimmunoassay directly.

Isolation of Total RNA and Northern Blot Analysis

Total RNA was isolated from smooth muscle cells using a commercially available kit (RNAzol, Biotex Laboratories Inc), quantified by absorbance at 260 nm, and stored at −70°C in a mercaptoethanol/ethanol/ammonium acetate solution. A unique 40-mer oligonucleotide probe (5’-ATGTACGGTTTGTGAACCATGCCCTGGAGCTGCTGGTGA-3’) for the 70 kD subunit (β) of the rat lung soluble guanylate cyclase (sGC) was synthesized on an automated DNA synthesizer using phosphoramidite chemistry based on the sequence published by Nakane et al.34 The probe was labeled with α-32P-deoxyxycytidine 5’-triphosphate (NEN Research Products) using a random primer labeling kit (Pharmacia LKB Biotechnology) to a specific activity of 10⁶ cpm/μg. Total RNA (10 μg each) was electrophoresed on a 1% agarose gel in 10 mmol/L NaPO₄, pH 6.5, and transferred to a nylon membrane in 20x SSC. Membranes were hybridized overnight at 42°C in 50% formamide, 5× SSC, 0.2% sodium dodecyl sulfate (SDS), 0.05 mol/L NaCl, 10% dextran sulfate, 50 μg poly-A RNA, 50 μg denatured salmon sperm DNA, and the 32P-labeled probe (100 000 cpm/cm² of the membrane). After the overnight incubation membranes were washed at room temperature twice with 2× SSC/0.1% SDS for 10 minutes and then once at 50°C with 0.1× SSC/0.1% SDS for 5 minutes before being exposed to x-ray film. For test for equal loading and transfer, blots were stripped and rehybridized with a 1.2-kb chicken β-actin complementary DNA probe. Densitometric analysis of the autoradiographic signals was performed on a dual-wave-length scanning densitometer (CS 9000, Shimadzu).

Protein Determination

Protein content of the supernatant of the centrifuged (2000 rpm for 5 minutes at room temperature) NaOH-solubilized samples was measured by the Bradford method.35 Sample aliquots were combined with the protein binding dye (Bio-Rad), and optical density was determined at 630 nm using a multowell plate reader (Dyntech Laboratories Inc). Bovine albumin, fraction V (Sigma), was used as standard.

Experimental Protocol

Single Rat Aortic Smooth Muscle Cell Cultures

cGMP accumulation in smooth muscle cell cultures from age-matched SHR and WKY rats was determined using various nitrovasodilators. Cells were washed with Earle’s balanced salt solution (ES) to remove serum traces and incubated with ES containing SNP (Sigma; 0.1 μmol/L to 1 mmol/L) or GTN (Nitrostat, Parke-Davis; 0.1 to 100 μmol/L) for 15 minutes in the absence or presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, Sigma; 0.3 mmol/L) to prevent cGMP breakdown. The putative EDRF 5-nitroso-L-cysteine (CYSNO) was prepared in our laboratory according to previously published procedures36 and used at 10 nmol/L to 10 μmol/L to stimulate cGMP accumulation in the presence of IBMX. After the 15-minute incubation with the nitrovasodilators, medium was rapidly aspirated, and 200 μL of 0.1N HCl was added to each well to stop enzymatic reactions and extract cGMP. Thirty minutes later, the HCl extract was deproteinized and cell remnant removed from the wells by adding hot 1.0N NaOH and scraping the well with a rubber policeman. The HCl extract was analyzed for cGMP by radioimmunoassay, and NaOH-solubilized samples were used for protein determination.

