Effect of Induced Hypercholesterolemia in Rabbits on Functional Responses of Isolated Large Proximal and Small Distal Coronary Arteries

Ulf Simonsen, Dolores Prieto, Michael J. Mulvany, Eva Ehrnrooth, Niels Korsgaard, and Niels C.B. Nyborg

We studied the effects of hypercholesterolemia on the vascular responses of proximal and distal parts of the rabbit coronary circulation in two consecutive studies. For 12 weeks, New Zealand White rabbits were fed a control diet or a diet with 1% cholesterol dissolved in either 3% coconut oil (study A) or ether (study B). Isolated proximal epicardial and distal intramyocardial coronary arteries from control and hypercholesterolemic rabbits were mounted for isometric tension recording in a double myograph. In study A for hypercholesterolemic rabbits (n=12), the maximal relaxation and sensitivity to acetylcholine (ACH) were significantly decreased in proximal coronary segments contracted with 30 mmol/l potassium solution compared with segments from control rabbits (n=13). The only change observed in distal coronary segments was a slight decrease in relaxation in response to low ACh concentrations (10^-4 and 3x10^-6 mol/l). However, in study B for proximal coronary and distal coronary segments from hypercholesterolemic rabbits (n=13), the area under the ACh relaxation curve was increased compared with that of control rabbits (n=12). Other parameters that were similarly affected in studies A and B include: 1) proximal coronary segments from hypercholesterolemic rabbits were more sensitive to sodium nitroprusside (SNP) than were those from control rabbits, but this was not true for distal coronary segments; 2) endothelial removal from arterial segments of control rabbits induced a significant increase in sensitivity and maximal relaxation to SNP of proximal coronary and distal coronary arteries; 3) in segments from hypercholesterolemic rabbits, the absence of endothelium did not alter the response of proximal coronary segments to SNP but did augment the relaxation of distal coronary segments to SNP; 4) the maximal response to 5-hydroxytryptamine in proximal coronary arteries from hypercholesterolemic rabbits was increased compared with those from control rabbits, whereas such changes were not observed in distal coronary arteries; and 5) histological examination showed the presence of atheromatous plaques in proximal coronary but not in distal coronary segments from treated animals. In conclusion, the present investigation demonstrates that induced hypercholesterolemia alters both the structure and function of proximal parts of the coronary circulation. In distal coronary arteries of hypercholesterolemic rabbits, the only change observed was an impaired endothelium-dependent cholinergic relaxation, but even this change appeared to be dependent on the manner in which cholesterol was added to the diet, although parallel studies are required to confirm this. (Arteriosclerosis and Thrombosis 1992;12:380-392)

KEY WORDS • coronary arteries • in vitro • endothelium • acetylcholine • 5-hydroxytryptamine • hypercholesterolemia • rabbits

Atherosclerotic large coronary arteries in humans and in various hypercholesterolemic animal models of atherosclerosis exhibit increased vasoconstriction.1-5 The pathophysiological basis for the increased vasoconstrictor response of atherosclerotic blood vessels to certain agonists could be an increased number of serotoninergic and α-adrenergic receptors6 or an increased cholesterol content of smooth muscle cell membranes augmenting the response to norepinephrine (NE),7 calcium, and potassium.8 Furthermore, the vasorelaxant role of the endothelium as measured by the response to endothelium-dependent vasodilators is impaired in atherosclerotic large coronary arteries of humans9-11 and hypercholesterolemic pigs.12,13 Recent evidence has suggested that the functional consequences of atherosclerosis in large coronary arteries may extend into the microcirculation.14,15 However, it is not clear at present whether alterations in the small coronary vessels themselves could precipitate ischemia associated with atherosclerosis. First, although clinical studies have reported augmented vasoconstrictor re-
sponses and impaired vasodilator capacity in the presence of angio-architecturally normal large proximal coronary arteries. These functional changes might be associated with the presence of lesions in small coronary arteries caused by other diseases, e.g., endothelial-dependent dilation of the coronary microcirculation in response to ACh is impaired in dilated cardiomyopathy in humans. 

