Aging and Chronic Hypertension Decrease Expression of Rat Aortic Soluble Guanylyl Cyclase

Stephan Klöß, Anne Bouloumié, Alexander Mülsch

Abstract—We analyzed the influence of aging and genetic hypertension on the function and expression of soluble guanylyl cyclase (sGC) in the aortas of prehypertensive and old spontaneously hypertensive rats (SHR) as well as in age-matched normotensive Wistar-Kyoto rats (WKY). The expression of heterodimeric sGC (α1 and β1) was assessed at the mRNA and protein level, and its function was assessed by the relaxant responses of phenylephrine- contracted endothelium-denuded aortic rings to the nitric oxide (NO) donor sodium nitroprusside. The vasodilator potency of sodium nitroprusside was significantly reduced (P<0.05) with age (3- to 6-fold increase in the EC50 in old WKY and SHR compared with their young counterparts) as well as with hypertension (3-fold increase in old SHR compared with age-matched WKY), whereas the vasodilator potency of sodium nitroprusside did not differ between young SHR and WKY. A similar influence of aging and hypertension on NO-stimulated GC activity was revealed at the GC expression level: Whereas the β1 protein content was similar in young rats of both strains, old WKY exhibited 60% lower and old SHR exhibited 80% lower β1 subunit protein compared with young rats (P<0.05). Moreover, the abundance of α1 and β1 mRNA (assessed by reverse transcriptase—polymerase chain reaction) was similar in young rats but was 2.5-fold (α1) and 4.3-fold (β1) lower in old SHR compared with old WKY. In conclusion, our findings show that both aging and hypertension decrease sGC expression and its NO-dependent activation in aortic tissue. Downregulation of sGC may therefore contribute to arterial dysfunction in senescence and chronic hypertension. (Hypertension. 2000;35:43-47.)

Key Words: aging ■ hypertension, genetic ■ guanylyl cyclase ■ aorta ■ nitric oxide

The hemoprotein soluble guanylyl cyclase (sGC) is the predominant intracellular nitric oxide (NO) receptor in vascular smooth muscle cells,1 which mediates NO signaling via formation of cGMP. This enzyme is a heterodimer and consists in most mammalian tissues of α1 (76- to 82-kDa) and β1 (70-kDa) protein subunits.2 Aging and chronic hypertension are associated with functional and morphological changes of the vessel wall, ie, the vascular endothelium and the smooth muscle. Endothelial dysfunction is characterized by a decreased responsiveness to endothelium-dependent vasodilators.3 Several studies have addressed the underlying mechanism(s) in different vascular beds at the level of endothelial NO formation and reported conflicting results with regard to activity and expression of endothelial NO synthase and NO bioavailability.4–6 However, endothelial dysfunction may also result from impaired signaling downstream from NO in the vascular smooth muscle. Thus, other studies emphasized a negative influence of aging7–9 and hypertension10,11 on the NO responsiveness of vascular smooth muscle cells. We found recently that compared with aortic rings of age-matched normotensive Wistar-Kyoto rats (WKY), aortas of 16-month-old genetically spontaneously hypertensive rats (SHR) exhibited a reduced vasodilator responsiveness to acetylcholine and sodium nitroprusside (SNP), although the expression of endothelial NO synthase protein and mRNA was not different between both strains.12 This finding suggested an impairment of NO-dependent vasodilator function either at the level of or downstream from sGC. Indeed, we observed a lower content of immunoreactive sGC β1 protein in aortic tissue of senescent SHR compared with age-matched WKY.12 The objective of the present study was to assess the influence of age on the expression and NO-dependent function of sGC in the aorta of normotensive and genetically hypertensive rats.

