Effect of Hypercholesterolemia on Expression and Function of Vascular Soluble Guanylyl Cyclase

Ute Laber, MD; Thorsten Kober, MD; Verena Schmitz, PharmD; Astrid Schrammel, PharmD; Wilfried Meyer, PhD; Bernd Mayer, PhD; Martina Weber, PharmD; Georg Kojda, PharmD, PhD

Background—Vasorelaxation to endothelial NO is mediated by activation of soluble guanylyl cyclase (sGC) and impaired by hypercholesterolemia in animals and humans. We investigated whether hypercholesterolemia impacts expression and function of sGC.

Methods and Results—White New Zealand rabbits (n=10 per group) received a standard diet for 16 weeks (SD16) (n=20) or 32 weeks (SD32) and a cholesterol diet (7.5 g/kg) for 16 weeks (CD16) (n=20) or 32 weeks (CD32), respectively. Another group received cholesterol diet for 16 weeks followed by standard diet for 16 weeks (CD/SD). Aortic expression of the α1-subunit of sGC (sGC-α1) and β1-subunit of sGC (sGC-β1) was assessed by Western blot. Function was measured by aortic relaxation to S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) and sGC activity in aortic cytosols. Hypercholesterolemia induced an upregulation of sGC-β1 in CD16 (3.5±0.4-fold, P<0.001 versus SD16) and CD32 (4.0±0.4-fold, P<0.001 versus SD32). A similar increase was found for sGC-α1. In striking contrast, basal and NO-stimulated sGC activities in aortic cytosols of CD16 were only slightly enhanced (1.4-fold, P<0.05). Furthermore, the vasodilator potency of SNAP (EC50 in −logM) was 10-fold lower in CD16 (6.76±0.09) than in SD16 (7.66±0.14, P<0.01). The increase of sGC expression was completely reversible, as indicated by comparable sGC-β1 amounts in SD32 and CD/SD (1.2±0.1-fold, P>0.05). Immunohistochemical analysis suggests that a great portion of the overexpressed sGC is located in intimal lesions. Additional experiments showed that increased vascular superoxide production induced by 6-anilino-5,8-quinolinedione (LY85385) reduces sGC-activity but increases sGC-expression.

Conclusions—These results suggest that hypercholesterolemia induces a reversible overexpression of a dysfunctional vascular sGC, which may contribute to the pathogenesis of atherosclerosis. (Circulation. 2002;105:855-860.)

Key Words: nitric oxide ▪ atherosclerosis ▪ cardiovascular diseases ▪ endothelium ▪ signal transduction

Hypercholesterolemia is a major risk factor for cardiovascular disease.1 It has been shown to promote a typical atherosclerotic remodeling of the vascular wall. One of the earliest manifestations is an inhibition of endothelium-dependent vasorelaxation often referred to as endothelial dysfunction.2 Most likely, the pathogenesis of endothelial dysfunction involves many different factors that cause either impaired generation or enhanced breakdown of NO. Besides vasodilation, NO induces several other effects in the vascular wall that are potentially vasoprotective.3 Of these, inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and apoptosis as well as antioxidative effects are of particular importance. Most of these actions are mediated by the second messenger cGMP. Thus, the efficacy of the signal transduction pathway for NO plays an important role for the vascular effects of NO. Once generated, NO activates the soluble guanylyl cyclase (sGC) to produce cGMP, which in turn stimulates cGMP-dependent protein kinases (PKGI and PKGII).4 The enzyme is a heterodimer consisting of an 73-kDa α1-subunit (sGC-α1) and a 71-kDa β1-subunit (sGC-β1), and activation by NO is strictly dependent on the heme moiety.5,6 The regulation of sGC expression is poorly understood, and little is known about changes of sGC expression in disease states. Recent investigations have shown a downregulation of sGC activity and expression induced by cAMP and hypertension,7-10 whereas conditions such as hypoxia and nitroglycerin treatment are associated with an upregulation of sGC.11,12 Changes of sGC expression under pathologic conditions such as hypercholesterolemia have not been investigated. In view of the major importance of the sGC in transducing the NO signal into cellular responses, we sought to determine the
effect of long-term hypercholesterolemia on expression and function of vascular sGC.