Second, the endothelium-dependent vasodilator effect of ACh in the perfused Langendorff heart preparation of hypercholesterolemic rabbits is converted to vasocostriction, and impaired vasodilation to ACh in the perfused hind limb and cremaster preparation of hypercholesterolemic rabbits is found. In direct contrast, others have reported unaltered endothelium-dependent vasodilation of the hind limb vasculature of the hypercholesterolemic rabbit and unaltered cholinergic vasodilatation of perfused kidneys of cholesterol-fed rabbits. The purpose of the present study was to investigate the effect of hypercholesterolemia on both large and small distal coronary arteries of rabbits by examining the influence of the endothelium on both vasodilator and vasoconstrictor responses and to determine if these responses were modulated or altered in cholesterol-fed rabbits. We chose to examine isolated proximal coronary (PC) and distal coronary (DC) arterial segments of the same rabbit in vitro, as perfusion studies do not permit separation of alterations in proximal and distal parts of the arterial circulation, and in vivo studies, increases in blood viscosity subsequent to high lipoprotein concentrations in plasma might alter the arterial blood flow and vascular responses. First, we performed one series of experiments in which we compared control rabbits to rabbits fed a 1% cholesterol-rich diet with the vehicle of 3% coconut oil (study A). However, we became aware that the above-mentioned conflicting results obtained in cholesterol-fed rabbits might be due to differences in diet composition, and a second series of experiments was subsequently performed (study B) to investigate whether modifications of the cholesterol-rich diet influenced the functional responses of the coronary circulation in cholesterol-fed rabbits. Furthermore, histological examination of the arteries was performed to see if the induced hypercholesterolemia was followed by atheromatous changes.

Methods

Animals

In a first series of experiments (study A), adult 24-week-old male New Zealand White rabbits, housed identically in individual cages, were fed either standard rabbit chow (n = 13) (Ewos, Södertalje, Sweden) or a 1% cholesterol-enriched diet (n = 12) for 12 weeks. The atherogenic diet was prepared every week by melting coconut oil (3%, wt/wt), dissolving the cholesterol at 140–150°C, and thoroughly mixing this with the standard chow pellets until they had absorbed the oil. The food was restricted to 120 and 110 g daily for the control and hypercholesterolemic groups, respectively, to obtain an isocaloric diet.

To avoid the influence of coconut oil, a second series of experiments (study B) was subsequently performed with New Zealand White rabbits of the same age. They were fed either a control diet (n = 12) or a cholesterol diet (n = 13), in which the cholesterol-rich diet was made by dissolving 1% cholesterol by weight in diethyl ether (Rathburn, 99% glass distilled), soaking the standard rabbit chow with this mixture, and allowing the ether to evaporate off under a hood. The food was restricted to 110 g in both groups. In both studies, the animals were provided with water ad libitum. The housing and experimental procedures were in accordance with Danish animal laws and regulations.

Blood samples for the determination of plasma cholesterol and triacylglycerol concentrations, which were measured enzymatically (CHOD-PAP and GPO-PAP high-performance methods, respectively; Boehringer Mannheim GmbH, Mannheim, FRG), were taken just before starting the cholesterol diet, after 6 weeks, and at the time the animals were killed. Heart rate was measured with a stethoscope while the animals rested quietly.

Tissue Preparation

After 12 weeks of receiving the respective diets, a control rabbit and a hypercholesterolemic rabbit were killed by cervical dislocation. The hearts were rapidly excised and placed in ice-cold physiological salt solution (PSS) to reduce cardiac metabolism and anoxia. Throughout the subsequent dissection, the hearts were bathed in cold PSS (4°C) of the following composition (mmol/l): NaCl 119, KCl 4.7, KH2PO4 1.18, MgSO4 1.17, CaCl2 2.5, EDTA 0.026, and glucose 5.5. The solution was gassed with 5% CO2 in 95% O2, to maintain pH at 7.4.

