Loss of Expression of the β Subunit of Soluble Guanylyl Cyclase Prevents Nitric Oxide–Mediated Inhibition of DNA Synthesis in Smooth Muscle Cells of Old Rats

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Abstract—We compared the effects of NO donors and cGMP analogues on the growth of aortic smooth muscle cells (SMCs) derived from newborn, adult (aged 3 months), and old (aged 2 years) rats. We found that the NO donor S-nitroso-N-acetylpenicillamine failed to block DNA synthesis in SMCs from old rats but was effective in SMCs from newborn and adult rats. However, cGMP analogues were inhibitory in all 3 SMC types. We demonstrated that in SMCs from old rats, NO was unable to increase the concentration of intracellular cGMP, suggesting that either cGMP synthesis was defective or cGMP degradation was enhanced. Western blot analysis revealed that SMCs from old rats do not express the β subunit of soluble guanylyl cyclase. To confirm the importance of this observation in vivo, we balloon-injured the carotid arteries of adult and old rats. Whereas soluble guanylyl cyclase was expressed at the same level in the media of injured vessels and uninjured vessels of both groups, its expression in the intimas of old rats was reduced by 70% compared with intimas from adult animals. Furthermore, Nω-nitro-L-arginine, an inhibitor of NO synthesis, enhanced the intimal thickening in injured vessels in adult rats but not in old rats. We conclude that the loss of NO responsiveness in aged rats is due to the lack of the β subunit of soluble guanylyl cyclase, and we speculate that this defect contributes to the enhanced intimal thickening in response to injury in old animals. (Circ Res. 2000;86:520-525.)

Key Words: cGMP ■ hyperplasia ■ nitric oxide ■ smooth muscle cells ■ soluble guanylyl cyclase

The principal function of NO in large arteries is to regulate vascular tone. This effect is mediated by the activation of the heme-containing soluble guanylyl cyclase (sGC) and the subsequent increase of cGMP levels.1–5 NO might also be an inhibitor of smooth muscle cell (SMC) proliferation and migration. NO donors or NO generated by the overexpression of NO synthase inhibits DNA synthesis and the growth of cultured SMCs.5–8 Oral delivery of NO donors or L-arginine (a NO precursor) and local transfer of endothelial constitutive NO synthase inhibit intimal formation after balloon injury.9–14 These results demonstrate that NO can blunt the injury response in vivo. Most studies have suggested that sGC and cGMP are the major mediators for the antiproliferative effects of NO on SMCs.2–15,16 One of the major downstream events after an increase in intracellular cGMP is the activation of cGMP-dependent protein kinase (PKG).15,16 It is reasonable to think that the NO-mediated inhibitory effect on SMC growth might be through PKG. However, the loss of PKG expression in passaged SMCs seems to cause the cells to adopt a “synthetic phenotype” even though the cells still respond to NO donors.17–20 This finding provides support for the possibility that NO can inhibit SMC growth by a pathway that does not depend on PKG. These results do not eliminate the possibility of involvement of cGMP in NO-mediated inhibition of SMC proliferation.

Aging might affect the ability of SMCs to proliferate in response to vascular injury.21 Several observations indicate that the response of SMCs to injury is greater in older rats compared with younger rats. SMC proliferation and intimal thickening in balloon-injured vessels are enhanced in older rats.22–24 Likewise, cultured aortic SMCs from old rats exhibit a more dedifferentiated phenotype and grow more rapidly in response to serum and platelet-derived growth factor than do SMCs from younger animals.25,26 Vessels from young and old rats have been cross-transplanted into syngeneic hosts of different ages; only the vessels from old rats exhibit a marked intimal thickness in response to injury that is independent of the age of the host.21 This finding indicates that the enhanced neointimal response is determined by the age of the vessel donor.