Methods

Materials
Bradford reagent was purchased from Bio-Rad; guanidine thiocyanate, from Sigma; reverse transcriptase and agarose, from GIBCO-BRL; and Tween 20, from Serva. Oligodeoxythymidine and Taq polymerase were from Pharmacia Biotech. All other chemicals were bought from Roth. The polyclonal peptide antibody directed against the β1 subunit of the rat lung sGC was kindly provided by Dr Peter Yuen, Memphis, Tenn.13

Animals
Investigations were performed with isolated aortic rings from 2-month-old prehypertensive and 16-month-old hypertensive male SHR and normotensive age-matched male WKY (n=7 in each age group).
and strain group, 28 rats in total). SHR and WKY were purchased from Møllergaard (S lessenved, Denmark) at the age of 1 month, when they exhibited equal body weights (75±5 g) and systolic blood pressures (SBPs). SBP was measured in conscious rats by tail plethysmography under light anesthesia. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1985).

**Vasodilator Responsiveness of Preconstricted Aortic Rings**

The thoracic aorta was isolated from anesthetized (60 mg/kg pentobarbital IP) rats, cleaned of connective tissue, cut into rings of equal length (5 mm), linear increase in optical density (correlation coefficients 1.0). One onefifth of the RNA was immediately frozen in liquid nitrogen for measurements of the mRNA and protein levels of sGC. Other rings (2) were mounted in an organ bath (Schuler-Organbad, Hugo Sachs Electronic) and suspended in Krebs-Henseleit solution (pH 7.4, 37°C) for measurements of isometric contractile force. The rings were equilibrated for 30 minutes under a resting tension of 2.5 g (achieved by 0.5-g steps) in carbon-gassed (95% O2/5% CO2) Krebs-Henseleit buffer12 in the presence of diclofenac (1 μmol/L). Rings were preconstricted 2 times with 60 nmol/L phenylephrine (PE) and thereafter with 1 μmol/L phenylephrine (PE). After development of a stable contraction to PE, the relaxant response to increasing cumulative concentrations of SNP (0.3 nmol/L to 1 μmol/L) was determined.

**Isolation of Total RNA From Rat Aorta and RT-PCR of α1 and β1 sGC mRNA**

The total RNA was extracted from aortic tissue ground in liquid nitrogen by the modified guanidine isothiocyanate method of Chomczynski and Sacchi. Total RNA (2 μg) was incubated with 200 U reverse transcriptase (RT), deoxy nucleotides (dNTPs, 125 μmol/L), 200 ng oligodeoxythymidine (dT), and reaction buffer in a final volume of 20 μL at 37°C for 1 h. Published sequences13 were used to synthesize primers for the sGC α1 subunit (forward, base position 1527 5′-GAAATCTTCAAGGTTATG-3′; reverse, base position 1525 5′-GACTGTCCTGGCAAGCGA-3′), β1 subunit (forward, base position 1491 5′-GGGTGCCAGAACCTTGATAACAC-3′; reverse, base position 1750 5′-GACGTCCCGCTGGTATAG-3′), and elongation factor II (225 bp), sGC α1 subunit (826 bp), and elongation factor II subunit (666 bp) at 94°C for 1 minute (denaturation), at 54°C to 58°C for 1.5 minutes (annealing), and at 72°C for 1.5 minutes (elongation). The final step was completed with 7 minutes of elongation at 72°C. The cDNA was amplified with 10 pmol of each primer, 2.5 U Taq polymerase, dNTPs (200 μmol/L), and MgCl2 containing reaction buffer (50 μL final volume). Ten microliters of this mixture was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator. Fluorescent bands were records by means of a fluorescent light–sensitive video camera, and negatives were evaluated by scanning densitometry (SCION IMAGE BETA II). Polymerase chain reaction (PCR) product sizes of the elongation factor II (225 bp), sGC α1 subunit (826 bp), and β1 subunit (284 bp) were controlled by use of DNA standards. With 0.5 μg to 2.5 μg total RNA, a linear increase in optical density (correlation coefficient, 0.998 for α1, 0.995 for β1, and 0.996 for elongation factor II) was observed between 20 to 35 cycles (β1, elongation factor II) and 30 to 40 cycles (α1).

