Decreased Flow-Induced Dilation and Increased Production of cGMP in Spontaneously Hypertensive Rats

Hong Ying Qiu, Daniel Henrion, Joëlle Benessiano, Christophe Heymes, Bruno Tournier, Bernard I. Levy

Abstract—We investigated flow (shear stress)– and agonist-induced cGMP release in mesenteric vascular beds of spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY). The mesenteric vascular bed was perfused in situ with Tyrode’s solution. Vascular relaxation and cGMP release in the perfusate were determined on stimulation by flow or by acetylcholine (0.1 μmol/L) or sodium nitroprusside (0.1 mmol/L). Flow-induced release of cGMP was significantly greater in SHR than in WKY (P<0.01), despite a lower flow-induced dilation in SHR. In both strains, Nω-nitro-L-arginine methyl ester (L-NAME) completely inhibited cGMP release in response to flow (P<0.001), although flow-induced dilation was not affected by L-NAME in SHR. Moreover, the activity of the constitutive nitric oxide synthase (NOS) was significantly greater in SHR (82±3.5 fmol/min) than in WKY (66±3.5 fmol/min; P<0.05) and was associated with increased expression of endothelial NOS mRNA in SHR. Sodium nitroprusside induced larger increases in cGMP release in SHR (3593±304 fmol/min) than in WKY (2467±302 fmol/min; P<0.05). The release of cGMP in response to acetylcholine was significantly lower in SHR (292±80 fmol/min) than in WKY (798±218 fmol/min; P<0.05) in parallel with smaller acetylcholine-induced relaxation in SHR. Despite increased cGMP production in response to flow and NOS activity, flow-induced dilation was decreased in SHR, suggesting an upregulation of the NO/cGMP pathway to compensate for the increased vascular tone in SHR. (Hypertension. 1998;32:1098-1103.)

Key Words: endothelium ■ cyclic GMP ■ nitric oxide synthase ■ mesenteric arteries

Nitrovasodilators produce vascular smooth muscle relaxation through activation of the soluble isofom of guanylate cyclase and increase cellular cGMP,1,2 which lowers intracellular Ca2+ levels.3,4 Nitric oxide (NO), a major endogenous vasodilator synthesized from l-arginine, is released by endothelial cells.5-7

In several models of hypertension, endothelium-dependent responses are altered8; the increase in peripheral resistance would be caused at least in part by a decrease in endothelium-dependent dilation and/or to increased release of endothelium-derived constricting factors.9 Endothelial dysfunction in large conduit arteries8,10 and in resistance arteries of hypertensive rats11-13 has also been shown. Similar alterations occur in hypertensive patients.14-16 Functional alterations of the endothelial l-arginine/NOS pathway may be important in cardiovascular disease, since depressed activity of this dilator mechanism would reduce local blood flow.

In most experimental and clinical studies, acetylcholine (ACh) was used to test the endothelial function. However, the main in vivo physiological stimulus of endothelial responses is the wall shear stress directly related to the blood flow rate, blood viscosity, and vessel diameter.17 Interestingly, in the coronary18 and forearm19 circulations of hypertensive subjects, flow-dependent vasodilatation was reported to remain intact, although the response to ACh was abnormal. However, we have previously reported that the arterial wall cGMP content was greater in spontaneously hypertensive rats (SHR) than in normotensive Wistar Kyoto rats (WKY).20 To test the hypothesis that response to flow may be primarily decreased in hypertension, leading to compensatory mechanisms such as an increased NO/cGMP pathway responsiveness, we measured cGMP release and vessel dilation on flow or agonist stimulation in mesenteric arteries from SHR and WKY.

Methods

In Situ Mesenteric Bed Perfusion and Effluent Collection
All experiments were performed on 12-week-old SHR andagematched WKY obtained from Center d’Elevage R. Janvier (St Berthevin, France). A medial laparotomy was performed after sodium pentobarbital (50 mg/kg IP) anesthesia was administered. The last loop of the small intestine was exposed and placed in a container, which allowed superfusion of the preparation as described previously.17 All second-generation branches of the mesenteric artery
were sutured except one irrigating a 3- to 4-cm segment of small intestine that was cut from the remaining small intestine laid on a glass container. This second-generation mesenteric artery branch was then perfused, and the effluent was collected. Samples of effluent were immediately frozen in liquid nitrogen and stored at −80°C until cGMP measurement.

