Comparison of Early Microcirculatory and Aortic Changes in Hypercholesterolemic Rats

Dale A. Schuschke, Irving G. Joshua, and Frederick N. Miller

The microcirculatory changes caused by hypercholesterolemia were studied in the rat cremaster muscle model by intravital microscopy and were compared with aortic ring segments from the same animals. Male Sprague-Dawley rats were fed either a normal chow diet or a chow diet supplemented with 1% cholesterol and 0.5% cholic acid for 3 or 5 weeks before experimentation. Three weeks of hypercholesterolemia produced a significantly decreased vasodilator response to serotonin in the arterioles. This response was also seen after 5 weeks on the hypercholesterolemia diet. Three weeks of hypercholesterolemia produced a significantly increased macromolecular leakage from postcapillary venules in response to serotonin. However, after 5 weeks of hypercholesterolemia, the serotonin-induced leakage was less than in control animals. Hypercholesterolemia for 3 weeks decreased the arteriolar dilation evoked by acetylcholine but did not change the arteriolar response to sodium nitroprusside. Contraction of the aortic rings induced by serotonin and aortic ring relaxation induced by sodium nitroprusside were not different between 3-week-control and 3-week-hypercholesterolemic animals. However, 3 weeks of hypercholesterolemia attenuated the aortic ring relaxation evoked by acetylcholine. These results suggest that hypercholesterolemia causes an early depression of endothelium-derived relaxing factor (EDRF)-mediated receptor responses in both microvessels and the aorta, whereas non-EDRF-mediated receptor responses are altered in the microcirculation but not in the aorta. (Arteriosclerosis and Thrombosis 1991;11:154-160)

In several animal models, hypercholesterolemia has been shown to cause abnormal aortic and coronary artery reactivity preceding the onset of atherosclerotic lesions. Diet-induced hypercholesterolemia significantly increased maximal aortic responses to norepinephrine, histamine, and serotonin after 4 weeks of high-cholesterol feeding in rabbits. However, after 8 weeks on the hypercholesterolemia diet, these responses became attenuated as lipid deposition occurred in the artery walls. The response to norepinephrine in canine coronary arteries was potentiated by 4-5 weeks of a high-cholesterol diet. In contracted porcine coronary arteries, endothelium-dependent relaxations caused by serotonin were reduced by hypercholesterolemia, while endothelium-independent relaxations caused by sodium nitroprusside were unaffected. Endothelium-derived relaxing factor (EDRF) relaxation evoked by acetylcholine was also decreased in the aortas of nonatherosclerotic rabbits fed a high-cholesterol diet for 4 weeks.

While there is a large body of literature that addresses the in vitro responses of large-conduit arteries, there are relatively few studies that describe the effects of high serum cholesterol on the microcirculation, and no work has been done comparing large-artery and microvascular changes in the same animal. Recently, we have shown in the rat microcirculation that after 3 weeks on a diet supplemented with cholesterol, there is a reduction in microcirculatory reactivity in response to norepinephrine and in macromolecular leakage produced by histamine and by compound 48/80 (Sigma Chemical Co., St. Louis, Mo.).

The objective of the present study was to determine whether, during the time that there is an absence of histological evidence of atherosclerosis, hypercholesterolemia alters receptor- or nonreceptor-mediated responses in the microcirculation and aorta of rats. Responses to serotonin, acetylcholine, and sodium nitroprusside were studied in vivo by direct observations of the microcirculation of the cremaster muscle and in vitro by using isolated thoracic aorta rings.

Methods

Male weanling Sprague-Dawley rats were divided into control and experimental groups and housed...
individually. The control animals were fed Purina 5001 Laboratory Chow Meal, 12 g/day, Purina, St. Louis, Mo. The experimental animals' diet (12 g/day) consisted of the control diet to which 1% cholesterol had been added. Cholic acid (0.5%) was also added to enhance the intestinal absorption of the dietary cholesterol. Experimentation was done 3 and 5 weeks after initiation of the diets.6

