Hypercholesterolemia and Atherosclerosis
Change Vascular Reactivity in Rabbits by Different Mechanisms

Jan Galle, Rudi Busse, and Eberhard Bassenge

Vasomotor reactivity was assessed in vitro in arterial segments obtained from rabbits with different stages of atherosclerosis. Rabbits were fed a standard chow diet (controls) or a cholesterol-enriched diet to induce hypercholesterolemia and atherosclerosis. A third group received the hydroxymethylglutaryl coenzyme A reductase inhibitor, lovastatin, simultaneously with the cholesterol diet. Contractile responses of thoracic aortas to norepinephrine, serotonin, and potassium-rich solution, as well as endothelium-dependent dilations to acetylcholine, were compared after 2 and 4 months on the respective diet. Additionally, plasma cholesterol levels and the amount of plaques covering the intimal surface (as a percentage of the intimal surface) were determined; transmission electron microscopy of atherosclerotic arteries was also performed. After 2 months, the only difference was an enhancement of contractile responses to serotonin in the cholesterol-fed versus the control group. After 4 months on the diet, contractile responses to serotonin were further enhanced, and norepinephrine- and potassium-induced vasoconstrictions were now also significantly enhanced in cholesterol-fed animals versus controls. Endothelium-dependent vasodilations were simultaneously reduced in cholesterol-fed animals. These alterations were partly prevented in cholesterol-fed and lovastatin-treated animals. Suppression of nitric oxide synthesis in control aortas by \( \mathrm{N}^\mathrm{G} \)-nitro-L-arginine did not reveal any significant increases in contractile responses. Contractile responses to serotonin were enhanced after 2 months on the diet but before the appearance of intimal plaques, whereas attenuation of endothelium-dependent dilations, as well as the further enhancement of contractile responses to serotonin and to other agonists, were closely correlated with the degree of intimal plaques after 4 months on the diet. The similarity of alterations in vascular reactivity after 4 months on the diet to the effects of isolated low density lipoproteins on vascular tone and the correlation of these changes with the degree of lipid-containing plaques support the hypothesis that lipoprotein accumulation in atherosclerotic arteries contributes to altered vascular reactivity. (Arteriosclerosis and Thrombosis 1991;11:1712–1718)

Numerous studies have shown that hypercholesterolemia is associated with the development of atherosclerosis.\(^1\)\(^2\) During this process various alterations in vascular reactivity have been observed, in particular, attenuation of endothelium-dependent vasodilations\(^3\)\(^–\)\(^5\) and increased responsiveness to different contractile agonists.\(^6\)\(^–\)\(^8\) The mechanisms responsible for the altered vascular reactivity are not fully understood. It is also not clear to what extent the observed alterations are related to hypercholesterolemia alone, to the progression of atherosclerosis, or to a combination of both. Possible explanations for the increased responsiveness to vasoconstrictors are a decreased release or efficacy of endothelium-derived relaxing factor (EDRF). However, in some studies attenuation of endothelium-dependent vasodilations preceded changes in contractile responsiveness,\(^9\)\(^,\)\(^10\) indicating that impaired endothelial function per se does not lead to enhanced vasoconstriction and that a mechanism independent of endothelial dysfunction may play a role. Recently, it has become apparent that native and oxidized low density lipoproteins increase the sensitivity of vascular smooth muscle to contractile agonists in vitro.\(^11\)\(^–\)\(^13\) There is now also evidence that these lipoproteins accumulate in atherosclerotic arteries,\(^14\)\(^–\)\(^16\) particularly in atherosclerotic plaques. Therefore, in this study we analyzed changes in the vascular...
reactivity of arteries obtained from hypercholesterolemic rabbits and determined whether these changes were correlated with the degree of plaque formation. Alterations in vascular reactivity similar to the effects of native and oxidized low density lipoproteins on isolated arteries would strengthen the hypothesis of a sensitization of the vasculature by lipoprotein accumulation during atherosclerosis.