Long- and Short-term Smooth Muscle–BAE Cocultures

EDRF-induced cGMP accumulation was investigated in long- and short-term cocultures as described.37 Briefly, for the long-term cocultures, BAE (50 000 cells per 2-cm² well) were seeded on a confluent SHR or WKY smooth muscle cell multilayer. After a 24-hour cocultivation period, the growth medium (M199) was aspirated, and the cells were washed with ES and incubated in ES containing 0.3 mmol/L IBMX for 15 minutes in the absence (basal) and presence of bradykinin or A23187 (agonist stimulated). For the short-term coculture experiments smooth muscle cells were grown in 24 multwell (2 cm² per well) plates, and BAE were grown on glass coverslips. The short-term cocultures were established by gently transferring, with the aid of fine forceps, the coverslips with the endothelial cells into wells containing smooth muscle. After a 10-minute equilibration period, the ES solution was aspirated and replaced with ES containing IBMX, with and without bradykinin or A23187. After 15 minutes, the IBMX solution was aspirated, the coverslips with BAE cells were removed from the wells, and intracellular cGMP content of the SHR or WKY smooth muscle cells was quantified as described above.
cGMP Determination in Aortic Rings

Aortas from 12- to 14-week-old SHR and WKY rats were removed and placed in Hanks' balanced salt solution. Polymyxin B (2 µg/mL), an antibiotic with anti-endotoxin activity, was included in all solutions bathing the aortas to eliminate undesirable increases in cGMP content caused by induction of nitric oxide synthase in the smooth muscle resulting from endotoxin contamination. Aortas were flushed with Hanks' solution to remove blood, cleaned of periadventitial fat, and cut in rings (3 to 4 mm long), and the endolumen was removed by forcing a glass rod through the lumen. Rings were then placed in ES (without polymyxin B) and allowed to equilibrate for 15 minutes before being treated with IBMX in the presence or absence of nitrovasodilators (10 µmol/L SNP, 10 µmol/L S-nitroso-N-acetylpenicillamine (SNAP)) for 20 minutes. Rings were then placed in 750 µL of 0.1N HCl, homogenized, and allowed to stand 30 minutes at room temperature for the cGMP extraction to be completed. After the 30-minute period samples were centrifuged at 12,000 rpm for 15 minutes, and the supernatant was removed for radioimmun assay determination of cGMP content. Pellets were resuspended in 1 mL of 1N NaOH and homogenized to redissolve and quantify proteins by the Bradford method.

Data Analysis

Data are presented as mean±SEM of the indicated number of individual cultures. Data are expressed either as picomoles cGMP per milligram protein per 15 minutes or as the percentage of the control value. Statistical comparisons between groups were performed using the one-way ANOVA or Student's t test, as appropriate. Differences among means were considered significant at a value of P<.05.

Results

Single Smooth Muscle Cell Cultures

Baseline cGMP values did not differ between WKY and SHR smooth muscle cells. Under control conditions cGMP levels were 1.48±0.13 versus 1.46±0.23 pmol/mg protein per 15 minutes for SHR and WKY cells, respectively, whereas in the presence of IBMX, cGMP levels were 2.52±0.10 versus 2.03±0.30 pmol/mg protein per 15 minutes for SHR and WKY cells, respectively. Exposure of smooth muscle cells of both SHR and WKY rats to 100 µmol/L SNP led to increased cGMP accumulation in the presence of IBMX (Fig 1). cGMP levels were greater in the presence of IBMX for both strains. Contrary to findings with cells derived from 5- to 6-week-old SHR and WKY rats displayed similar cGMP accumulation in response to SNP (Fig 2A), with maximal increases of 77±14-fold and 91±11-fold from baseline for SHR and WKY cells, respectively. Stimulation of smooth muscle cultures with the putative EDRF CYSN0 (Fig 2B) yielded results similar to those with SNP treatment. cGMP accumulation was greater in cells from 12- to 14-week-old SHR compared with age-matched WKY rats (Fig 2B), whereas it was similar in cells from 5- to 6-week-old animals (Fig 2D).