Animals were prepared for in vivo microcirculatory observations by the following method. Anesthesia was induced with pentobarbital (50 mg/kg i.p.), and airway patency was maintained with a tracheal cannula. The carotid artery was cannulated, and the blood pressure was monitored with a polygraph (model 2200, Gould, Inc., Glen Burnie, Md.). Heart rate was calculated from the frequency of the pressure pulse. The skin of the right scrotum was opened and the cremaster muscle incised longitudinally, keeping the nerves and blood vessels to the muscle intact. The cremaster muscle was spread with sutures over a coverslip in the bottom of a specially designed Plexiglas bath. The bath was filled with 60 ml modified Krebs' solution, which was replaced every 15 minutes with fresh solution. pH (7.35–7.45) was maintained by bubbling nitrogen and carbon dioxide into the bath. An indwelling heater coil with a negative-feedback system was used to keep the bath at 35–36°C. Animals were placed on a heating pad to maintain rectal temperature at 35–37°C.

The animal and tissue bath were positioned on a modified stage of a Leitz fluorescence microscope, so that the cremaster muscle, which is approximately 200–250 μm thick, could be observed by transmitted light or fluorescence microscopy. A closed-circuit television system was used to monitor the experiments, which were recorded on videotape for later analysis. The magnification of the system was determined using a stage micrometer to allow for vessel diameter measurements. The camera system was adjusted to provide a uniform output for a known quantity of fluorescent standard. The emission intensity of fluorescein isothiocyanate tagged to bovine serum albumin (FITC-BSA) was used to assess macromolecular leakage. All animal experimentation was in compliance with institutional animal care and use committee guidelines.

After the surgical preparation and preceding each experiment, there was a 1-hour equilibration period. After equilibration, one of three experimental protocols was followed. The first protocol was performed to determine a dose–response curve to serotonin in six hypercholesterolemic and six control animals at 3 weeks after initiation of the diets. The same protocol was used in six hypercholesterolemic and six control animals after 5 weeks on the diet. FITC-BSA (0.2 ml/100 g) was injected intra-arterially and allowed to circulate for 30 minutes. Immediately before administration of serotonin, the diameter of a third-order arteriole (20–30 μm) was measured, and the interstitial fluorescence adjacent to a third-order venule (30–40 μm) was recorded. Successive concentrations of serotonin (10^-9 to 10^-3 M) were added to the tissue bath at 10-minute intervals. Higher concentrations caused vasoconstriction of the larger arterioles in the cremaster muscle and thus were not used in the current studies.7 Arteriolar diameters were measured each minute, and interstitial fluorescent intensity adjacent to the venule was recorded at 5, 7, and 10 minutes for each dose. Fluorescent intensity images were recorded during 10-second exposures to the blue light (2 J/cm² each). This light dose has no effect on macromolecular leakage in the cremaster muscle.8

The second experimental protocol compared the response of third-order arterioles (20–30 μm) of 3-week-hypercholesterolemic (n=5) and control (n=5) animals to two vasodilators. After the equilibration period, successive concentrations of acetylcholine (10^-7 to 10^-4 M) were added to the bath at 10-minute intervals, and arteriolar diameters were measured each minute. The cremaster muscle was then washed, and the bath was filled with fresh Krebs' solution. A 15-minute equilibration allowed the arterioles to return to basal diameters. A dose response to sodium nitroprusside was then determined by adding successive concentrations of sodium nitroprusside (10^-8 to 10^-4 M) to the bath at 5-minute intervals. The arteriolar diameters were measured each minute during the protocol.

The third protocol tested for changes in basal levels of EDRF caused by 3 weeks of hypercholesterolemia. The diameter of third-order arterioles (20–30 μm) in hypercholesterolemic animals (n=6) and controls (n=6) was measured before and after the addition of 5×10^-4 M hydroquinone9 to the tissue bath to determine the role of EDRF in the maintenance of vascular tone. At the end of all in vivo experimentation, a 5-ml blood sample was collected (no anticoagulant) via an intracardiac puncture and analyzed for total cholesterol (ASTRA Systems Cholesterol Chemistry, Beckman Instruments Inc., Brea, Calif.).