Methods

Drugs

Acetylcholine (ACH), norepinephrine (NE), and serotonin (5-HT) were obtained from Sigma Chemical Co., Munich, FRG. Nω-Nitro-L-arginine (LNNAn) was purchased from SERVA, Heidelberg, FRG. Stock solutions of these drugs were further diluted in Tyrode’s solution. Lovastatin was provided by Merck Sharp & Dohme, Rahway, N.J., and given to the animals by mouth. For investigation of isolated aortic segments, lovastatin was dissolved in dimethyl sulfoxide and further diluted in Tyrode’s solution. Potassium (K⁺)-rich Tyrode’s solution was prepared by isomolar replacement of sodium by potassium.

Animals

Rabbits (8 weeks old, weighing 2.3 ±0.2 kg) were housed individually, and six animals were fed a standard rabbit chow diet (controls), a diet enriched with 0.5% cholesterol, or a cholesterol diet plus 25 mg lovastatin/day. Another group of six rabbits was fed standard chow or standard chow plus 25 mg lovastatin/day. The standard and the atherogenic chows were purchased from Altromin, Lage, FRG. Plasma cholesterol was determined by an enzymatic method (Boehringer Mannheim, Mannheim, FRG) at the beginning of the study, after 4 weeks on the respective diet, and when the animals were killed. Animals of the first group (n=18) were killed after 8 weeks and those of the second group (n=18), after 17 weeks. The animals of the control group (standard chow versus standard chow plus lovastatin) were investigated 10 weeks after the experiment was begun.

Vessel Preparation

Thoracic aortas were carefully removed, cleaned of connective tissue, and cut into segments about 2 cm long. These segments were mounted on steel cannulas, placed in organ baths, and perfused intraluminaly under isobaric conditions with oxygenated Tyrode’s solution. The outer vascular diameter of the segments was measured with a photoelectric device that permitted diameter measurements of opaque and transparent blood vessels within the bath solution. The resolution of the gauge was better than 0.5 μm.† Resting diameter of unstimulated segments was 4.3 ±0.2 mm. Full details of this experimental setup have been described elsewhere.18 Contractile agonists (5-HT, NE, and K⁺-rich Tyrode’s solution, in that order) were added to the intraluminal perfusion in cumulative doses. Contractile responses are expressed as percentage of resting diameter. Endothelium-dependent vasodilations, elicited by adding cumulative doses of ACh to 5-HT–preconstricted segments, are expressed as percentage of preconstriction. In another series with normocholesterolemic rabbits, we tested the effect of EDRF inhibition by LNNAn on contractile responses to NE, 5-HT, and K⁺ depolarization. Cumulative dose–response relations to these agonists were compared between segments preincubated with LNNAn (30 μM, 30 minutes, six animals) and control segments obtained from the same animals. Endothelium-dependent vasodilations to 1 μM ACh were 21 ± 4% in LNNAn-treated segments versus 74 ± 8% in the untreated segments.

Plaque Determination

After diameter recordings, the segments were opened longitudinally and placed on a glass plate for transillumination photography of atherosclerotic plaques. By this method, plaques appeared as black spots on a white background. The extent of surface covered by plaques was determined by placing a frame on the magnified photos and scanning the black spots in relation to the normal surface.

Electron Microscopy

Transmission electron microscopy was performed at the Anatomical Institute of the University of Freiburg after fixation of the aortic segments in glutaraldehyde.

Statistical Analysis

All data are presented as mean±SEM. Student’s t test for unpaired data was used to evaluate statistical significance of differences. For multiple comparisons of data, Bonferroni’s correction was applied. Linear correlation was calculated according to the least-squares method. A probability value less than 0.05 was considered statistically significant.

Results

Diet-Induced Increases in Plasma Cholesterol and Atherosclerotic Plaques

Plasma cholesterol levels of the animals at the start, after 4 weeks on the diet, and before investigation of vascular reactivity (at 8 or 17 weeks) are shown in Tables 1 and 2. Simultaneous treatment with orally administered lovastatin significantly reduced plasma cholesterol. The differences in the cholesterol levels between the 8-week group and the 17-week group at 4 weeks after the start of the diet might be due to differences in the susceptibility of the 8-week group and the 17-week group to the diet. Because plasma cholesterol levels had not reached steady-state levels at 4 weeks, the influence of these different susceptibilities on the rise of cholesterol levels is more evident at this time.

