Hypercholesterolemia Is Associated With a Reduced Response of Smooth Muscle Guanylyl Cyclase to Nitrovasodilators

Kurt Schmidt, Peter Klatt, and Bernd Mayer

A diminished relaxant response of atherosclerotic arteries to nitrovasodilators has been frequently observed in advanced stages of hypercholesterolemia. In the present study, we investigated whether this effect might be a result of reduced activity of smooth muscle guanylyl cyclase. Experimental atherosclerosis was induced by feeding rabbits a cholesterol-rich diet (1%) over a period of 4 months. Aortas were removed and homogenized, and guanylyl cyclase activity was measured in the 100 000g supernatants. Sodium nitroprusside, which stimulated cyclic GMP (cGMP) formation in control tissues almost 200-fold (from 3 to 585 pmol cGMP • mg"1 • min"1), increased enzyme activities in atherosclerotic aortas only ~90-fold (from 3 to 257 pmol cGMP • mg"1 • min"1). Similarly, the maximal stimulatory effects of S-nitrosoglutathione were reduced from 200-fold (controls) to 114-fold in atherosclerotic tissues. Basal guanylyl cyclase activities were identical in both atherosclerotic and control vessels. Hypercholesterolemia also reduced the activity of smooth muscle adenyl cyclase. In control aortas, basal and NaF-stimulated enzyme activities were 24 and 349 pmol cAMP • mg"1 • min"1, respectively, whereas cAMP formation was reduced in atherosclerotic aortas to 7 (basal) and 96 (NaF) pmol cAMP • mg"1 • min"1. The stimulatory effect of NaF (=14-fold) remained unchanged. Since adenyl and guanylyl cyclase have important functions in regulating vascular tone, reduced activities of both enzymes may contribute to the diminished relaxant and/or enhanced vasoconstricting effects of vasoactive compounds in atherosclerotic blood vessels. (Arteriosclerosis and Thrombosis 1993;13:1159-1163)

KEY WORDS - atherosclerosis • vascular smooth muscle • nitrovasodilators • soluble guanylyl cyclase

The pathogenesis of atherosclerosis is associated with various changes in the structure and properties of arterial blood vessels. Monocytes penetrate the endothelium, express scavenger receptors, and become tissue macrophages, which take up oxidatively modified low-density lipoprotein (Ox-LDL) and form foam cells and fatty streaks (for review see Reference 1). Depending on the degree of fatty streak formation, the dilatory response of such arteries to vasodilators is differentially affected. Whereas in the early stages of atherosclerosis, only endothelium-dependent relaxation is reduced,2-6 the endothelium-independent relaxation to nitrocompounds may additionally be impaired in more severely affected arteries.3,5,7,9 Since the relaxant effects of these nitrovasodilators (eg, nitroglycerin or nitric oxide [NO]) are caused by stimulation of soluble guanylyl cyclase,10 a diminished activity of this enzyme in atherosclerotic blood vessels may provide an explanation for the impaired relaxant response of these arteries. In previous reports, we demonstrated that Ox-LDL, which is found in atherosclerotic lesions but not in normal areas of human and rabbit arteries,11,12 reduces the responsiveness of soluble guanylyl cyclase to nitrovasodilators.13-15 Recently, it was shown that preincubation of isolated smooth muscle strips with Ox-LDL diminished not only the relaxant response to sodium nitroprusside (SNP) but also the SNP-induced accumulation of cyclic GMP (cGMP).16 To investigate whether such impaired response of guanylyl cyclase also occurs during hypercholesterolemia in vivo, we compared the activities of guanylyl cyclase obtained from aortas of cholesterol-fed rabbits with those obtained from control tissues. Since the regulation of vascular tone is also modulated by cyclic AMP (cAMP), we also measured adenyl cyclase activities in atherosclerotic and control aortas.