**Immunodetection of sGC β1 Protein in Rat Aorta**

Total protein was precipitated by alcohol from the phenol phase of the RNA extraction. Proteins (50 μg per lane) were separated on Laemmli gels and electroblotted. The blots were blocked at 4°C overnight and then incubated for 2 hours at room temperature with a polyclonal peptide antibody directed against the β1 subunit of rat sGC (1:1000 dilution in blocking buffer). The blots were washed and incubated with a peroxidase-linked anti-rabbit IgG, and immunoreactive protein bands were visualized by chemiluminescence.

**Influence of Aging and Chronic Hypertension on Vasodilator Responses to SNP**

The NO donor SNP applied in cumulative concentrations (0.3 nmol/L to 1 μmol/L) elicited a concentration-dependent relaxation of endothelium-denuded PE (1 μmol/L)–contracted aortic rings of all animal groups studied. Complete offset of active tension (100% relaxation) was obtained in each group at the highest concentration of SNP applied (1 μmol/L) (Figure 1). However, the vasodilator potency of SNP was significantly decreased in aortas of old WKY (EC50 3±0.3 nmol/L) compared with their young counterparts (EC50 1.0±0.2 nmol/L, n =6). The vasodilator potency of SNP was similar in prehypertensive young SHR (EC50 1.5±0.2 nmol/L) and normotensive young WKY. These findings indicate a significant influence of age on NO-induced vasorelaxation.

In addition, chronic hypertension was associated with a further loss of SNP responsiveness independent of age. Thus, the EC50 for SNP-induced aortic relaxation was 3-fold higher (9±1 nmol/L, n=6) in old SHR than in old WKY (Figure 1). These results suggest that genetic hypertension and age are associated with a defect in NO-dependent smooth muscle relaxation at the level of or downstream from sGC.

**TABLE 1. SBP and BW in SHR and WKY**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young Rats</th>
<th>SHR</th>
<th>Old Rats</th>
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<tr>
<td>SBP, mm Hg</td>
<td>102.7±2.9</td>
<td>109.7±2.7*</td>
<td>115±8</td>
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<tr>
<td>BW, g</td>
<td>198.5±5</td>
<td>157.6±8.3</td>
<td>650±15</td>
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</table>

Values are mean±SEM (n=6 per group). BW indicates body weight. Young rats were aged 2 months and old rats were aged 16 months.

*P<0.05 vs WKY.

exposure to x-ray film. The autoradiographs were analyzed by scanning densitometry. To check for equal protein loading and transfer, Ponceau staining and α-actin immunostaining were performed with stripped blots.

**Statistics**

Data are expressed as mean±SEM of n rats. Direct comparisons between 2 animal groups (RT-PCR and Western blot) were performed by the Student t test. A value of P<0.05 was considered significant. Relaxant responses were expressed as percent relaxation relative to the level of precontraction. EC50 values were estimated by fitting individual concentration-relaxation curves to a 3-parameter logistic equation by use of nonlinear regression analysis (Slide Write Plus software). Differences between EC50 values were tested by 1-way ANOVA, followed by the Tukey correction for multiple comparisons (Instat software).

**Results**

**SBP and Body Weight of SHR and WKY**

The 16-month-old SHR presented a significantly higher mean SBP than did age-matched normotensive WKY (Table 1). The 2-month-old prehypertensive SHR also exhibited a higher SBP than did young WKY; however, the difference between the young rats was less marked than between the old rats. Furthermore, the body weight was significantly higher in WKY than in age-matched SHR (Table 1).
Influence of Aging and Hypertension on Expression of sGC \( \alpha_1 \) and \( \beta_1 \) mRNA