Segments (~2 mm long) of the proximal and distal parts of the left anterior descending coronary artery were dissected as previously described for rat PC and DC arteries. The vessels were subsequently mounted as ring preparations on two 40-μm wires in an isometric double myograph by fixing one of the wires to a force transducer and the second wire to a length-displacement device. All the experiments were performed with an artery from a cholesterol-fed rabbit and a corresponding segment from the respective control animal, thus allowing the two arteries to be tested and compared simultaneously.

The vessels were allowed to equilibrate in PSS at 37°C, pH 7.4, for about 30 minutes. The relation between resting wall tension and internal circumference was determined, and from this the internal circumference, L100, corresponding to a transmural pressure of 100 mm Hg for a relaxed vessel in situ, was calculated. The vessels were set to the internal circumference L1, given by L1 = 0.9 × L100. Preliminary experiments showed that at this internal circumference, the force production is close to maximal. The effective internal lumen diameter was determined as l1 = L1/π. The lumen diameter l1 of the proximal epicardial part of left descending coronary segments of control rabbits (studies A and B,
n=24) was 1,282±27 µm and of the distal intramyocardial coronary segments was 427±18 µm, compared with proximal segments for cholesterol-fed rabbits (n=25) of 1,372±44 µm (p=0.05 versus control; NS) and with DCs of 369±20 µm (p<0.05 versus control).

Protocol

The experiments with coronary arteries were initiated by activating the segments with control activating solution to check their mechanical condition. For this purpose, vessels were successively activated three times with 125 mmol/l K+PSS, which is PSS with KCl exchanged for NaCl on an equimolar basis. Tissue maximal force development, \( \delta T_{\text{max}} \), was obtained by stimulating the vessels with 125 mmol/l K+PSS containing 10^{-5} mol/l prostaglandin F_2 (PGF_2). Study A. In the first part of the experiments, the PCs and DCs were exposed to the same protocol. For determination of relaxation responses, the normal PSS was replaced by 30 mmol/l K+PSS. The bath volume was 14 ml, and when a plateau was reached, the following cumulative relaxation curves were made by adding aliquots of the relaxing agonist: 1) a concentration–response curve to ACh (10^{-2}–10^{-4} mol/l) and 2) a concentration–response curve to sodium nitroprusside (SNP; 10^{-5}–3x10^{-5} mol/l). In addition, cumulative concentration–response curves were made with 3) 5-hydroxytryptamine (serotonin [5-HT]; 10^{-4}–3x10^{-5} mol/l) and 4) NE (10^{-5}–3x10^{-5} mol/l) in the presence of 10^{-5} mol/l propranolol to block the \( \beta \)-receptors. The endothelium was removed from the PC segments by rubbing a horse hair back and forth across the lumen several times, and from the DC segments in the same manner, but by use of a thin human hair; the protocol was then repeated from steps 1–4.

In other experiments, instead of removing the endothelium, the vessels were contracted with 30 mmol/l K+PSS and concentration–relaxation curves were constructed in response to 5) isoproterenol (10^{-2}–10^{-4} mol/l), 6) NE in the presence of 10^{-5} mol/l propranolol and 10^{-6} mol/l prazosin, and 7) 5-HT in the presence of 10^{-5} mol/l ketanserin. Finally, the segments were fixed for histological studies as described below.

The preparations were washed by changing the bath solution several times with PSS, and the segments were allowed to equilibrate between each concentration–response curve. The order of concentration–relaxation curves was randomized to minimize the influence of nonpharmacological tissue alterations. Study B. The PC and DC arteries were exposed to a similar protocol as in study A. The vessels were contracted with 30 mmol/l K+PSS, and the following cumulative relaxation curves were made: 1) a concentration–response curve to ACh; 2) a concentration–response curve to SNP; 3) a concentration–response curve to isoproterenol; and 4) the thromboxane mimetic U46619 (3x10^{-7} mol/l) was added to the bath, and if the vessels contracted, a concentration–response curve to ACh was made. Cumulative contraction–response curves were constructed to 5) 5-HT; 6) NE in the presence of propranolol, and finally the vessels were contracted with 30 mmol/l K+PSS, and concentration–relaxation curves were made in response to 7) NE in the presence of propranolol and prazosin; and 8) 5-HT in the presence of ketanserin. The vessel segments were fixed for histological studies as described below.