Methods

Materials

Stock solutions of S-nitrosoglutathione (GSNO) (10 mmol/L) were prepared by adding 0.1 mL of an aqueous solution of 0.1 mol/L NaNO₂ to 0.9 mL of a solution of 11.1 mmol/L glutathione in 0.01N HCl. These stock solutions were stable for several hours and were diluted with 0.01N HCl before the experiment. [α-32P]GTP and [α-32P]ATP were obtained from DuPont de Nemours, Bad Homburg, FRG. Buffer salts, biochemicals, and cholesterol were purchased from Sigma, Deisenhofen, FRG.
Animal Model of Atherosclerosis

Rabbits of either sex (1.5 to 2 kg) were randomly assigned to control and treatment groups (eight rabbits each). The control group was fed with a standard rabbit diet (120 g per day), and the treatment group received the same diet enriched with 1% cholesterol. The plasma levels of cholesterol in the control and treatment groups were measured after 4 weeks with a diagnostic kit from Sigma and were 84 ± 8 and 1944 ± 204 mg/dL, respectively. After 4 months, all rabbits were killed, and the thoracic aortas, including aortic arches, were removed from each animal. The complete intimal surface of the aortic arch and at least 80% of the thoracic aorta of all cholesterol-fed rabbits were covered by fatty streaks. In the aortas of control rabbits, no fatty streaks were visible. All aortas were carefully cleaned from connective tissues and immediately used for enzyme preparation.

Preparation of Guanylyl and Adenylyl Cyclase

Each aorta (1.2 to 2.1 g wet weight) was minced with scissors and homogenized in a threefold volume of a 50-mmol/L triethanolamine/HCl buffer (pH 7.4) containing 2 mmol/L dithiothreitol (DTT) and 0.5 mmol/L EDTA for 45 seconds with a microdismembrator (Braun, Melsungen, FRG). The homogenates were centrifuged for 60 minutes at 100 000g, and the supernatants were removed and stored in 0.5-mL aliquots at −70°C for measurement of guanylyl cyclase activity. The pellets were resuspended in 10 mL of homogenization buffer and centrifuged for 15 minutes at 10 000g. The supernatants were discarded, and the final pellets were resuspended in 1 to 2 mL of homogenization buffer and stored in 0.5-mL aliquots at −70°C for measurement of adenylyl cyclase activity. The protein concentration of each preparation was determined according to the method of Bradford and ranged between 1 and 2 mg/mL for the soluble fractions and between 2 and 3 mg/mL for the particulate fractions.

Guanylyl Cyclase Assay

Soluble guanylyl cyclase activity was measured as previously described. Briefly, 20-μL aliquots of the supernatants were incubated in the presence of 1 mmol/L MgCl₂ (or, if indicated, 1 mmol/L MnCl₂), 0.15 mmol/L [α-32P]GTP (=300 000 cpm), 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 1 mmol/L cGMP, 3 mmol/L DTT, 0.5 mg/mL bovine serum albumin, 5 mmol/L phosphocreatine, 10 U/mL creatine phosphokinase, and 50 mmol/L triethanolamine/HCl, pH 7.4, in a total volume of 0.1 mL for 15 minutes at 37°C. Supernatants were incubated in the presence of 10 mmol/L MgCl₂, 0.1 mmol/L [α-32P]ATP (=800 000 cpm), 1 mmol/L IBMX, 1 mmol/L cAMP, 0.5 mg/mL bovine serum albumin, 5 mmol/L phosphocreatine, 10 U/mL creatine phosphokinase, and 50 mmol/L triethanol-

Adenylyl Cyclase Assay

Adenylyl cyclase activity was measured as previously described. Briefly, 30-μL aliquots of the particulate fractions were incubated in the presence of 10 mmol/L MgCl₂, 0.1 mmol/L [α-32P]ATP (=800 000 cpm), 1 mmol/L IBMX, 1 mmol/L cAMP, 0.5 mg/mL bovine serum albumin, 5 mmol/L phosphocreatine, and 50 mmol/L triethanol-

<table>
<thead>
<tr>
<th>Addition</th>
<th>Control aortas</th>
<th>Atherosclerotic aortas</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>3±1.6</td>
<td>3±1.3</td>
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<tr>
<td>SNP (0.3 mmol/L)</td>
<td>585±77.4</td>
<td>257±27.3</td>
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<td>GSNO (0.3 mmol/L)</td>
<td>676±65.8</td>
<td>332±29.1</td>
<td>&lt;.01</td>
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</table>

Values are in pmol cyclic GMP·mg⁻¹·min⁻¹ (mean±SEM) (n=8).