Smooth Muscle-Endothelium Cocultures

Long-term coculture of aortic smooth muscle cells with BAE led to increased cGMP accumulation in the presence of the phosphodiesterase inhibitor IBMX. Pretreatment of the cocultures with the L-arginine analogue N⁵-monomethyl L-arginine (L-NMMA) (300 µmol/L) for 30 minutes decreased cGMP levels to almost baseline smooth muscle cell levels (Table). The inhibition of cGMP accumulation by L-NMMA (17±1% of the coculture cGMP levels) could be reversed by the addition of 1 mmol/L L-arginine but not D-arginine (79±11% and 28±3%, respectively). Preincubation of the cocultures for 30 minutes with methylene blue (10 µmol/L) and oxyhemoglobin (10 µmol/L) decreased cGMP content of the coculture to 46±4% and 28±6%, respectively. Data shown in the Table are from SHR/BAE cocultures; similar results were observed in WKY/BAE cocultures. Treatment of single smooth muscle or endothelial cell cultures with L-NMMA, L-arginine or D-arginine, methylene blue, and oxyhemoglobin had no effect on smooth muscle or endothelial cGMP levels. Similar results were ob-
FIG 2. Line graphs show concentration response of sodium nitroprusside (SNP)-induced (A and C) and S-nitroso-L-cysteine (CYSNO)-induced (B and D) cyclic GMP (cGMP) accumulation in cultured smooth muscle cells from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Rat aortic smooth muscle cells from SHR and WKY rats were exposed to SNP (0.1 μmol/L to 1 mmol/L) or the putative endothelium-derived relaxing factor CYSNO (10 nmol/L to 1 μmol/L) for 15 minutes in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (0.3 mmol/L). A and B, Cells isolated from 12- to 14-week-old animals; C and D, cells isolated from young, prehypertensive (5- to 6-week-old) animals (YWKY or YSHR). cGMP was extracted with HCl and measured by radioimmunoassay. Mean±SEM; n=4 wells. *P<.05 compared with WKY or YWKY rats; #P<.05 compared with YSHR.

Table: Cyclic GMP Accumulation in Rat Aortic Smooth Muscle-Bovine Aortic Endothelial Cell Cocultures

<table>
<thead>
<tr>
<th></th>
<th>Basal cGMP, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without L-NMMA</td>
</tr>
<tr>
<td>Control</td>
<td>100±21.5</td>
</tr>
<tr>
<td>L-Arginine (1 mmol/L)</td>
<td>94.2±13.5</td>
</tr>
<tr>
<td>d-Arginine (1 mmol/L)</td>
<td>93.9±7.6</td>
</tr>
<tr>
<td>Methylene blue (10 μmol/L)</td>
<td>45.6±3.9*</td>
</tr>
<tr>
<td>Oxyhemoglobin (10 μmol/L)</td>
<td>27.6±5.6*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Agonist-Stimulated cGMP, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without L-NMMA</td>
</tr>
<tr>
<td>Control</td>
<td>100±7</td>
</tr>
<tr>
<td>A23187 (1 μmol/L)</td>
<td>520.7±51*</td>
</tr>
<tr>
<td>L-Arginine (1 mmol/L)</td>
<td>...</td>
</tr>
<tr>
<td>d-Arginine (1 mmol/L)</td>
<td>...</td>
</tr>
</tbody>
</table>

L-NMMA indicates N⁰-monomethyl L-arginine, cGMP, cyclic GMP; and L-NMMA, N⁰-nitro-L-arginine methyl ester. Data are from cocultures of aortic smooth muscle cells from spontaneously hypertensive rats and bovine aortic endothelial cells; similar results were observed in cocultures using cells from Wistar-Kyoto rats. Data are mean±SEM; n=4-8 wells.

*P<.05 compared with control.
Basal cGMP accumulation in long-term cocultures of SHR/BAE was greater than in WKY/BAE cocultures (Fig 3). Similar results were observed in short-term cocultures, in which BAE were grown on glass coverslips, transferred onto a confluent smooth muscle cell culture for 15 minutes, and then carefully removed. EDRF-induced cGMP accumulation was calculated by subtracting L-NAME cGMP values from control values. Mean±SEM; n=8 wells. *P<.05 compared with WKY rats.

Levels) of cGMP accumulation were reversed by the addition of L-arginine but not D-arginine (498±37% and 54±3%, respectively).