To clarify whether the reduction in nitrovasodilator potency by aging and hypertension was due to a reduced expression of sGC, total RNA was extracted from rat aortas, and sGC mRNA was amplified by RT-PCR using specific oligonucleotide primers for \( \alpha_1 \) and \( \beta_1 \) subunits. According to densitometric analysis of the RT-PCR products of \( \alpha_1 \) (826-bp) and \( \beta_1 \) (284-bp) subunits, the abundance of both transcripts was similar between young SHR and age-matched WKY (Figure 2A and 2B, and Table 2) but was significantly lower in old SHR (\( \alpha_1 \) 67% and \( \beta_1 \) 57%) and old WKY (\( \alpha_1 \) 80% and \( \beta_1 \) 70%) than in young rats (Figure 2C and 2D, Figure 3A and 3B, and Table 2). In contrast, the mRNA levels of elongation factor II were not significantly different in both young and old SHR and WKY (Figures 2 and 3). This finding shows that age is associated with a reduced expression of sGC subunit mRNAs in rat aorta and thus provides an explanation for the age-dependent loss in nitrovasodilator responsiveness of these blood vessels (Figure 1). Furthermore, the expression of both sGC transcripts was influenced by chronic hypertension independent of age. It was 2.5-fold (\( \alpha_1 \) mRNA) and 4-fold (\( \beta_1 \) mRNA) lower in old SHR than in old WKY (Figure 3C and 3D). As summarized in Table 2, these results provide a rationale for the loss in NO-dependent sGC function in the rat aorta by age and chronic hypertension.

Influence of Aging and Chronic Hypertension on Expression of sGC Protein

Finally, to investigate whether the reduced mRNA levels are translated into reduced expression of sGC protein, the concentration of the sGC \( \beta_1 \) subunit was determined by Western blot analysis. Immunoblotting of total protein extracts from rat aortic tissue with a polyclonal antibody raised against the \( \beta_1 \) subunit of sGC revealed 2 positive bands, one that comigrated with the single 70-kDa band of the \( \beta_1 \) subunit of purified sGC from bovine lung used as a standard and another (unspecific) that migrated at 45 kDa (not shown). The densitometric analysis of the 70-kDa band revealed no difference in the \( \beta_1 \) protein content between young SHR and WKY (Figure 4A). However, the expression of the \( \beta_1 \) subunit was markedly reduced in old SHR and WKY compared with young rats of either strain (Figure 4B and 4C), thus confirming that the age-induced loss of nitrovasodilator responsiveness is due to decreased sGC protein expression. There was also a significant decrease in the \( \beta_1 \) protein content in old SHR compared with old WKY (Figure 4D).

Discussion

To identify the role of the NO receptor sGC in vascular dysfunction associated with aging and chronic hypertension,

<table>
<thead>
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<th>Comparison</th>
<th>Ratio ( \alpha_1 ) Subunit</th>
<th>Ratio ( \beta_1 ) Subunit</th>
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<tr>
<td>WKY (young) vs WKY (old)</td>
<td>4.4:1*</td>
<td>3.4:1*</td>
</tr>
<tr>
<td>SHR (young) vs SHR (old)</td>
<td>3.1:1*</td>
<td>2.3:1*</td>
</tr>
<tr>
<td>WKY (young) vs SHR (young)</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>WKY (old) vs SHR (old)</td>
<td>2.5:1*</td>
<td>4.3:1*</td>
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</table>

Values are ratios of sGC subunit mRNA abundance (normalized for EF II mRNA abundance) in both animal groups compared. Combined results are from semiquantitative RT-PCR (Figures 2 and 3).

*Significant deviation of the ratio from unity (\( P<0.05 \)).
we assessed NO donor–dependent (SNP-dependent) relaxations and expression of sGC mRNA and protein in aortic tissue of senescent and young SHR and their normotensive counterparts, age-matched WKY.

We observed a downregulation of sGC expression at the mRNA (\(a_1\) and \(b_1\) transcripts) and protein (\(b_1\) subunit) levels induced by aging (old versus young WKY). Consistently, the decreased aortic sGC expression in senescent WKY and SHR translated functionally into a blunted vasodilator response of endothelium-denuded PE-contracted aortic rings to SNP (Figure 1). Strain differences did not account for the effect of aging, in view of the fact that prehypertensive young SHR and age-matched WKY exhibited similar sGC expression and NO vasodilator responsiveness. This is in accordance with previous reports of a normal nitrovasodilator response and NO formation in conduit and resistance vessels of SHR before the onset of hypertension.6,7