**Drugs**

The following pharmacological agents were used: ACh (Fluka AG, Buchs SG), ADP (Sigma Chemical Co., St. Louis, Mo.), isoprenaline (Sigma), propranolol (Frekven, Ferrosan, Copenhagen, Denmark), NE HCl (Sigma), 5-HT creatinine sulfate complex (Sigma), SNP dihydrate (Merck), ketanserin (a gift from Janssen Pharmaceuticals), prazosin HCl (Sigma), and 9,11-methanoepoxy PGG_2 (U46619, Sigma).

**Histological Examination**

On completion of the mechanical experiments (n=8), the solution was changed to calcium-free PSS for 10 minutes to obtain complete relaxation. The vessels were then fixed for histology, while mounted on the myograph and still at internal circumference L_i, using 5% glutaraldehyde in Sørensen buffer adjusted to pH 7.4. The vessels were demounted and postfixed in glutaraldehyde. Furthermore, 3x3x2-mm blocks of myocardium were cut and fixed in glutaraldehyde. The vessels were preembedded in agar to maintain orientation, dehydrated by being placed in graded concentrations of ethanol, and embedded in historesin. Ten 5-µm transverse sections were made to obtain a general view of the structure in the segments. The sections were mounted on glass slides and stained with either Giemsa or hematoxylin and eosin for light microscopy.

**Data and Statistical Analysis**

The mechanical responses of the vessels were measured as force and expressed as active wall tension, \( \delta T \), which is the increase in measured force divided by twice the segment length. For each concentration–response curve, the concentration required to give a half-maximal response (EC_{50}) was determined by computerized iteration (GRAPHPAD Software, version 2.0, San Diego, Calif.) and fitting the responses and logarithmic concentrations to the Hill equation. EC_{50} values are expressed as the negative logarithm of the molar concentration, pD_2=−\log(EC_{50}). The responses to the relaxing agonists were normalized to the initial tone in the vessel induced with 30 mmol/l K+PSS or U46619, and the concentration to give a half-maximal response was calculated and expressed as pIC_{50} (i.e., the negative logarithm of the molar concentration). The results are expressed as mean±SEM (number of animals). The area under the concentration–response curve, or indicated parts thereof for each experiment (i.e., area in arbitrary values), was used for comparison. Significance of differences between the control and cholesterol-fed groups was assessed either by Student's two-tailed t test or by paired observations as indicated. Probability levels under 5% were considered significant.

**Results**

**General Parameters**

The hypercholesterolemia resulting from feeding rabbits a diet enriched with 1% cholesterol dissolved either in coconut oil (study A) or in ether (study B) was similar (Table 1). During the 12-week feeding period, the
Maximal Tissue Response and Contractile Responses of Coronary Segments to Serotonin, U46619, and Norepinephrine

The responses to vasoconstrictors were affected similarly by hypercholesterolemia in studies A and B and are therefore reported together (Table 2). The maximal active tension $\Delta T_{\text{max}}$ and the response to 125 mmol/l K+PSS of endothelium-intact PC segments from cholesterol-fed rabbits were significantly reduced compared with those of control rabbits, but this was not the case in DC segments (Table 2). The response of control PC arterial segments with endothelium and after endothelial removal in the same segments was not different. However, the procedure applied to remove the endothelium reduced the $\Delta T_{\text{max}}$ of control DC segments ($p<0.001$, paired t test; Table 2). Similarly, $\Delta T_{\text{max}}$ of PC segments from hypercholesterolemic rabbits remained unaffected by removal of the endothelium, while $\Delta T_{\text{max}}$ of DC segments was significantly diminished ($p<0.05$, paired t test; Table 2).