Soluble guanylyl cyclase was assayed in the absence or presence of sodium nitroprusside (SNP) and S-nitrosothioglutathione (GSNO) as described under "Methods." Complete concentration-response curves are shown in Fig 1.

Statistics

The enzymatic activities of each preparation were derived from at least two identical experiments performed in triplicate, and mean values for every single preparation were calculated. Analysis of variance was used to test for differences between the mean values of preparations obtained from control and cholesterol-fed rabbits, and differences with values of P<.01 (Scheffe’s F test) were considered to be significant. Median effective concentration (EC₅₀) values were extrapolated from individual concentration-response curves and are expressed as geometric means with 95% confidence limits calculated as the product of SEM times Student’s t test values.

Results

Soluble guanylyl cyclase obtained from aortas of control rabbits showed a basal activity of 3±1.6 pmol cGMP·mg⁻¹·min⁻¹ and was stimulated with SNP almost 200-fold (585±77.4 pmol cGMP·mg⁻¹·min⁻¹). A similar stimulatory effect was observed with GSNO, which increased the enzyme activity up to 676±65.8 pmol cGMP·mg⁻¹·min⁻¹. Guanylyl cyclase isolated from aortas of cholesterol-fed rabbits was stimulated up to 257±27.3 and 332±29.1 pmol cGMP·mg⁻¹·min⁻¹ with SNP and GSNO, respectively. Basal activities, however, were not affected by the cholesterol-rich diet (Table 1). Since these activities were barely detectable (limit, 1 to 2 pmol cGMP·mg⁻¹·min⁻¹), we also measured basal guanylyl cyclase activities in the presence of Mn²⁺ instead of Mg²⁺ as the cosubstrate. Under these conditions, guanylyl cyclase activities were 87±9.6 and 82±6.8 pmol cGMP·mg⁻¹·min⁻¹ in control and atherosclerotic vessels, respectively (P>.5).

Although atherosclerosis reduced the maximal stimulatory effects of nitrovasodilators on soluble guanylyl cyclase, the potencies of these compounds were apparently not affected. As shown in Fig 1, enzymes obtained from control and atherosclerotic aortas were activated by SNP over a closely similar concentration range, with EC₅₀ values and 95% confidence limits of 32 (17 to 58)

TABLE 1. Guanylyl Cyclase Activities in Control and Atherosclerotic Aortas
μmol/L and 31 (23 to 41) μmol/L, respectively. Similar results were obtained when soluble guanylyl cyclase was stimulated with GSNO. The EC50 values derived from these experiments were 25 (19 to 33) μmol/L for the control enzyme and 23 (14 to 38) μmol/L for the enzyme obtained from atherosclerotic vessels.

In contrast to its effect on guanylyl cyclase, hypercholesterolemia did not reduce the stimulatory effects of vasodilators on adenylyl cyclase but diminished basal and stimulated enzyme activities to similar extents. As shown in Table 2, adenylyl cyclase obtained from the aortas of control rabbits showed a basal activity of 24±1.3 pmol cAMP·mg⁻¹·min⁻¹, which was increased with NaF up to 349±21 pmol cAMP·mg⁻¹·min⁻¹. Adenylyl cyclase activities from atherosclerotic arteries were 7±0.6 and 96±8.1 pmol cAMP·mg⁻¹·min⁻¹ in the absence and presence of NaF, respectively. Thus, cholesterol feeding reduced neither the stimulatory effects of NaF (=14-fold) nor, as shown in Fig 2, the concentrations of sodium fluoride (NaF) on adenylyl cyclase from control (open circles) and atherosclerotic (filled circles) aortas. Values represent mean±SEM (n=8).

There is evidence that only the endothelium-dependent relaxations are reduced in the early stages of atherosclerosis, whereas endothelium-independent relaxations induced by nitrovasodilators seem to be addition-ally impaired in later stages of the disease. This dysfunction of atherosclerotic arteries can be mimicked in vitro by preincubation of intact arterial strips with Ox-LDL, indicating that atherogenic lipoproteins may contribute to this process. As shown by recent findings, Ox-LDL may initially interact with the endothelium and reduce the biosynthesis of endothelium-derived relaxing factor. Later, when arteries become more severely affected, Ox-LDL may also interact with the smooth muscle and inhibit vascular relaxation either by potentiating the actions of vasoconstrictors or by antagonizing the effects of vasodilators.