To investigate whether age could modify the response of SMCs to NO, we compared the effects of NO donors and
cGMP analogues on the growth of aortic SMCs derived from newborn, adult (aged 3 months), and old (aged 2 years) Fischer rats. We investigated the downstream events in NO signaling, including intracellular cGMP levels and activation of cGMP-dependent protein kinase (PKG) in these 3 types of SMCs. We also examined the expression of the β subunit of sGC (sGCβ) in these 3 types of SMCs in vitro and in rat balloon-injured carotid arteries in vivo.

Materials and Methods

Materials

Rabbit polyclonal anti–vasodilator-stimulated phosphoprotein (anti-VASP), anti–PKG β sera, and mouse monoclonal anti–VASP phosphoserine 239 antibody were generous gifts from Drs Ulrich Walter and Suzanne M. Lohmann (University of Würzburg, Würzburg, Germany). The rabbit anti-sGC serum was purchased from Alexis Co. Other chemicals were purchased from Sigma Chemical Co.

Cell Cultures

Adult SMC cultures were prepared by enzymatic digestion of aortas from 3-month-old Fischer 344 rats (Simonsen Laboratories, Gilroy, Calif) as described. SMCs from newborn (aged <5 days) and old (aged 2 years) Fischer rats were prepared as described.26 The cells were propagated in DMEM containing 10% FBS (GIBCO Laboratories) in 5% CO2 at 37°C and were used between passages 6 and 15.

DNA Synthesis

DNA synthesis was measured as described by use of [3H]thymidine incorporation in response to stimulation of 10% FBS for 24 hours in the presence or absence of appropriate reagents (S-nitroso-N-acetylpenicillamine [SNAP] or cGMP analogues). The passage numbers of SMCs from animals of different ages were the same in each experiment.

Measurement of Intracellular cGMP Levels

The cells were seeded at 4×10^5 cells per well in 6-well tissue culture plates. The cells were extracted, and the intracellular cGMP concentration was determined by a cGMP enzyme immunoassay system according to the manufacturer’s protocol (Amersham Co).

Protein Extraction and Western Blotting

Cultured SMCs and frozen tissues were extracted in HEB buffer (25 mmol/L HEPES [pH 7.5], 5 mmol/L EDTA, 5 mmol/L EGTA, 150 mmol/L NaCl, 100 mmol/L Na4 P2O7, 50 mmol/L NaF, 1 mmol/L benzamidine, 1% Triton X-100, 10% glycerol, 0.1% β-mercaptoethanol, 1 μg/mL pepstatin-A, 5 μg/mL leupeptin, and 5 μg/mL aprotinin).27 The same amount of protein was heated for 5 minutes at 95°C, then subjected to 10% SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). The blots were submerged in 5% milk in TTBS (25 mmol/L Tris-HCl, 5 mmol/L NaCl, and 0.1% Triton X-100), followed by an incubation at 4°C overnight in 0.1% milk in TTBS containing the appropriate primary antibody in concentrations recommended by the manufacturer or references. The proteins were detected by horseradish peroxidase–labeled secondary antibody with the use of a standard enhanced chemiluminescence protocol provided by the manufacturer (Amersham Co). Relative protein quantification was performed by scanning autoradiographs.

Animal Experiments and Tissue Preparation

Male Fischer 344 rats, aged 3 months (220 to 330 g) from Simonsen Laboratories (Gilroy, Calif) or aged 24 months (370 to 500 g) from the National Institutes of Health (NIH, Bethesda, Md), were anesthetized, and the left carotid arteries were surgically exposed. The distal half of the common carotid arteries was isolated, and the endothelium was stripped by the passage of a 2F balloon catheter (V, Mueller) introduced through an arteriotomy in the external branch. The external carotid arteries were then ligated after removal of the catheter, the blood flow was restored, and the wound was closed. Animals were fed with either H2O or H2O plus N*-nitro-L-arginine (L-NA), an inhibitor of NO synthesis (10 mg/kg per day), for 2 weeks. Systolic arterial blood pressures were measured in conscious restrained rats by tail-cuff plethysmography (Norco Biosystems). After 2 weeks, the animals were euthanized, and the arteries were flushed clear of blood with Ringer’s lactate solution. Arteries intended for Western blotting analysis were excised. The neointima and media in injured carotid arteries were separated under a microscope, immediately frozen in liquid nitrogen, and stored at −70°C. Arteries intended for histological analysis were flushed clear of blood with Ringer’s lactate solution and fixed by perfusion with 10% neutral buffered formalin, pH 7.4, at 100 mm Hg and embedded in paraffin for histology.8 Measurement of luminal, intimal, and medial areas was made on 2 cross sections per carotid taken from the middle of each carotid segment. All surgical procedures were performed according to the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