Basal cGMP accumulation in long-term cocultures of SHR/BAE was greater than in WKY/BAE cocultures (Fig 3). Similar results were observed in short-term cocultures, in which BAE were grown on glass coverslips and cGMP levels were measured in the smooth muscle after removal of the BAE. EDRF-induced cGMP accumulation (calculated as the L-NAME-inhibitable cGMP levels) was significantly greater in SHR versus WKY smooth muscle cells in both long- and short-term cocultures (80±17 versus 11±2 and 53±12 versus 17±5 pmol/mg protein per 15 minutes, for long- and short-term cocultures, respectively). A23187 produced larger increases in cGMP levels in both long- and short-term SHR/BAE compared with WKY/BAE cocultures (Fig 4A). For the long-term cocultures, EDRF-induced cGMP accumulation was 152±12 versus 80±26 pmol/mg protein per 15 minutes for SHR/BAE and WKY/BAE cocultures, respectively. In the short-term cocultures cGMP levels of 171±45 versus 30±11 pmol/mg protein per 15 minutes were observed for SHR/BAE and WKY/BAE, respectively. Another endothelium-dependent vasodilator, bradykinin (Fig 4B),
produced results similar to those for A23187. EDRF-induced bradykinin-stimulated cGMP accumulation was 163±21 versus 41±12 pmol/mg protein per 15 minutes for long-term SHR/BAE and WKY/BAE cocultures and 178±27 versus 67±25 pmol/mg protein per 15 minutes for short-term SHR/BAE and WKY/BAE cocultures, respectively.

cGMP Determination in Aortic Ring Homogenates

To confirm the cell culture findings, we determined cGMP accumulation in aortic rings from 12- to 14-week-old animals. Stimulation of the rings with the exogenous nitrovasodilators SNP and SNAP yielded cGMP levels that were greater in homogenates of aortas from SHR than WKY rats (Fig 5).

Northern Hybridization

Northern blot analysis of total RNA (Fig 6) from cultured aortic smooth muscle cells revealed a single 3.4-kb signal for the β subunit of sGC containing double the amounts of the sGC message in cells isolated from 12- to 14-week-old SHR compared with WKY cells, as assessed by densitometry.

Discussion

An increase in peripheral vascular resistance, thought to result from increased vasoconstriction and/or decreased vasodilation, is one of the hallmarks of hypertension. cGMP, formed by the conversion of GTP by sGC, has been recognized to play an important role in vasorelaxation. To investigate whether defective cGMP formation in smooth muscle cells from hypertensive animals may account for the elevated tone seen in vivo, we determined cGMP levels in response to nitrovasodilators in smooth muscle cells from SHR and WKY rats. The major findings of this study were the following: (1) exogenous nitrovasodilators (SNP, GTN, CYSNO) produced a dose-dependent cGMP accumulation in aortic smooth muscle cells from both SHR and WKY rats; (2) cGMP levels after SNP stimulation were higher in 12- to 14-week-old SHR compared with WKY smooth muscle cells in both the absence and presence of phosphodiesterase inhibition; (3) cGMP accumulation in cultured cells in response to SNP, GTN, and CYSNO was greater in 12- to 14-week-old SHR compared with age-matched WKY smooth muscle cells throughout the concentration range used; cGMP accumulation on stimulation with SNP and SNAP was also greater in endothelium-denuded aortic rings of 12- to 14-week-old SHR versus WKY rats; (4) smooth muscle cells from 5- to 6-week-old SHR responded similarly to exogenous nitrovasodilators (SNP, CYSNO) as did cells isolated from age-matched WKY rats; (5) cGMP accumulation was greater in smooth muscle from 12- to 14-week-old SHR in response to basal and agonist-stimulated EDRF.
released from BAE compared with cells from age-matched WKY rats; and (6) steady-state mRNA levels for the 70-kD subunit of sGC were greater in smooth muscle cells from 12- to 14-week-old SHR than WKY rats.