The degree of intimal plaque formation was obviously a function of both the cholesterol-feeding
duration and the absolute plasma cholesterol level. It was significantly lower in the lovastatin-treated animals (Table 2).

**Endothelium-Dependent Vasodilation**

After 8 weeks of cholesterol feeding, vasodilations to ACh were not impaired compared with those of controls (Figure 1, right panel). However, after 17 weeks on the diet, ACh induced vasodilations that were significantly impaired in the hypercholesterolemic animals. The attenuation was observed only in arteries with visible intimal plaques and was partly prevented by lovastatin treatment (Figure 1, left panel).

**Contractile Responses to Serotonin, Norepinephrine, and Potassium**

Contractile responses to cumulative doses of 5-HT, NE, and potassium (in this order) were assessed after 8 and 17 weeks on the respective diet. After 8 weeks, contractile responses to NE and potassium did not differ among the three groups. However, contractile responses to 5-HT were significantly enhanced in segments obtained from the cholesterol-fed animals compared with the control group (Figure 2) and were further enhanced after 17 weeks on the cholesterol diet. After 17 weeks on the diet, contractile responses to high doses of NE and potassium were also significantly enhanced versus those in the control group. Simultaneous treatment with the hydroxymethylglutaryl coenzyme A reductase inhibitor partially prevented this enhancement (Figure 3).

**Effect of Lovastatin on Contractile Responses**

To analyze whether lovastatin itself modified vascular responsiveness, we administered the drug to animals fed a standard diet for 10 weeks and compared the vascular responsiveness of segments obtained from these animals with controls. Contractile responsiveness of segments from lovastatin-fed rabbits to cumulative doses of NE, 5-HT, and K⁺-rich solution did not differ significantly from controls (12 segments obtained from three animals of the respective groups; data not shown).

In a further series of experiments, we added lovastatin (1 mg/ml) to the intraluminal perfusion of isolated arteries obtained from control rabbits and studied its influence on contractile responses to cumulative doses of NE and ACh. Vascular reactivity of the segments in the presence of lovastatin did not differ from vasomotor responses of segments without lovastatin treatment (seven segments, data not shown).

**Effect of N⁶-Nitro-L-Arginine on Contractile Responses**

To study whether attenuation of endothelium-dependent vasodilations in atherosclerotic segments was causative for the enhanced responsiveness to the contractile agonists, we performed control experiments with the specific EDRF inhibitor LNNN. Contractile responses to cumulative doses of NE, 5-HT, and K⁺ depolarization in segments preincubated with LNNN (30 μM, 30 minutes, six segments) were compared with those of control segments obtained from the same animals. Although endothelium-dependent vasodila-

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**TABLE 2. Plasma Cholesterol and Atherosclerotic Plaques (17-Week Diet Group)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control*</th>
<th>Cholesterol+lovastatin†</th>
<th>Cholesterol alone‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At start</td>
<td>42±4</td>
<td>46±5</td>
<td>48±6</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>46±7</td>
<td>440±57</td>
<td>695±45</td>
</tr>
<tr>
<td>17 Weeks</td>
<td>54±6</td>
<td>714±72</td>
<td>1,314±137</td>
</tr>
<tr>
<td>Plaques (% of intimal surface)</td>
<td>0</td>
<td>8±5</td>
<td>37±16</td>
</tr>
</tbody>
</table>

There were six animals in each group.

*Control animals were fed a standard rabbit chow diet.
†Lovastatin (25 mg/day), by mouth.
‡Diet enriched with 0.5% cholesterol.
Intimal Plaques Covering the Arterial Surface

All aortas of the animals on the diets for 8 weeks were free of plaques. In the group of animals on the diets for 17 weeks, plaques could be detected in five of six aortas from the animals fed cholesterol only and in two of six aortas from rabbits with simultaneous lovastatin treatment. Furthermore, in the lovastatin-treated group, plaques were less pronounced (Tables 1 and 2).