Methods

Animals Studied
We investigated a total of 70 New Zealand White rabbits (Harlan-Winkelman, Borchon, Germany). The mean body weight was 237 ± 37 g, and age was 10 to 12 weeks. The animals were housed individually in stainless-steel cages at a temperature of 18 to 20°C and humidity of 50% to 60%, had a day/night rhythm of 12 hours, and received water ad libitum. Rabbits were randomly divided in 5 groups of 10 animals each and were fed a standard diet for 16 weeks (SD16) (n = 20) or 32 weeks (SD32) and a diet supplemented with 0.75% (wt/wt) cholesterol for 16 weeks (CD16) (n = 20) or 32 weeks (CD32), respectively. The fifth group received a standard diet for 16 weeks and a cholesterol diet for the following 16 weeks (SD/CD). The body weight of every animal was determined weekly. After the feeding period, the acute experiments and determination of plasma cholesterol levels were performed according to standard methods. Additional experiments were performed with segments of thoracic aorta taken from 6 male 3- to 4-month-old Wistar rats. Permission for this study was provided by the regional government, and the experiments were performed according to the guidelines for the use of experimental animals as given by Deutsches Tierschutzgesetz and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the United States.

Vasorelaxation Studies
Aortas from the rabbits were rapidly excised and immersed in cold oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit buffer (pH 7.4, 37°C) of the following composition (in mmol/L): Na⁺ 143.07, K⁺ 5.87, Ca²⁺ 1.6, Mg²⁺ 1.18, Cl⁻ 125.96, HCO₃⁻ 25, H₂PO₄⁻ 1.18, SO₄²⁻ 1.18, and glucose 5.05. The aorta was divided and used for vasorelaxation studies and measurements of the expression of the sGC-α₁ and sGC-β₁ by Western blot densitometry. Four ring segments (5-mm width) of the thoracic aorta were mounted between stainless steel triangles in a water-jacketed organ bath for measurement of tension development, as recently described.14 Resting tension was 2 g.15 After equilibration (1 hour), contractile function of aortic segments (5-mm width) of the thoracic aorta was mounted between stainless steel triangles in a water-jacketed organ bath for measurement of tension development, as recently described.14 Resting tension was 2 g.15 After equilibration (1 hour), contractile function of aortic segments was tested by application of KCl (80 mmol/L). acetyl-D, L-penicillamine (SNAP) (1 nmol/L to 10 mol/L) was added to the organ bath, and cumulative concentration-effect curves were determined. The contractile response was normalized to the response to phenylephrine (100 μmol/L).

Statistics
The concentrations of the half-maximal vasorelaxant effect of acetylcholine and SNAP (EC₅₀ in −logM) were calculated from the individual concentration effect curves, as proposed previously.21 Vasorelaxant responses are expressed as percentage of the contractile response achieved with 0.2 μmol/L phenylephrine at the beginning of the experiments. All data were analyzed by standard computer programs (GraphPad Prism PC Software version 3.0, ANOVA) and are expressed as mean values ± SEM. Significant differences were evaluated using either Newman-Keuls multiple comparison test (ANOVA) or 2-way ANOVA. P < 0.05 was considered significant.

Results
The plasma cholesterol concentrations in animals receiving normal chow were 62.4 ± 6.0 mg/dL (SD16) and 57.5 ± 4.8 mg/dL (SD32). All animals fed the cholesterol chow had strongly increased levels of 1631 ± 112 mg/dL (CD16) and 1703 ± 183 mg/dL (CD32). In CD/SD, the value was 63.7 ± 7.5 mg/dL. The cholesterol chow significantly increased aortic superoxide production (in counts/mg) from 229 ± 22 (SD16) to 360 ± 56 (CD16, P = 0.0244).

Determination of sGC Activity
Specific activity of sGC was measured by the formation of [³²P]-cGMP from [α-³²P]-GTP, as described previously.8-18 Briefly, sGC of aortic cytosol (20 to 40 μg of protein) was incubated in a total volume of 200 μL of a triethanolamine-HCL buffer (50 mmol/L, pH 7.4, 37°C) containing [α-³²P]-GTP (5 mmol/L, 0.4 μCi/mL), GTP (100 μmol/L), cGMP (1 mmol/L), 3-isobutyl-1-methylxanthine (1 mmol/L), MgCl₂ (1 mmol/L), and DTNB (1 mmol/L) in presence of 2,2-diethyl-1-hydroxy-1-nitrosohydrazin (DEA/NO, 100 μmol/L), 3-(5-Hydroxymethyl-2-furyl)-1-benzylindazole (YC-1, 200 μmol/L), or 1 mmol/L MnCl₂ instead of MgCl₂.