Some reports in the literature demonstrate that atherosclerosis affects cyclic nucleotide levels in smooth muscle cells. In aortas of cholesterol-fed pigs, cAMP levels were about fivefold lower than in control aortas. Similarly, in primary cultures of vascular cells obtained from atherosclerotic human aortas, cAMP levels were twofold to eightfold lower than in control cells. In all these studies, the reduced cAMP levels correlated with enhanced protein biosynthesis. Furthermore, dibutyryl cAMP was reported to inhibit the enhanced proliferation rates that were observed in smooth muscle cells cultured from the intima of atherosclerotic arteries. These data indicate that reduced levels of cAMP may be responsible for the enhanced formation of the neointima during the progress of atherosclerosis. Since we found that adenylyl cyclase obtained from the aortas of cholesterol-fed rabbits was 3.5-fold less active than the enzyme from control animals, the reduced cAMP levels in atherosclerotic tissues are apparently the result of a diminished formation rather than an enhanced degradation of cAMP in the vessel wall. Similar results reported for the human aorta showed that adenylyl cyclase activities in fatty streaks and plaques were twofold to sixfold lower than in unaffected tissues. Since cholesteryl hemisuccinate was reported to reduce both

### TABLE 2. Adenylyl Cyclase Activities in Control and Atherosclerotic Aortas

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<tr>
<th>Addition</th>
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<th>Atherosclerotic aortas</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>24±1.3</td>
<td>7±0.6</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>NaF (10 mmol/L)</td>
<td>349±21.4</td>
<td>96±8.1</td>
<td>&lt;.01</td>
</tr>
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</table>

Values are in pmol cyclic AMP·mg⁻¹·min⁻¹ (mean±SEM) (n=8).

Adenylyl cyclase was assayed in the absence or presence of NaF as described under "Methods." Complete concentration-response curve is shown in Fig 2.
basal and NaF-stimulated adenyl cyclase activities in cell-free preparations of rat lung, enhanced contents of cholesterol may be responsible for the diminished formation of cAMP in atherosclerotic arteries. In addition to its effect on cAMP formation, hypercholesterolemia also markedly reduced the stimulation of smooth muscle guanylyl cyclase by nitrovasodilators. It could be argued that the reduced enzyme activities might result from the proliferation of the intima during atherosclerosis, so that the ratio of (normally active) guanylyl cyclase vs total protein is decreased. Several observations indicate, however, that this is not the case: (1) Basal, i.e., unstimulated, guanylyl cyclase activities were not affected by the cholesterol treatment of the animals; (2) comparable basal guanylyl cyclase activities were found in the intima and media of both atherosclerotic and normal blood vessels; and (3) the present findings are in good agreement with our previous data, demonstrating that Ox-LDL inhibits stimulation of soluble guanylyl cyclase by NO but not the basal formation of cGMP. Therefore, we suggest that enzyme stimulation by NO-containing compounds is reduced in atherosclerotic arteries because of an interaction of smooth muscle guanylyl cyclase with the Ox-LDL accumulating in the pathologically affected blood vessels. The mechanism by which NO stimulates soluble guanylyl cyclase is not fully understood; therefore, we can only speculate about how Ox-LDL might interfere with this process. It is currently thought that increased cGMP formation results from binding of NO to ferroporphyrin IX, a prosthetic group of soluble guanylyl cyclase. Accordingly, Ox-LDL could antagonize the interaction of NO with the enzyme by making the heme group less accessible to NO or by removing heme from its binding site at the enzyme. However, further work, above all the measurement of heme in Ox-LDL-treated soluble guanylyl cyclase, is necessary for definitive conclusions.

In summary, our findings may provide a conclusive explanation for the reduced responses of atherosclerotic blood vessels to nitrovasodilators, as has been observed in hyperlipidemic rabbits and in patients using isolated human arteries, and in patients with severe hypercholesterolemia.

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References