The observed higher cGMP levels in the SHR smooth muscle cells on stimulation with exogenous nitrovasodilators can be the result of increased production of cGMP by sGC and/or decreased degradation of cGMP by phosphodiesterase. However, it is unlikely that a lower phosphodiesterase activity contributes significantly to the elevated cGMP levels seen in SHR because cGMP accumulation in response to SNP was enhanced in cells from SHR in both the presence and absence of the phosphodiesterase inhibitor IBMX. Moreover, baseline (unstimulated) cGMP levels were not different between SHR and WKY cells in the presence of IBMX. These observations are in agreement with previously published results in which a twofold higher KM for cGMP in the SHR compared with the WKY rat was offset by a twofold increase in V_{max} for the hydrolysis of cGMP by the low-KM cGMP phosphodiesterase, leading to an equal rate of hydrolysis for cGMP in the two strains. In addition, it is unlikely that differences in the time course of cGMP accumulation between the two strains could account for the enhanced cGMP accumulation in response to SNP in cells from SHR, because cGMP levels in SHR were consistently higher compared with WKY rats at different time points (1 and 15 minutes) after SNP exposure.

Nitric oxide is released from SNP in a dose-dependent manner by either photolysis or after a one-electron reduction of SNP in the presence of reducing agents.38 The latter mechanism is thought to operate in vivo at low SNP concentrations. More recently in bovine coronary arterial smooth muscle cells, evidence was presented for a membrane-associated nitric oxide–generating activity for SNP that was distinguished from the previously identified nitric oxide–generating activity for GTN.39 To establish the fact that increased cGMP levels in SHR versus WKY cultured smooth muscle cells were not restricted to SNP, cGMP formation was studied using three additional agents—GTN, CYSNO, and SNAP—with different mechanisms of nitric oxide generation. GTN requires metabolic activation in order to yield nitric oxide, but the enzymatic pathway responsible for the metabolism of GTN in smooth muscle remains unidentified. In the liver, GTN denitrification is mainly carried out by glutathione-S-transferase, glutathione reductase, and cytochrome P-450.40,41 On the other hand, most primary nitrosothiols undergo a spontaneous time- and pH-dependent decomposition to nitric oxide in physiological buffers, as reflected by the loss of absorbance at 540 nm.42 Stimulation of cultured smooth muscle cells from both rat strains with all three exogenous nitrovasodilators tested (SNP, CYSNO, and GTN) led to a dose-dependent increase in cGMP levels, although the increases observed in response to GTN were much smaller than those seen with SNP and CYSNO. This is consistent with the notion that GTN is more of a venous dilator, whereas SNP is equally effective in dilating the arterial and venous sides of the circulation. Cultured cells from SHR were hyperresponsive to all three exogenous nitrovasodilators used (SNP, GTN, and CYSNO) compared with smooth muscle cells from WKY rats at a stage of established hypertension (12 to 14 weeks of age). Cell culture observations were reproduced using aortic rings in which cGMP formation was stimulated with SNP or SNAP. Cys-cGMP accumulation in response to nitrovasodilators was greater in aortic ring homogenates of 12- to 14-week-old SHR versus WKY rats. These findings are in agreement with those of Fukuda et al,44 in which extracellular cGMP levels in SNP-perfused mesenteric arteries were higher in 12-week-old SHR compared with WKY rats. On the contrary, cells from prehypertensive (5- to 6-week-old) SHR responded similarly to both SNP and CYSNO compared with WKY cells.

cGMP accumulation in cocultures of BAE with smooth muscle cells from SHR and WKY rats was EDRF dependent because it could be blocked by L-arginine analogues (L-NMMA and L-NAME) and the known inhibitors of the nitric oxide–sGC pathway methylene blue and oxyhemoglobin.45 L-Arginine analogue inhibition of cGMP accumulation could be reversed by the addition of L-arginine but not D-arginine. cGMP accumulation in the long-term cocultures is not likely to be due to a rise in the BAE cGMP levels because these cells lack the soluble isofrom of guanylate cyclase (as suggested by the lack of responsiveness of these cells to SNP); instead, it is likely to be due to cGMP accumulation in the smooth muscle cells caused by BAE-released EDRF that stimulates the sGC of the underlying smooth muscle.