Incubation of Rat Aorta
Rat aorta was incubated with LY 83583 (10 μmol/L) for 4 hours, frozen in liquid nitrogen, and prepared as described above to determine expression and activity of sGC. Determination of aortic superoxide production induced by LY 83583 was determined with 5 μmol/L lucigenin, as described previously.9

Immunohistochemistry
Small sections of aortic rings were fixed in buffered formol (4%) solution and embedded with Technovit 7100 ( Heraeus Kulzer). Cross-sections were immunostained with the sGC-β₁ antibody (Alexis). Control experiments showed no specific staining in the absence of this antibody.

Substances and Solutions
SNAP was synthesized in our laboratory according to Field et al.,19 as described previously.20 All other chemicals were obtained from Merck or Sigma in analytical grade. The stock solutions of acetylcholine (10 mmol/L) and phenylephrine (10 mmol/L) were prepared in distilled water. The stock solution of SNAP (200 mmol/L) 10 mmol/L was prepared in dimethylsulfoxide. All stock solutions were prepared daily, diluted with Krebs buffer as required, kept on ice, and protected from daylight until use. All concentrations indicated in the text and figures are expressed as final bath concentrations.

Preparation of 100 000g Supernatants
Frozen aortic rings were homogenized with 2 mL Tris buffer (5 mmol/L) supplemented with dithiothreitol (5 mmol/L), containing the proteinase inhibitors leupeptin, benzamidine, aprotinin, phenylmethylsulfonylfluoride, and antipain (10 μg/mL). The tissues were homogenized using a polytron, and the homogenate was centrifuged at 4°C at 100g (10 minutes), 10 000g (15 minutes), and 100 000g (1 hour). Removal of plasma globulins from the supernatant was achieved using a Sepharose column (HiTrap, Amersham/Pharmacia). Total protein levels were determined using the Bradford method.16 This cytosolic preparation was used for enzyme activity assays and Western blotting.

Western Blotting
Western blotting was performed as described previously.19 Briefly, each lane was loaded with 10 μg total protein, and blots were incubated with polyclonal antibodies to either the sGC-α₁ (generously provided by Dr H.-P. Stasch, Bayer AG, Wuppertal, Germany) or sGC-β₁ (Cayman, Alexis, Gruenberg, Germany). After incubation with a horseradish peroxidase–conjugated antibody (Calbiochem, Darmstadt, Germany), blots were developed using ECL (Roche) and exposed to x-ray films. The autoradiographs were analyzed by densitometry (Geldoc, Bio-Rad). Additional experiments were done with another sGC antibody staining both sGC-α₁ and sGC-β₁ (Alexis) and yielded comparable results (data not shown).
Effect of Hypercholesterolemia on sGC Expression

Hypercholesterolemia induced a significant upregulation of sGC-β1 in CD16 (3.5 ± 0.4-fold, \( P < 0.001 \) versus SD16) and in CD32 (4.0 ± 0.4-fold, \( P < 0.001 \) versus SD32, Figure 1). The expression of sGC-α1 was measured in CD16 and SD16 and found to be similarly upregulated (2.9 ± 0.4-fold, \( P < 0.001 \) versus SD16, Figure 2). The effect of hypercholesterolemia on sGC expression was fully reversible, because 16 weeks of normal food given to hypercholesterolemic rabbits (CD/SD) normalized the increased sGC-β1 expression to values found in age-matched SD32 rabbits (1.2 ± 0.1-fold, \( P < 0.05 \)). Hypercholesterolemia did not change the pattern of sGC distribution in the media and the adventitia. However, we found a strong staining of intimal lesions, suggesting that a great portion of the overexpressed sGC is located in intimal lesions (Figure 3).

Effect of Hypercholesterolemia on NO-Induced Vasodilation

Endothelium-Dependent Vasodilation

There was a profound inhibition of endothelium-dependent vasodilation to 0.5 \( \mu \text{mol/L} \) acetylcholine in CD16 (25.8 ± 12%) versus SD16 (67.4 ± 6.1%, \( P < 0.001 \)) and in CD32 (−1.6 ± 5.9%) versus SD32 (70.7 ± 4.3%, \( P < 0.001 \)). This inhibition was fully reversible. Aortic rings of CD/SD showed an endothelium-dependent relaxation of 73.7 ± 8.0%.