Our finding of a reduced aortic sGC expression in senescent WKY and SHR translated functionally into a blunted vasodilator response of endothelium-denuded PE-contracted aortic rings to SNP (Figure 1). Strain differences did not account for the effect of aging, in view of the fact that prehypertensive young SHR and age-matched WKY exhibited similar sGC expression and NO vasodilator responsiveness. This is in accordance with previous reports of a normal nitrovasodilator response and NO formation in conduit and resistance vessels of SHR before the onset of hypertension.6,7

Our finding of a reduced aortic sGC expression in senescent WKY and SHR provides an explanation for the decreased SNP-induced relaxation of aortas from aged Wistar rats observed previously.7,8 Thus, it appears that aging worsens the NO-dependent vasodilator mechanism of the rat aorta not only by eliciting endothelial dysfunction (ie, decreasing agonist-induced endothelial NO release and bioavailability)5 but also by decreasing the expression of sGC in aortic smooth muscle cells. Interestingly, aging also decreases nitrovasodilator responsiveness of nonvascular smooth muscle in guinea pigs,16 suggesting that downregulation of vascular and nonvascular smooth muscle sGC may be a common response to aging throughout the animal species.

Furthermore, we demonstrated that in addition to aging, chronic hypertension decreases sGC expression in the rat aorta at the mRNA level, thus corroborating our previous observation of a lower sGC protein level in aortic tissue of aged SHR compared with aged WKY.12 This finding is in line with the loss of nitrovasodilator responsiveness in hypertensive SHR observed by other investigators9,11 and the reduced sGC activity in lung homogenates of old SHR.17 However, it is still unclear whether hypertensive patients suffer from reduced vascular sGC expression in addition to endothelial dysfunction. Either reduced10 or unaltered18 forearm blood flow responses to nitrovasodilators have been observed. Interestingly, sGC subunit gene loci cosegregate with blood pressure–controlling genes in Dahl rats with salt-sensitive hypertension.19

In apparent conflict with the present findings, in one recent study the level of sGC \(b_1\) mRNA (detected by Northern blot) in cultured aortic smooth muscle cells from hypertensive (14-week-old) SHR was found to be 2-fold higher than in cultured
cells from age-matched WKY. In that study the cGMP response to NO donors was higher in cultured cells and aortic rings from SHR compared with WKY, whereas there was no difference in NO responsiveness between cultured cells from prehypertensive 5- to 6-week-old SHR and normotensive WKY. One explanation for the discrepancy with our findings is that smooth muscle cells change their phenotype in culture and therefore do not express the same protein pattern as in the vessel wall in situ. However, we cannot exclude the possibility that sGC expression differs between adult (14-week-old) and more aged (16-month-old) SHR.

The mechanisms underlying the reduced expression of sGC in chronic hypertension and aging are still unknown. Several conditions that lead to decreased sGC protein expression have been identified in vitro: In cultured cells, cAMP-eliciting agonists and exposure to high NO levels, achieved either by nitrovasodilators or by cytokine-elicited NO synthase II, reduce the stability of the sGC αi and βi mRNA. Furthermore, nerve growth factor reduces the abundance of βi mRNA in PC-12 cells via a p21ras-dependent pathway. Adaptation to hypertension promotes morphological changes in the aorta characterized by wall thickening due to media hypertrophy, and enhanced levels of various growth factors seem to account for this adaptive morphological response. For instance, SHR exhibit increased plasma levels of endothelin-1, angiotensin II, thrombin, and platelet-derived growth factor, all of which stimulate proliferation of rat aortic vascular smooth muscle cells via activation of p21ras. It is tempting to speculate that growth signals in general decrease the expression of sGC in smooth muscle cells.

We have shown that aging and chronic hypertension decrease the expression of sGC at the mRNA and protein level, thus attenuating NO-dependent vasodilator function in aortas of senescent WKY and SHR. The reduced NO-dependent vasodilator capacity at the level of the vascular smooth muscle will contribute to vascular dysfunction in aging and hypertension, in addition to endothelial dysfunction.

Acknowledgments

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References