For analysis of macromolecular leakage, the fluorescent image was digitized by a PC VISION PC PLUS image analysis system, Woburn, Mass. The digitized image is composed of pixels of varying gray levels, depending on the light intensity (gray levels range from zero to 255, with zero representing a black image and 255 representing a white image). A single area of interest, which was adjacent to a venule, was used for analysis throughout the entire experiment. The mean pixel gray level in the area of interest was determined at each analysis time. The data are plotted as the mean±SEM fluorescent intensity units. A unit is the recorded gray level multiplied by a known quantity of fluorescent standard. The emission intensity of fluorescein isothiocyanate tagged to bovine serum albumin (FITC-BSA) was used to assess macromolecular leakage. All animal experimentation was in compliance with institutional animal care and use committee guidelines.

To perform isolated vascular ring studies, segments of the thoracic aorta from animals used in the
TABLE 1. Characteristics of Rats Fed Control and Cholesterol-
Supplemented Diets

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Week 3</th>
<th>HC Week 3</th>
<th>Control Week 5</th>
<th>HC Week 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>56±1</td>
<td>104±6*</td>
<td>56±2</td>
<td>115±8*</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>163±10</td>
<td>162±8</td>
<td>198±6</td>
<td>200±12</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>117±5</td>
<td>123±4</td>
<td>129±4</td>
<td>140±4</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>404±16</td>
<td>402±15</td>
<td>400±7</td>
<td>393±25</td>
</tr>
</tbody>
</table>

HC, hypercholesterolemic. All values are mean±SEM. *p<0.05 compared with control values.

in vivo studies (n=7 control and n=6 hypercholesterolemic animals) were excised and placed in a physiological salt solution (PSS) of the following composition (mM): NaCl 130, KCl 4.7, NaHCO 3 14.9, KH 2PO 4 1.17, CaCl 2 1.6, MgSO 4 0.7, dextrose 5.5, and Ca, Na 2EDTA 0.03. The vessel segments were cleaned of excess connective tissue and cut into 4-mm ring segments. Two wires were passed through the lumen, taking care not to damage the endothelium. After the rings were mounted in the bath, a passive tension of 3 g was applied to each ring, and isometric tension was measured with a force transducer. The PSS in the tissue bath was aerated with 95% O 2/5% CO 2 and maintained at a temperature of 37°C. The aortic rings were allowed to equilibrate for 60 minutes before the experiment.

The relaxation responses to either acetylcholine (10^-9 to 3×10^-5 M) or nitroprusside (10^-9 to 10^-5 M) were quantified in ring segments that had been preconstricted with norepinephrine (10^-6 M). These responses are reported as percent relaxation. The contractile responses to serotonin (10^-9 to 10^-4 M) were also determined and are reported as a percent of the constriction produced by 10^-6 M norepinephrine. In all studies, the bath concentrations of drug were increased threefold after a plateau in the constrictor or dilator response had been reached.

Statistical analysis of comparisons between groups was performed by Student's t test for unpaired data. Differences were considered significant at p<0.05.

Results

Rats fed the diet high in cholesterol and cholic acid for 3 and 5 weeks had serum cholesterol levels approximately two times higher than control rats for the same time periods (Table 1). Body weight, blood pressure, and heart rate were not affected by the dietary cholesterol (Table 1).

In the cremaster muscle preparation, successive additions of increasing concentrations of serotonin to the tissue bath caused arteriolar dilation in the 3- and 5-week-control animals (Figure 1), which were not different from each other. The same doses of serotonin had no significant effect on arteriolar diameters of 3- or 5-week-hypercholesterolemic animals (Figure 1). The difference in arteriolar diameters between control and hypercholesterolemic animals was significant at doses of 10^-3 M serotonin and greater.

The effects of serotonin on macromolecular leakage is shown in Figure 2. Serotonin (10^-6 M) caused significant venular macromolecular leakage in control and hypercholesterolemic groups 3 and 5 weeks after starting their respective diets. However, in the 3-week-hypercholesterolemic animals, 10^-6 and 10^-5 M serotonin caused greater leakage than in the control animals, while in the 5-week animals, the response in hypercholesterolemic animals was significantly attenuated in comparison to the control animals.

The aortic ring protocol, concentration-response curves to serotonin (Figure 3) demonstrated that no difference in the aortic contractile response to this agonist between hypercholesterolemic and control groups. There was also no difference in the aortic contractile response to 10^-6 M norepinephrine in control (1,214±114 mg) versus the 3-week-hypercholesterolemic (1,242±182 mg) rats.