NO Donor–Induced Vasodilation

Despite the substantial upregulation of sGC expression, the vasorelaxant activity (EC\textsubscript{50} in \(-\log \text{M}\)) of SNAP was 10-fold lower in aortic rings of hypercholesterolemic rabbits (Figure 4A). This inhibition of SNAP-induced vasodilation was almost completely reversible, because the EC\textsubscript{50} of SNAP (in
Effect of Hypercholesterolemia on sGC Activity
The activity of sGC was measured in 100 000 g supernatants of the aortas with and without various enzyme activators, such as MgCl₂ (basal activity), MnCl₂ (increased basal activity), DEA/NO (NO-dependent activation), and YC-1 in the presence of oxyhemoglobin (NO-independent activation). As shown in Figure 4B, none of these activation procedures produced an increase in sGC activity that would match the increase in sGC expression (Figures 1 and 2). According to 2-way ANOVA, the effect of cholesterol on sGC activity accounted for ~1.49% of the total variance and was barely significant (P<0.05). An additional analysis of the difference between the specific sGC activities found in normocholesterolemic and hypercholesterolemic rabbits showed that the small effect of cholesterol on sGC activity in CD16 versus SD16 was not dependent on the different sGC stimulators (P=0.229; one-way ANOVA).

Figure 4. Function of sGC in aortic rings of rabbits fed normal chow (SD16 and SD32), cholesterol chow (CD16 and CD32), and cholesterol chow followed by normal chow (CD/SD). A, Concentration for half-maximal vasorelaxation induced by SNAP (pD₂ values in −logM). Aortic rings of cholesterol-fed rabbits showed strongly impaired vasodilator response as indicated by the reduction of the pD₂ value (*Significant difference; P<0.001). B, Specific activity of sGC in 100 000g supernatants of aortas from SD16 and CD16. None of the stimuli (100 μmol/L DEA/NO, 200 μmol/L YC-1, or 10 μmol/L oxyhemoglobin) used to activate sGC produced a difference between the specific activities in SD16 and CD16 that would match the difference in sGC expression (Figure 1). Analysis of the difference between the specific sGC activities found in normocholesterolemic and hypercholesterolemic rabbits showed that the small effect of cholesterol on sGC activity in CD16 versus SD16 was not dependent on the different sGC stimulators (P=0.229; one-way ANOVA).

Effect of LY 83583 on sGC Expression and Activity in Rat Aorta
To evaluate the effects of increased vascular superoxide production on the expression and activity of sGC, we incubated rat aortic rings with 10 μmol/L LY 83583, which significantly enhanced the superoxide-specific lucigenin signal (Figure 5A). In contrast, LY 83583 showed no effect in the absence of tissue (control, 1131±34 counts; LY 83583, 1095±45; n=6). Incubation with LY 83583 for 4 hours resulted in a significant upregulation of sGC-β₃ expression (Figure 5B), whereas the specific activity measured in the same cytosols was greatly reduced (Figure 5C).
Discussion

The aim of this study was to determine the influence of hypercholesterolemia on the expression and function of vascular sGC. Our main result is that hypercholesterolemia induces a substantial overexpression of sGC that is not accompanied by a similar increase in the activity of the enzyme and is associated with marked inhibition of endothelium-dependent and SNAP-induced vasodilation. These data suggest that hypercholesterolemia induces a disturbance of the NO signal-transduction pathway by initiating the expression of a dysfunctional sGC.

The sGC is a heterodimeric protein that can exist in different isoforms. Hypercholesterolemia induced an overexpression of the 2 subunits ($\alpha_1$ and $\beta_1$) that form the vast majority of vascular sGC.6,22 Little is known about the regulation of expression of sGC. The parallel change in the expression of the $\alpha_1$- and $\beta_1$-subunit is consistent with the suggested tandem organization of these genes that might induce a coordinated transcription.23 However, the expression of the $\alpha_1$- and $\beta_1$-subunit of mouse sGC is suggested to be regulated independently, and the level of sGC expression also involves changes of mRNA stability.24,25

It was shown that NO itself can modulate the expression of sGC in rat vascular smooth muscle cells and aortic ring preparations.25,26 In both studies, this effect of NO was detectable as early as 4 hours after starting the treatment. Thus, it seems that changes in the production of vascular NO will regulate sGC expression in a negative feedback manner. Upregulation of sGC would then be obvious in conditions associated with a reduced bioavailability of endogenous NO, which is a cornerstone of the pathophysiological changes occurring in hypercholesterolemia, both in rabbits and humans.3 In accordance, we found an increased aortic superoxide production and a strong impairment of endothelium-dependent vasodilation in cholesterol-fed rabbits. It seems conceivable that the reduction of the bioavailability of endogenous NO is one of the triggers that has contributed to the upregulation of sGC expression by hypercholesterolemia in our study.