In a first group of experiments, the isolated mesenteric arterial beds were perfused at flow rates ranging from 0.5 to 4 mL/min with Tyrode’s solution containing 0.2 mmol/L IBMX. Each flow step was maintained for 6 minutes, and 2 mL of effluent was collected. The same procedure was performed 20 minutes after addition of N^6-nitro-L-arginine methyl ester (L-NAME; 1 mM/mL), an inhibitor of NO synthase (NOS), to the perfusate. Flow rates from 0.5 to 4 mL/min are compatible with the in vivo flow shown previously.31 There is no difference in blood flow between WKY and SHR.21

In a second series of experiments, we measured mesenteric arterial diameter changes and cGMP release in response to ACh or sodium nitroprusside (SNP) stimulation. After preconstriction of the mesenteric arteries with phenylephrine (PE, 10 μmol/L), ACh (n=8 in each strain, 0.1 μmol/min) or SNP (n=10 in each strain, 0.1 μmol/L) was added to the perfusate for 10 minutes. The mesenteric arterial diameter was continuously measured. Samples of effluent were collected under control conditions or after a 10-minute perfusion with ACh (n=8 per strain, 0.1 μmol/min) or SNP (n=10 per strain, 0.1 μmol/L).

In a third group of experiments, the mesenteric vascular bed was perfused with Tyrode’s solution at a constant flow (500 μL/min) and then dissected and removed from the gut for measurement of constitutive NOS (cNOS) activity (21 SHR and 21 WKY) and for determination of NOS mRNA expression (5 SHR and 5 WKY).

**Mesenteric Artery Perfused and Pressurized In Vitro**

A segment of mesenteric artery, ~400 μm ID, was isolated, cannulated at both ends, and mounted in a video-monitored perfusion system as previously described.32,33 Briefly, the artery was bathed in a 5-mL organ bath containing Tyrode’s solution. Pressure in the proximal end of the artery segment was monitored by use of a pressure transducer and controlled by a servoperfusion system. Arterial diameter was recorded by use of a video-monitoring system (Living System Instrumentation Inc). Diameter changes were measured under no flow, when intraluminal pressure was set at 25, 50, 75, 100, 125, and 150 mm Hg. Flow rate in the artery ranged from 0 to 150 μL/min (under a pressure of 75 mm Hg). Step increases in pressure and flow were subsequently repeated after addition of either L-NAME (10 μmol/L) or indomethacin (3 μmol/L) to the perfusate, and superfusate. Arteries were then perfused and superfused with Ca^2+-free PSS containing EGTA (2 mM/mL) and SNP (10 μmol/L) to determine the passive diameter of the vessel after full relaxation. Results are given in micrometers for diameters of arteries. Myogenic tone was expressed as active tone (passive diameter − diameter). Flow-induced relaxation was expressed as increases in diameter induced by flow.

**Determination of cGMP Production in Mesenteric Vascular Bed**

Samples of effluent were treated with 6% trichloroacetic acid and centrifuged at 20,000g for 15 minutes at 4°C. Supernatant fractions were extracted 4 times with 5 volumes of water-saturated diethyl ether; the organic phase was discarded each time. Residual aqueous phase was lyophilized and assayed for cGMP after acetylation by use of an immunoenzymatic assay as previously described.24 Levels of cGMP in the effluents were expressed as fentomoles per milliliter, and production of cGMP by the mesenteric bed (fmol/min) was calculated as the product of cGMP concentration and the mesenteric flow value.

**Determination of cNOS Activity in the Mesenteric Arterial Bed**

cNOS activity was determined by measuring the conversion of [3H]-arginine to [3H]-citrulline.25 Tissues were homogenized at 4°C in 500 μL of homogenization buffer.26 The assay was terminated by the addition of 1 mL of 30% HEPS and 3 mM/L EDTA, pH 5.5 ice-cold buffer. The terminated reaction was applied to a Dowex AG50W-X8 (Na^+ form) column, and [3H]-citrulline was eluted with 2 mL of distilled water. Radioactivity was measured in a liquid scintillation counter. Ca^2+-dependent NOS activity was evaluated by the difference in activity in between samples assayed in the presence of CaCl2 and those assayed in the presence of EDTA.

**Reverse Transcriptase–Polymerase Chain Reaction of NOS Isoforms**

Total RNA was extracted according to Trizol reagent protocol (Life Technologies Inc). Purified RNA was dissolved in water, and the concentration was measured by absorbance at 260 nm. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed for NOS type I, type II, and type III (NOS I, neuronal NOS; NOS II, inducible NOS; and NOS III, endothelial NOS, respectively) and for GAPDH gene expression. The primers chosen for sense were 5'-CTGGCTCAACAGAATAACAGG-CT-3' for NOS I, 5'-AAAGCACCAGTGCTCTTTT-3' for NOS II, and 5'-TTCCCCGCTGCACTGATCCCTAA-3' for NOS III. For antisense, the primers chosen were 5'-ACATGTGACGCTTCCTCGGAG-3' for NOS I, 5'-CGCAAACATAGGGTGCCC-3' for NOS II, and 5'-AACATGTGCTCTGTGGAGGCA-3' for NOS III, thus amplifying a 293-bp, a 388-bp, and a 340-bp fragment, respectively. For GAPDH, the primers chosen were: sense 5'-TGAAGGCGGTGTCACGGAGTTTTG-3' and antisense 5'-CATGAGCCCATGAGGTCCACC-3', resulting in a 982-bp band.