The effect of successive additions of increasing concentrations of acetylcholine was to produce a concentration-dependent increase in arteriolar diameters in both 3-week-control and 3-week-hypercholesterolemic animals (Figure 4, upper panel). However, the dilatory response in the hypercholesterolemic group was significantly less than the response of the
control group at all but the lowest concentrations. After norepinephrine-induced contraction, receptor-mediated aortic ring dilation induced by acetylcholine was significantly reduced in the hypercholesterolemic animals (Figure 4). There appeared to be a shift of the concentration-response curve to the right in the hypercholesterolemic animals.

**The dose–response relation to sodium nitroprusside was tested to determine if the smooth muscle dilatory mechanisms of hypercholesterolemic animals had been altered.** Successive doses of sodium nitroprusside also caused a concentration-dependent increase in arteriolar diameter in both control and hypercholesterolemic groups. However, there were no differences in response at any concentration between the two groups (Figure 5, upper panel). Sodium nitroprusside also induced a concentration-dependent dilation of the aortic rings. However, this non–receptor-mediated dilation was not different between the two groups (Figure 5, lower panel).

The role of EDRF in regulating basal microvascular tone was determined by inhibiting EDRF with hydroquinone. In control animals, the diameter of third-order arterioles decreased, on average, by 40±6% (mean±SEM) from initial values 5 minutes after 5X10^-4 M hydroquinone was added to the tissue bath. This was not different from the mean arteriolar diameter decrease in the hypercholesterolemic group (38±5%). There was also no difference in the initial third-order arteriolar diameters between the groups before treatment with hydroqui-
FIGURE 5. Line plot showing effect of increasing concentrations (M x 10^{-7}) of nitroprusside on arteriolar diameter (% change, upper panel) and on aortic relaxation (% , lower panel) 3 weeks after initiation of control (•—•) or high-cholesterol (○—○) diet. Dilation is presented as percentage of the maximal response to 10^{-6} M norepinephrine.

Discussion

In the present studies of hypercholesterolemia, there are changes in the vascular reactivity that are unique to the small arterioles, while other changes occur in both arterioles and the aorta. In previous reports, hypercholesterolemic rat models have attained blood cholesterol levels as high as 1,000 mg/dl.12,13 In this study of 3-week- and 5-week-hypercholesterolemic animals, blood cholesterol concentrations were 104 ±6 and 115 ±8 mg/dl, respectively. Thus, the changes in vascular reactivity we have observed occur in animals whose cholesterol levels are in the moderate range (100-400 mg/dl) as described by Rogers and Karnovsky.12

In these hypercholesterolemic rats, we have demonstrated that the vasodilator microvascular responses to serotonin (Figure 1) change without concomitant changes in the aortic constrictor response to serotonin (Figure 3). We have previously reported that the microcirculatory constrictor response to norepinephrine is attenuated by 3 weeks of high-cholesterol feeding.6 However, in our current study with aortic rings, the 3-week hypercholesterolemic and control aortic rings constricted to the same degree in response to 10^{-6} M norepinephrine. The EDRF-dependent relaxation stimulated by acetylcholine14 is reduced in both the microcirculation and the aorta (Figure 3) after 3 weeks of the high-cholesterol diet. However, smooth muscle relaxation induced by sodium nitroprusside is not affected by hypercholesterolemia in either the microcirculation or the aorta (Figure 5). These vascular response changes in both the arterioles and the aorta all occur during the time in which no histological changes occur in this hypercholesterolemic model.6

The present data, when combined with our previous microvascular study showing decreased responses to norepinephrine, histamine, and mast cell degranulation in 3-week-hypercholesterolemic rats,6 suggest that receptor-mediated microvascular responses are attenuated by hypercholesterolemia. This attenuation of responses in the microcirculation by hypercholesterolemia includes receptor responses to acetylcholine (Figure 4, upper panel), which are EDRF mediated,14 and receptor responses to serotonin (Figures 1 and 2). Preliminary results in the rat cremaster muscle microcirculation suggest that serotonin-induced dilation does not involve EDRF mediation.15