To evaluate whether the degree of plaque formation was correlated with the changes in vascular reactivity, ACh-induced vasodilation and contractile responses to each agonist were plotted against the percentage of intimal surface covered by plaques of the respective aorta. There was a close correlation between the degree of plaque formation and the attenuation of endothelium-dependent vasodilations as well as the increase in contractile responsiveness (Figure 5). Transmission electron microscopy of aortic segments from cholesterol-fed rabbits showed typical lipid droplets in the subendothelial space, both intracellularly and extracellularly (not shown).

Discussion

This study describes changes in vascular reactivity in arteries obtained from hypercholesterolemic rabbits. Dependent on the degree of atherosclerotic lesions of the arterial intima, contractile responses to NE, 5-HT, and K⁺ depolarization were enhanced and endothelium-dependent vasodilations to ACh were attenuated. Before the appearance of atherosclerotic lesions, the only change in vascular reactivity was an increased responsiveness to 5-HT. However, at a later stage contractile responses to 5-HT were further enhanced, and these were also correlated with plaque formation. Treatment with the hydroxymethylglutaryl coenzyme A reductase inhibi-
tor, lovastatin, lowered plasma cholesterol and the extent of atherosclerotic lesions and partly prevented the attenuation of endothelium-dependent vasodilations and the enhancement of contractile responses.

The finding that endothelium-dependent vasodilations were attenuated in arteries from cholesterol-fed rabbits is in agreement with previous studies. However, in contrast to some studies, in our model hypercholesterolemia alone did not cause impairment of endothelial function but occurred only in arteries with visible intimal thickening. Furthermore, the attenuation was closely correlated with the degree of these lesions. To explain this impairment, either decreased EDRF release from the endothelial cells or accelerated inactivation of EDRF must be considered. In a recent study with the same animal model, release of nitric oxide, which is likely to be identical with EDRF, from atherosclerotic arteries was not reduced, despite a loss of its vasodilator activity. This observation argues in favor of decreased efficacy of EDRF in some animal models. In fact, native and oxidized low density lipoproteins inactivate the labile EDRF in vitro. Because these lipoproteins accumulate in atherosclerotic plaques, an accelerated inactivation of EDRF by these lipoproteins is conceivable.

The enhancement of contractile responses to 5-HT in arteries from hypercholesterolemic rabbits is also in accordance with previous studies. It has been proposed that a loss of EDRF activity unmasks the vasoconstricting properties of 5-HT. However, because vasoconstrictions to 5-HT were enhanced before endothelium-dependent vasodilations were attenuated, a different mechanism seems to underlie this phenomenon. Furthermore, attenuation of en-

**Figure 3.** Contractile (contr.) response curves of rabbit aortas to cumulative doses [μM] of norepinephrine, serotonin [μM], and potassium-enriched [mM] solution after 17 weeks on the control, the cholesterol (chol.)-enriched, and the cholesterol plus lovastatin (lova.) diet. Responses are measured as percent diameter (D). Filled symbols indicate statistically significant difference vs. controls with p<0.05, n=18 (obtained from six animals in each group).

**Figure 4.** Contractile (contr.) response curves to cumulative doses [μM] of norepinephrine, serotonin [μM], and potassium-enriched [mM] solution of rabbit aortic segments preincubated with the stereospecific inhibitor of nitric oxide synthesis, N^G^-nitro-L-arginine (L-NNA). Endothelium-dependent vasodilations to acetylcholine (1 μM) were 21±4% in norepinephrine-preconstricted segments treated with L-NNA and 74±8% in untreated control segments. Responses are measured as percent diameter (D). n=6 paired segments, obtained from three rabbits.
Figure 5. Scatterplots of endothelium-dependent vasodilations (vasodil., as percent of preconstricted [precontr.] values) to 1 μM acetylcholine (left panel) and contractile (contr.) responses to 1 μM norepinephrine (right panel) plotted against percentage of intimal surface covered by atherosclerotic plaques in eight aortas from hypercholesterolemic rabbits. Vasodilations and contractile responses are mean±SEM of diameter (D) recordings in three or four segments of the respective aortas. Calculated correlation coefficients are $r=0.90$ ($p<0.01$) for acetylcholine, $r=0.90$ ($p<0.01$) for norepinephrine, and $r=0.75$ ($p<0.05$) for serotonin (not shown), and $r=0.69$ ($0.1>p>0.05$) for $K^+$ depolarization (not shown).