Statistics

All values are expressed as mean±SD. Comparisons among the groups were made by the Mann-Whitney nonparametric test.

Results

NO Donor SNAP Fails to Inhibit DNA Synthesis and to Increase cGMP Levels in Old SMCs

To investigate the influence of age on NO-mediated growth inhibition, we compared the effect of the NO donor SNAP and cGMP analogues 8-bromo-cGMP and dibutyryl-cGMP on DNA synthesis in rat aortic SMCs derived from newborn, adult (aged 3 months), and old (aged 2 years) animals. DNA synthesis in response to 10% FBS was inhibited by both cGMP analogues in all 3 groups by 40% (Figure 1A). The NO donor SNAP, however, had no effect on SMCs from old rats but decreased DNA synthesis in SMCs from newborn and adult rats by 25% (Figure 1B). This observation supported the conclusion that NO failed to increase cGMP levels in SMCs from old rats.

To confirm this conclusion, we measured the induction of cGMP on SNAP treatment. In SMCs from newborn and adult rats, SNAP increased the cGMP concentration 25- and 10-fold, respectively, but it failed to elevate cGMP levels in SMCs from old rats (Figure 2).

cGMP-Dependent Protein Kinase Is Expressed and Functional in All 3 SMC Groups

Because it has been suggested that cultured SMCs may lose expression of the cGMP-dependent protein kinase (PKG),17–20 we tested whether PKG was functional and could account for the inhibitory effect of cGMP in all 3 rat SMC groups. To monitor the PKG activity in cells, we used the shift assay with the PKG substrate VASP. VASP is a 46-kDa protein of yet unknown function that has been identified on the basis of its phosphorylation by PKG.28,29 The phosphorylated form of VASP migrates in SDS-polyacrylamide gels as a 50-kDa protein.29 On treatment of SMCs with 8-bromo-cGMP, a 50-kDa band of VASP was detected by Western blotting in all 3 SMC types (Figure 3A). Consistent with our observation that NO does not increase cGMP levels in SMCs from old rats, NO induced VASP phosphorylation in SMCs from newborn and adult rats only (Figure 3A). VASP was
phosphorylated in SMCs from newborn and adult rats on SNAP treatment in a dose-dependent manner (Figure 3B). SNAP failed to induce VASP phosphorylation in SMCs from old rats even at the highest concentration (250 μmol/L, Figure 3A) and over a long period of time (up to 24 hours, data not shown). To confirm that cGMP analogue and SNAP-induced cGMP activate PKG, which, in turn, phosphorylates VASP, we used a Western blotting assay with the monoclonal antibody against VASP phosphoserine 239. This site is specifically phosphorylated by PKG but not protein kinase A (PKA), even though PKA is also capable of phosphorylating VASP. The results indicate that VASP is phosphorylated by PKG in response to 8-bromo-cGMP in all 3 cell types and in response to SNAP in SMCs from newborn and adult rats (Figure 4A). VASP phosphorylation induced by a cAMP analogue, dibutyryl-cAMP, a PKA activator, could not be detected by the antibody to phosphoserine 239 (Figure 4A), even though VASP phosphorylation in response to cAMP analogues could be detected with an antibody to the other 2 PKA-dependent VASP phosphorylation sites. Furthermore, the presence of PKG protein in all 3 SMC types could be demonstrated by Western blotting (Figure 4B). These results indicate that PKG was expressed and functional in all 3 types of SMCs, but NO was not able to activate PKG in SMCs from old rats.