In the current study, microvascular arteriolar dilation induced by serotonin is attenuated in both the 3- and 5-week-hypercholesterolemic animals (Figure 1). Macromolecular leakage induced by serotonin is augmented in 3-week-hypercholesterolemic animals but is attenuated by 5 weeks on the cholesterol diet (Figure 2). These microvascular changes in the rat occur in the absence of aortic reactivity changes (Figure 3), suggesting that the microvasculature is more sensitive to elevated levels of serum cholesterol. These changes in microvascular responses are likely caused by changes in sensitivity of the serotonin receptors. In other preparations, serotonin 5-HT_{1} receptors have been shown to cause vascular dilation,16 while 5-HT_{2} receptors cause vasoconstriction17 and mediate macromolecular leakage.18 The current results suggest that vasodilation is attenuated in the rat microcirculation very early in the development of hypercholesterolemia. At the same time, vasoconstriction and macromolecular leakage are potentiated initially but attenuated with time on the high-cholesterol diet. This sequence of changes is similar to the early increased constrictor response in rabbit aorta, which decreases with time as reported by Heric and Tackett,1 although their preparations had much higher serum cholesterol levels (1,600-2,100 mg/dl versus 104-115 mg/dl in the current study).

Several authors have attributed a loss of selective endothelial cell receptor-mediated relaxation in large arteries of pigs3,4 and rabbits5 to high serum cholesterol levels. Jayakody et al19 proposed that the impairment of endothelium-dependent relaxation may be due to several factors including impairment of the relaxation of medial smooth muscle cells and
the impairment of the synthesis, release, and/or diffusion of EDRF. Our studies of vascular smooth muscle cell relaxation with sodium nitroprusside would suggest that, at an early stage of hypercholesterolemia for both large arteries and the microcirculation from the same animals, the smooth muscle relaxation mechanism is intact (Figure 5).

Impairment of endothelium-dependent relaxation has also been shown to occur in vessels with regenerated endothelium. Since endothelial injury and regeneration are elements of the response-to-injury hypothesis, it is possible that this is a factor in the attenuated response to acetylcholine in both the microvasculature and the aorta. However, as we have reported earlier, there are no histological changes that occur in this model of hypercholesterolemia during the time that the in vivo experimentation was done. Therefore, it is likely that at this time, hypercholesterolemia causes a dysfunction at the receptor–endothelial level of mediation.

Previously, Shimokawa and Vanhoutte reported a depressed release of EDRF in hypercholesterolemic porcine coronary arteries. The current study demonstrates that EDRF-dependent relaxations in the rat aorta and dilation in the cremaster arterioles (Figure 4) produced by acetylcholine are depressed in the hypercholesterolemic rat. However, our testing of the role of EDRF in the regulation of microvascular tone would indicate that basal levels of EDRF are not affected by hypercholesterolemia, since hydroquinone caused the same percent contraction in control and hypercholesterolemic animals. Thus, our data for the microcirculation are consistent with microcirculatory data that suggest that there may be a decrease in EDRF release after stimulation.

Hypercholesterolemia attenuates the receptor-mediated microvascular responses of serotonin (Figures 1 and 2) in addition to norepinephrine and histamine as previously reported. These agonist-mediated responses do not involve EDRF, and the attenuation may be due to decreased membrane fluidity caused by the accumulation of cholesterol in the lipid bilayer. The decreased fluidity has been reported to decrease transmembrane transport and may restrict receptor protein movement within the membrane, producing a nonspecific depression of receptor stimulation.

In these current studies, diet-induced hypercholesterolemia in the rat caused both microvascular and large-artery dysfunction during a time when there were no histological changes associated with atherosclerosis. This dysfunction involves changes of endothelium-mediated responses without affecting vascular smooth muscle contraction or relaxation mechanisms. The progression of changes may appear first as a depressed EDRF receptor response, which manifests itself in both arterioles and in the aorta. Concurrently, our data suggest that there is also a non–EDRF-mediated receptor response, which at this early stage of hypercholesterolemia, is only present in the microcirculation. Whether the microcirculation is more susceptible to the perturbations that eventually lead to atherosclerotic lesions or whether microcirculatory dysfunctions have a role in the progression of atherogenesis have yet to be determined.

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