5-HT (10^-5–10^-3 mol/l) induced weak contractions in control PC segments (Table 2). Endothelial removal potentiated the maximum response to 5-HT of 10 control PC segments ($p<0.05$, paired t test). DC segments with (n=24) or without (n=9) endothelium did not evoke significant contractions to 5-HT (Table 2). In PC rings (n=5) and DC rings (n=5) incubated with ketanserin (10^-5 mol/l) and constricted with 30 mmol/l K+PSS, 5-HT (10^-6–3×10^-4 mol/l) only caused further constriction.

The concentration–response curve of PC arteries from hypercholesterolemic rabbits to 5-HT was bell shaped, with the maximum response reached at 10^-4 mol/l. Higher cumulative concentrations of 5-HT were followed by decreases in the response (Figure 1). The maximum response to 5-HT was increased about 10 times compared with segments of control rabbits (Figure 1, Table 2; $p<0.001$). Removal of the endothelium did not affect the response to 5-HT of PC segments (n=5) from hypercholesterolemic rabbits (maximum response, 78±8% of $\Delta T_{\text{max}}$ and pD2 of 6.82±0.13 with endothelium versus 68±11% of $\Delta T_{\text{max}}$ and pD2 of 6.49±0.09 in the same vessel without endothelium; $p>0.10$, paired t test).

### Table 1. Plasma Lipid and Other Parameters in Control and Hypercholesterolemic Rabbits

<table>
<thead>
<tr>
<th>Time/variable</th>
<th>Study A</th>
<th>Study B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Cholesterol-fed</td>
<td>Control</td>
</tr>
<tr>
<td>0 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPC (mmol/l)</td>
<td>1.9±0.3 (13)</td>
<td>2.4±0.7 (12)</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>0.76±0.08 (13)</td>
<td>0.75±0.07 (12)</td>
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<td>6 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPC (mmol/l)</td>
<td>1.0±0.1 (13)</td>
<td>59.1±5.5 (12)*</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>0.62±0.05 (13)</td>
<td>2.9±0.6 (12)*</td>
</tr>
<tr>
<td>12 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPC (mmol/l)</td>
<td>1.0±0.14 (13)</td>
<td>48.6±6.5 (12)*</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>0.72±0.07 (13)</td>
<td>5.0±1.4 (12)*</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>135±3 (13)</td>
<td>134±6 (12)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>3.6±0.4 (13)</td>
<td>3.6±0.6 (12)</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>0–12-Week exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPC (mmol/l)×day</td>
<td>102±12 (13)</td>
<td>3,554±335 (12)*</td>
</tr>
<tr>
<td>TG (mmol/l)×day</td>
<td>54.9±3.5 (13)</td>
<td>241±50 (12)*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. (n), Number of animals examined.

Total plasma cholesterol (TPC) and plasma triacylglycerol (TG) levels and body, heart, and liver weights of control and cholesterol-fed New Zealand White rabbits were measured at the beginning of the study (0 weeks), after 6 weeks, and at the time the animals were killed (12 weeks). TPC and TG exposures are expressed as areas under the plasma concentration–time curves determined over the 12-week period for each animal. The cholesterol-fed rabbits were fed a 1% cholesterol-rich diet for 12 weeks either supplemented by 3% coconut oil (study A) or dissolved in ether (study B).

*Statistically significant difference between control and hypercholesterolemic rabbits ($p<0.05$, Student’s t test for unpaired observations).
Potassium (30 mmol/l) contracted the vessels to a steady level of force. With this preconstriction, 10−8−10−4 mol/l ACh caused concentration-dependent relaxations in both PC and DC arteries from control rabbits with intact endothelium but not in those without endothelium (Figures 2a and 2b); at higher concentrations of ACh (3×10−6−10−4 mol/l), the arterial segments began relaxing, 18±5%; n=12, p>0.20 versus control), while NE did not induce relaxation in DC segments (n=11).