Results similar to those observed with the exogenous nitrovasodilators were obtained when BAE were used as a source of the endogenous nitrovasodilator EDRF. Cys-cGMP accumulation, elicited by basal and agonist-stimulated (bradykinin and A23187) EDRF release, in long-term cocultures of SHR/BAE was greater than in WKY/BAE cocultures. These observations are unlikely to result from modulation of EDRF release from the BAE due to prolonged exposure and cocultivation of BAE with smooth muscle cells from SHR, because similar results were obtained in the short-term cocultures, in which BAE were grown on glass coverslips and allowed to interact with muscle cells for only 15 minutes. Increased cGMP accumulation in response to EDRF in cultured smooth muscle cells can explain the increased levels of cGMP in the homogenates of carotid arteries from SHR versus WKY rats observed by Mourlon-le-Grand et al.46 Nevertheless, other investigators47 have reported that whereas basal and acetylcholine-stimulated cGMP levels in homogenates of aortas from 5-week-old SHR were similar to those from WKY rats, cGMP levels in 15- to 18-week-old SHR were decreased compared with age-matched WKY rats. cGMP accumulation in the aortic rings was attributed mainly to EDRF released from the endothelium, because methylene blue, a selective inhibitor of the EDRF–cGMP pathway but not the atrial natriuretic peptide–cGMP pathway, reduced the cGMP content of the homogenates by approximately 90%.

sGC is a heterodimer composed of a large (α) and small (β) subunit. Recently, cDNAs for both subunits of rabbit and human sGC have been cloned and sequenced.48,49 Northern blot analysis of steady-state mRNA yielded a higher amount for the β message in cells from 12- to 14-week-old SHR versus WKY rats. Increased formation of cGMP in smooth muscle cells from SHR with established hypertension may be due to
an increased sensitivity of sGC to nitric oxide. Two lines of evidence favor the former: (1) EC50 values for nitrosodilators were similar in cells from SHR and WKY rats (10 μmol/L for SNP and GTN and 50 nmol/L for CYSNO), indicating similar sensitivity of the sGC from the two strains to nitric oxide; and (2) steady-state mRNA levels for the β1 sGC subunit were increased in SHR compared with WKY rats. Provided that β1 subunit message levels are indicative of the amount of protein present and that the α subunit is similarly increased (expression of enzymatic activity requires both subunits9), SHR smooth muscle cells possess higher amounts of sGC than WKY cells.

In summary, cGMP formation in response to both exogenous and endogenous nitrosodilators in cultured smooth muscle cells is greater in SHR compared with WKY cells. It is not clear whether the biochemical pathway linking rises in cGMP levels to vasorelaxation operates as effectively in the SHR as in the normotensive animals. An indication of the integrity of this pathway is offered by the observation that membrane-permeable analogues of cGMP are equipotent in producing relaxation of aortas and carotid arteries from SHR and WKY rats.22,23 Increased vascular tone encountered in hypertension may be at least partly explained by a decreased production and/or increased degradation of EDRF. Some investigators suggest50 that overproduction of superoxide anions (O2·−) in hypertensive arteries may underlie the pathogenesis of hypertension.

We conclude that our data do not appear to support the hypothesis that defective cGMP formation underlies in-creased vascular tone in vivo. The higher cGMP levels observed on stimulation of the soluble isoform of guanylate cyclase in SHR compared with WKY cells seem to correlate with the development of hypertension and might be the result of upregulation of guanylate cyclase to compensate for the increased total peripheral resistance and/or endothelial dysfunction and chronic EDRF deficiency encountered with the development of hypertension.

Acknowledgments

This study was supported by grant HL-31422 from the National Institutes of Health, Bethesda, Md. We are pleased to acknowledge the expert technical assistance of Mr Jim Parkinson, Ms Jennifer Barrett, and Ms Livia Marczin. We thank Ms Annie Cruz for the preparation of the manuscript.

References


Smooth muscle cell responsiveness to nitrovasodilators in hypertensive and normotensive rats.

A Papapetropoulos, N Marczin, M D Snead, C Cheng, A Milici and J D Catravas

_Hypertension_. 1994;23:476-484

doi: 10.1161/01.HYP.23.4.476

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://hyper.ahajournals.org/content/23/4/476

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/