The most important finding was the enhancement of contractile responses to all agonists tested in control segments. On the other hand, a generalized sensitization of the vasculature in hypercholesterolemia before the onset of atherosclerosis is unlikely because in the animals without visible lesions, contractile responses to NE and $K^+$ depolarization remained unchanged. An increased expression of 5-HT receptors, as discussed, could explain the enhanced contractile responses at this early stage, but elucidation of this hypothesis was beyond the scope of this study.

The most important finding was the enhancement of contractile responses to all agonists tested in the atherosclerotic compared with the normocholesterolemic group. This observation is in contrast to a recent report. However, that study investigated animals of different age and strain, whereas in the present study animals of one strain and of the same age were used for comparison of vascular reactivity. This procedure may be important because contractile responsiveness decreases with the age of the animals, independent of diet. The enhanced responsiveness to the different contractile agonists raises the question of a more general mechanism than that responsible for the enhancement of contractile responses to 5-HT in the hypercholesterolemic group. When all segments of the hypercholesterolemic group were compared with controls, the enhancement was only significant at higher doses. However, the increase in responsiveness became striking when contractile responses were related to the degree of plaque formation. Thus, plaque formation was the crucial step for the more generalized alteration of vascular reactivity. Because attenuation of endothelium-dependent vasodilations was also correlated with the degree of plaque development, it should again be emphasized that inhibition of endothelium-dependent vasodilations with LNNA did not significantly enhance vasoconstrictions to the different vasoconstrictors. Thus, a mechanism different from impairment of endothelial function must be responsible. In cultured smooth muscle cells, incorporation of cholesterol into the cell membrane changes its fluidity and renders the vascular smooth muscle more sensitive to NE, a mechanism that could also play a role in the cholesterol-fed animals. Source for cholesterol could be the cholesterol-carrying lipoproteins, which accumulate in atherosclerotic plaques. These lipoproteins, in particular oxidatively modified low density lipoproteins and β-very low density lipoproteins, could also by themselves influence vascular tone because in vitro perfusion with oxidized low density lipoproteins potentiates vasoconstrictions. Evidence for the assumption of a sensitization of the vasculature in atherosclerotic arteries by accumulated oxidized lipoproteins is the finding that in these arteries contractile responses were enhanced to the same agonists that were used in the in vitro perfusion experiments.

Lovastatin lowered plasma cholesterol in the cholesterol-fed animals and partly prevented plaque formation, attenuation of endothelium-dependent vasodilations, and enhancement of contractile responses. In animals fed a standard diet, lovastatin had no influence on vascular reactivity. Therefore, the beneficial effect of lovastatin on the maintenance of normal vascular reactivity is likely to be due to the prevention of atherosclerotic plaque formation, which in this model seemed to be essential for provoking pronounced alterations of vascular reactivity and which was a function of both the absolute cholesterol level and the duration of hypercholesterolemia.

In summary, this study provides evidence that several different mechanisms are responsible for the alterations in vascular reactivity in experimental hypercholesterolemia and atherosclerosis in rabbits. We found 1) a selective enhancement of contractile responses to 5-HT, unrelated to visible atherosclerotic lesions or impairment of endothelial function; 2) a generalized increase of contractile responsiveness, closely related to the degree of plaque formation; and 3) an impairment of endothelial function, also closely related to the degree of plaque formation, but not the cause of an increased contractile responsiveness. Lowering plasma cholesterol with the hydroxymethylglutaryl coenzyme A reductase inhibitor, lovastatin, partly prevented atherosclerotic plaque formation and the alterations in vascular reactivity.

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References


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