First-strand cDNA was performed on 50 ng of total RNA. The single-strand cDNA synthesis was carried out in 20 μL of reaction buffer (20 mM/L Tris-HCl (pH 8.3), 50 mM/L KCl, 1.6 mM/L MgCl2, 1 mM/L dNTP, 10 mM/L DTT, 0.2 mM/L oligo-dT(21)). The reaction mixture was incubated 10 minutes at 25°C and then 60 minutes at 7°C. The resultant cDNA was amplified using 2.5 μL of Taq DNA polymerase (Boehringer, Meylan, France) and 0.5 μmol/L of the sense and antisense primers in 50 μL of 50 mM/L KCI, 10 mM/L Tris-HCl (pH 8.3), 3 mM/L MgCl2, 1 mM/L dNTP, and 0.01% gelatin. Thirty-two, 26, 25, and 25 amplification cycles for NOS I, NOS II, NOS III, and GAPDH, respectively, were carried out as follows: denaturation at 94°C for 1 minute, annealing at 62°C for NOS isoforms and at 60°C for GAPDH for 1 minute, and extension at 72°C for 1 minute. The final extension was carried out for 10 minutes.

The linear phase of the amplification was determined for each primer set to allow semiquantitative PCR analysis. To quantify NOS isoforms and GAPDH mRNA levels, a trace amount of [32P]dCTP was included in the PCR reaction. The PCR products were then electrophoresed on a 5% polyacrylamide gel, and radioactive signals were visualized and quantified in a computer-based imaging system (Fuji Bas 1000, Fuji Medical Systems). To normalize signals for NOS I, NOS II, and NOS III, the value was divided by the signal for GAPDH, a widely invariant and highly expressed gene.

**Drugs**

[3H]-arginine (44.2 Ci/mmol) was purchased from Amersham, and Dowex AG50-X8 cation exchange resin and protein assay reagent were obtained from Bio-Rad Laboratories. All other reagents were obtained from Sigma Chemical Co.

**Statistical Analysis**

Results are expressed as mean±SEM. The experimental design allowed us to perform 2-way ANOVA with factorial and repeated measurement to provide evidence of differences related to strains and experimental conditions (flow, agonist application). Differences were considered significant when P<0.05.

**Results**

Mean arterial pressure was 104±11 in WKY and 154±15 in SHR. The cross-sectional area in mesenteric arteries from...
SHR is significantly greater (19 033 ± 342 μm²) than that from WKY (14 563 ± 208 μm²; P < 0.001).

Flow-Induced cGMP Release

Figure 1 shows the relation between flow rate and cGMP release in mesenteric vascular beds from SHR (n = 7) and WKY (n = 11). Under control conditions, a significant linear flow-dependent release of cGMP was observed in SHR (from 232 ± 42 fmol/min at 0.5 mL/min to 969 ± 271 fmol/min at 4 mL/min; P < 0.001) and in WKY (from 123 ± 17 fmol/min at 0.5 mL/min to 627 ± 95 fmol/min at 4 mL/min; P < 0.001). Flow-dependent release of cGMP was significantly greater in SHR than in WKY (P < 0.01). L-NAME significantly inhibited the release of cGMP at all flow rates in both strains.

Flow-Induced Dilation and Myogenic Tone

In isolated mesenteric arteries, step increases in intraluminal pressure induced development of myogenic tone that was greater in SHR than in WKY (Figure 2). Passive arterial diameter in the absence of tone ranged from 257 ± 6 to 395 ± 16 μm in WKY and from 252 ± 2 to 368 ± 2 μm in SHR (P < 0.01 versus WKY, n = 6 per group) for pressure steps from 25 to 150 mm Hg. Step increases in flow, under a pressure of 100 mm Hg, induced significant dilation in WKY (Figure 3). In SHR, flow-induced dilation was significantly attenuated compared with WKY (P < 0.001). L-NAME (100 μmol/L) significantly attenuated flow-induced dilation in WKY but not in SHR (Figure 3).