Old SMCs Do Not Express sGCβ

The fact that NO failed to induce cGMP production in SMCs from old rats suggests either that sGC is not functional or that cGMP is rapidly degraded. sGC is a heterodimer consisting of α and β subunits, both of which are catalytically active and targeted by NO. When we measured the expression levels of

**Figure 1.** Inhibition of DNA synthesis by SNAP and 8-bromo-cGMP SMCs from newborn, adult, and old rats. Cells (1×10⁵) were seeded in 12-well plates and cultured overnight in DMEM with 10% FBS at 37°C with 5% CO₂. Cells were starved in DMEM for 2 to 3 days before medium was replaced by 1 mL DMEM containing 10% FBS, 1 μCi [³H]thymidine, and 50 μmol/L 8-bromo-cGMP (A) or 2.5 μmol/L SNAP (B) as indicated. Cells were harvested after 24 hours, and [³H]thymidine incorporation was determined as described in Materials and Methods. Values (mean of triplicates±SD) are expressed as percent inhibition of controls. Experiments in which 8-bromo-cGMP was replaced by dibutyryl-cGMP showed similar results (data not shown). The graphs are representatives of 3 independent experiments, and each sample has 3 replicates in each experiment. *P<0.01 (n=3).

**Figure 2.** cGMP production on stimulation with NO donor SNAP in SMCs from newborn, adult, and old rats. Cells (4×10⁵) were seeded in 6-well plates and cultured overnight before stimulation with 250 μmol/L SNAP for 5 minutes. Cells were extracted, and intracellular cGMP concentrations were measured. Values (mean of triplicates±SD) are expressed as fold increase over unstimulated controls. Open bars represent the unstimulated controls; stippled bars represent SNAP-stimulated levels. The graphs are representatives of 3 independent experiments, and each sample has 3 replicates in each experiment. *P<0.01 (n=3).
by Western blotting, we detected the protein in SMCs from newborn and adult rats but not in SMCs from old rats (Figure 5A). To rule out the possibility that SMCs from old rats do express a NO-responsive sGC that we failed to detect, we measured cyclic nucleotide levels on SNAP treatment in the absence and presence of 3-isobutyl-1-methylxanthine (IBMX), a general cyclic nucleotide phosphodiesterase inhibitor. The addition of IBMX increased cAMP but not cGMP levels whether or not SNAP was present (Figure 5B). This observation is consistent with the hypothesis that NO fails to produce cGMP in SMCs from old rats because they lack a functional sGC. The loss of guanylyl cyclase activity in SMCs from old rats seems to be specific for the heme-containing sGC. The atrial natriuretic peptide that binds and activates the type A membrane guanylyl cyclase was able to increase the intracellular cGMP level by 5-fold (from 2.8 ± 0.8 to 14.0 ± 2.0 fmol/mg, n=3) in SMCs from old rats as well as in SMCs from newborn and adult rats. As expected, we also observed atrial natriuretic peptide–induced VASP phosphorylation in all 3 cell types (data not shown).

In Old Rats, Intimal SMCs Exhibited Decreased Expression of sGCβ After Balloon Injury
We investigated the possibility that an age-dependent loss of sGC expression plays a role in the response to injury in rat carotid arteries. The left carotid arteries of adult and old rats were balloon-injured and harvested after 2 weeks. The media and intima of the injured carotid were separated, and the proteins were extracted and analyzed by Western blotting for the expression of sGC. As a control, the noninjured right carotid artery was processed in the same way. Both adult and old rats expressed similar levels of sGCβ in the uninjured carotid arteries. After injury, however, intimal SMCs from old but not adult animals exhibited an average 70% decrease in sGC levels (Figure 6). No differences between adult and old rats were observed in the media of the injured carotid arteries.