To evaluate whether oxidative stress might act on sGC expression independently of inhibition of NO-induced cGMP generation, we investigated isolated aortic rings of normal rats, which have a low basal production of NO because of the absence of shear stress. These rings were subjected to 10 $\mu$mol/L LY 83583, a compound that generates superoxide intracellularly.27 We found that 4 hours of exposure to LY 83583 significantly increased the expression of sGC but strongly inhibited its catalytic activity on stimulation with the NO donor SNAP. Our data suggest that intracellularly generated superoxide might increase sGC expression even at low levels of cGMP usually found in these isolated ring preparations.20 However, it is likely that many other mechanisms, such as inflammation and cytokines, which are present in atherosclerotic aorta, may contribute to the changes of sGC expression in atherosclerosis. For example, an increase of vascular superoxide production induced by nitroglycerin in the absence of atherosclerosis in rats resulted in a less pronounced overexpression of sGC.12

Another observation from this study is that the overexpressed sGC protein is obviously dysfunctional. This conclusion results from the striking disparity between sGC expression and sGC activity in cytosolic fractions from normocholesterolemic and hypercholesterolemic aortas. The small increase in the activity of the isolated sGC protein in hypercholesterolemia does by no way match the magnitude of protein overexpression detected under the same conditions. This holds true for basal activity and for different activation mechanisms as well. Whereas stimulation with DEA/NO is strictly NO dependent,28 stimulation with YC-1 is mediated by both NO-dependent and NO-independent activating mechanisms.29 Recently, a series of new stimulators of sGC has been described that are not NO-donors and activate sGC by a different mechanism.30,31 It is not known whether these activators of sGC may be capable of stimulating the dysfunctional sGC and may serve for an attractive therapeutic option to increase vascular cGMP in hypercholesterolemia.

The reasons for the impairment of sGC function in hypercholesterolemia are not known. Many different possibilities, such as inhibition of dimerization, oxidative modification, generation of an endogenous inhibitor, or reduced binding of substrate and cofactors, might contribute. There are some studies suggesting that oxidative stress might be of particular importance. It has been shown that oxidized LDL can inhibit NO-dependent activation of isolated sGC.32 Furthermore, we have found recently that peroxynitrite, a marker of oxidative stress that has been detected in a variety of pathologically changed vascular material from animal and man, impairs the activity of sGC.26

Despite markedly increased sGC expression and unchanged enzyme activity, relaxations to the NO donor SNAP were clearly impaired in hypercholesterolemic aortas. The reduced vascular sensitivity to SNAP agrees well with numerous previous studies describing similar results for a variety of vascular beds from different species, including humans.3 This apparent reduction of NO bioavailability may result from increased oxidative stress and subsequent inactivation of NO by reactive oxygen species, such as superoxide, in the atherosclerotic blood vessels. Alternatively, other mechanisms, such as decreased vascular responsiveness to cGMP or increased cGMP metabolism, might contribute.

Our immunohistochemical data suggest that hypercholesterolemia does not change the distribution pattern of sGC in medial and adventitial cells. In striking contrast, we found a strong sGC staining in intimal lesions. These data suggest that a great portion of the overexpressed and dysfunctional sGC is located in intimal plaques, where vascular oxidative stress, inflammation, and cytokine concentrations are comparably high.1 Thus, the proliferative and migratory activity and the loss of specific function of vascular smooth muscle cells might be facilitated by the loss of the sGC-dependent anti-proliferative and antimigratory responses to endothelial NO. Interestingly, it was shown recently that a gene transfer of the $\alpha_1$- and $\beta_1$-subunit of sGC increases NO responsiveness and reduces neointima formation after balloon injury in rats.33

In summary, we suggest that hypercholesterolemia creates an environment that inhibits the proper function of readily expressed sGC and induces a compensatory increase of sGC.
expression. It is conceivable that this pathomechanism facilitates the loss of the sGC-dependent antiproliferative and antimigratory responses of vascular smooth muscle cells to endothelial NO.

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