Agonist- or Exogenous Nitrovasodilator–Induced Relaxation and cGMP Release From Perfused Mesenteric Vascular Bed

Under control conditions (2 mL/min flow rate), cGMP release by the mesenteric vascular bed was significantly greater in SHR than in WKY (792 ± 76 versus 394 ± 44 fmol/min; P < 0.001) (Figure 4). Under stimulation by ACh, cGMP release reached similar levels in SHR (916 ± 126 fmol/min) and in WKY (1062 ± 273 fmol/min). However, the increase in cGMP release induced by ACh was significantly less (P < 0.05) in SHR (292 ± 80 fmol/min) than in WKY (798 ± 218 fmol/min). In parallel, relaxation to ACh was significantly less in SHR than in WKY (43 ± 9% versus 87 ± 3%; P < 0.01). After SNP stimulation, cGMP release was significantly greater in SHR than in WKY (3593 ± 304 versus 2000 ± 200 fmol/min; P < 0.05).
2467±302 fmol/min; P<0.02), whereas the relaxation obtained was similar in both strains (126±19% in SHR versus 114±3% in WKY).

cNOS Activity in Mesenteric Arterial Bed
cNOS activity was significantly greater in SHR (85±3.5 fmol/min) than in WKY (66±3.5 fmol/min; P<0.05), and inducible NOS activity of the mesenteric arterial bed (citrul-line produced in absence of calcium) was not detectable in either strain.

NOS mRNA in Mesenteric Arterial Bed
RT-PCR of NOS isoforms revealed the presence of NOS I, NOS II, and NOS III in mesenteric arteries from SHR and WKY (Figure 5). Expression of all 3 isoforms of NOS was significantly greater (P<0.01) in SHR than in WKY.

Discussion
The main finding of the present study is that flow-induced dilation was impaired in SHR mesenteric resistance arteries despite greater NO-dependent cGMP production and greater NOS activity in SHR than in WKY.

An increase in peripheral vascular resistance, thought to result from increased vasoconstrictor and/or decreased vasodilator tone, is one of the hallmarks of hypertension. Functional alterations of the endothelial l-arginine/NO pathway could contribute to regulation of peripheral resistance and, in turn, of blood pressure. Earlier experimental and clinical studies have reported endothelium dysfunctions in large arteries and in resistance arteries of hypertensive animals and patients.

In the present study, cGMP released by the mesenteric circulation was proportional to flow rate and was completely blocked by L-NAME in both WKY and SHR. These findings support the concept that flow induces a shear stress–dependent stimulation of the vascular l-arginine/NO/cGMP pathway in both strains. Flow-induced cNOS activity and cGMP release were significantly greater in SHR than in WKY despite markedly decreased flow-induced dilation in SHR. Increased cNOS activity in SHR was confirmed by increased mRNA expression of endothelial NOS. Beside endothelial NOS mRNA, neuronal NOS mRNA and inducible NOS mRNA were also increased in SHR. Together, these results are consistent with a marked increase in cGMP production in SHR after stimulation of the l-arginine/NO pathway by flow. A possible explanation could be that shear stress is greater in resistance arteries of SHR than in those of WKY because, in the present study, flow rate was set at the same value in both strains. Arterial rarefaction and narrowing have been reported in genetic hypertension and could be responsible for greater shear stress in the resistance arteries of SHR. Another possible explanation for increased cGMP production in SHR...
could be related to increased cNOS activity. The greater flow-induced cGMP release in SHR could be the consequence of an upregulation of the NO/cGMP pathway to compensate for an increase in vascular tone and arterial resistance observed with the development of hypertension, as shown in the present work and in previous studies. Another possible explanation for the increase in flow-induced cGMP production and the decrease in flow-induced dilation could be that cGMP is less efficient in SHR in inducing a smooth muscle relaxation; thus, a full relaxation may be obtained but more cGMP is required. In fact, flow may trigger not only a NO/cGMP-dependent dilation but also a release of contractile substances such as vasoconstrictor cyclooxygenase products and/or endothelin-[32] and/or endothelin-[33]. Moreover, because of the presence of a phosphodiesterase inhibitor (IBMX) in the perfusate, we can exclude that increased vascular tone and upregulation of the NO/cGMP pathway could be unrelated phenotypes of the SHR strain.

In conclusion, in genetically hypertensive rats, the mesenteric arterial network exhibited an overactivated endothelium-dependent t-arginine/NO/cGMP pathway associated with decreased flow-induced dilation. Although we may hypothesize that an increase in NO/cGMP activity occurred to compensate for an elevated constrictor tone, we cannot exclude that increased vascular tone and upregulation of the NO/cGMP pathway could be unrelated phenotypes of the SHR strain.

References
Decreased Flow-Induced Dilation and Increased Production of cGMP in Spontaneously Hypertensive Rats
Hong Ying Qiu, Daniel Henrion, Joëlle Benessiano, Christophe Heymes, Bruno Tournier and Bernard I. Levy

Hypertension. 1998;32:1098-1103
doi: 10.1161/01.HYP.32.6.1098

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 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/32/6/1098

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/