In Old Rats, Intimal Formation After Balloon Injury Was Not Affected by L-NA, an Inhibitor of NO Synthesis
To establish the relevance of decreased sGC expression in intimal formation in old rats, we investigated the effect of L-NA feeding on intimal formation after balloon injury in old and adult rats. Blood pressures were significantly increased in both age groups receiving L-NA (at 2 weeks, old rats: control 115 ± 8.0 mm Hg, n=8; L-NA 150 ± 6.3 mm Hg, n=11; adult rats: control 122 ± 8.2 mm Hg, n=11; L-NA 157 ± 9.3 mm Hg, n=11). The ratio of intima to media in old rats was not
expression of sGC

Figure 6. Expression of sGCβ in balloon-injured carotid arteries of adult and old rats. Balloon injury of old and adult rat carotid arteries was performed as described in Materials and Methods. Two weeks after the procedure, carotid arteries (injured and uninjured control arteries) were harvested, and the protein was extracted from the intima and media of the injured vessels. Equal amounts of protein were subjected to Western blotting with an anti-sGCβ antibody. The top panel shows a representative blot. The arrow indicates the position of 70-kDa sGCβ. Blots were scanned, and relative expression of sGCβ was determined. Values (mean of 6 animals ± SD) representing expression of sGCβ in intima and media of injured carotid arteries are expressed as percent of control (the respective uninjured contralateral carotid artery). *P<0.01 (n=6).

significantly changed by L-NA (0.95±0.17 [control, n=8] versus 0.92±0.22 [L-NA, n=11], P=0.75), whereas this ratio was significantly increased in adult rats (0.61±0.18 [control, n=11] versus 0.82±0.23 [L-NA, n=11], P=0.03). This result suggests that NO release in adult rat arteries inhibited intimal thickening, whereas it had no effect in old rat arteries. A likely explanation for this result is that intimal SMCs of adult rats express sGCβ and, therefore, are responsive to NO, whereas the old rat SMCs lacking sGCβ cannot respond to NO and, therefore, are unaffected by L-NA.

Discussion

NO is a vasodilator and a negative regulator of SMC function. On the basis of the observation that in the rat carotid artery subjected to balloon injury, older Fischer rats develop a bigger neointima than do younger rats, we investigated whether the SMC response to NO is lost as the animals grow older. The proliferation of SMCs derived from newborn and adult SMCs, but not SMCs from old rats, was inhibited by NO (Figure 1B). In contrast, all 3 SMC types were inhibited when the cGMP analogue was used (Figure 1A). It has previously been reported that cultured SMCs exhibit reduced expression of the cGMP-dependent protein kinase.17–20 Our data, however, support the conclusion that the lack of sGCβ in intimal SMCs renders these cells unresponsive to locally synthesized NO. Furthermore, the observation that intimal thickening in adult rats is increased by L-NA treatment confirms the importance of NO in limiting the extent of intimal thickening after injury. It is not clear why intimal SMCs in the old animals do not have a functional sGC inasmuch as they are derived from the media in which the enzyme is apparently present. Phenotypic heterogeneity and clonality has been reported for SMCs in the rat vessel wall.34,35 Because medial SMCs are permanently exposed to endothelium-derived NO, it is possible that aging favors the appearance of SMC clones lacking a functional sGC. Those clones may be the ones that migrate and proliferate after vessel injury. Another possibility is that the old SMCs are losing sGC in the course of forming the neointima. Future experiments will be designed to distinguish between these 2 possibilities and to delineate the molecular mechanism of how the expression of sGCβ is switched off.

In summary, we present a novel mechanism to explain how SMCs become insensitive to the growth-inhibitory effects of NO. It is an interesting possibility that a loss of NO inhibition caused by a loss of a functional sGC may also occur in humans and contribute to the higher risk in elderly people of developing restenosis after angioplasty.

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