The maximum contractions to NE in PC segments from hypercholesterolemic rabbits tended to be increased compared with those of control rabbits (0.05<p<0.10), but as in control segments, NE did not evoke contractions in DC segments of hypercholesterolemic rabbits (Table 2). NE relaxed the PC segments of the hypercholesterolemic rabbits to the same degree as control segments (maximum relaxation, 18±5%; n=12, p>0.20 versus control), while DC segments (n=12) did not relax to NE.

**Responses of Proximal and Distal Coronary Arterial Segments to Acetylcholine, ADP, Nitroprusside, and Isoproterenol**

Potassium (30 mmol/l) contracted the vessels to a steady level of force. With this preconstriction, 10−8−10−4 mol/l ACh caused concentration-dependent relaxations in both PC and DC arteries from control rabbits with intact endothelium but not in those without endothelium (Figures 2a and 2b); at higher concentrations of ACh (3×10−6−10−4 mol/l), the arterial segments began to contract weakly (Figure 3). However, contractions to ACh were not observed in endothelium-intact segments contracted with the thromboxane mimetic U46619 (Figure 4). ACh (10−8−10−4 mol/l) relaxed both PC and DC segments of hypercholesterolemic rabbits (n=4) was 45±25% of ΔTmax versus 32±19% of ΔTmax (n=5) for cholesterol-fed rabbits (p<0.5).

After incubation and in the presence of the β-adrenergic receptor antagonist propranolol (10−5 mol/l), NE induced weak contractions of control PC segments but caused no significant contractions of DC segments with or without endothelium (Table 2). The contractions of PC segments (n=10) were enhanced after removal of the endothelium, although not significantly, compared with the segments with endothelium (0.05<p<0.10, paired t test; Table 2). PC segments (n=12) taken from control rabbits incubated with propranolol (10−5 mol/l) and the α1-adrenergic antagonist prazosin and contracted with 30 mmol/l K+PSS relaxed only weakly, 23±6%, to NE (10−8−10−4 mol/l), whereas NE did not induce relaxation in DC segments (n=11).

Three of 25 DC segments of hypercholesterolemic rabbits contracted to 5-HT with a maximum response close to ΔTmax, but overall the maximum response was not different from that of control segments (p>0.10; Table 2).

The concentration response to the thromboxane mimetic U46619 of both PC and DC segments was variable: either there was no increase in isometric force or there was contraction close to the maximum of the segments. Five of eight PC segments and two of eight DC segments contracted to single doses of 3×10−7 mol/l U46619. Variability was also seen in segments from hypercholesterolemic rabbits; six of eight PC segments and five of eight DC segments contracted to 3×10−7 mol/l U46619. Maximum response to a cumulative concentration of U46619 in DC segments of control rabbits (n=4) was 45±25% of ΔTmax versus 32±19% of ΔTmax (n=5) for cholesterol-fed rabbits (p<0.5).

FIGURE 1. Cumulative concentration–response curves to 5-hydroxytryptamine (serotonin; 5-HT) in proximal coronary arteries (panel a) and distal coronary arteries (panel b) obtained from control (C) and 12-week-hypercholesterolemic (O) rabbits. Responses are relative as a percentage of the maximal tissue active tension obtained with 125 mmol/l potassium physiological salt solution and 10−5 M prostaglandin F2α. Each point is the mean of 24–25 coronary segments±SEM. 5-HT induced potent contractions in atheromatous proximal coronary arteries but not in distal coronary arteries of hypercholesterolemic rabbits or in coronary segments of control rabbits.

### TABLE 2. Evaluation of Contractile Responses

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hypercholesterolemic</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>DC</td>
<td>PC</td>
</tr>
<tr>
<td><strong>Intact endothelium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTmax (N/m)</td>
<td>8.3±0.6 (24)</td>
<td>5.2±0.4 (24)</td>
</tr>
<tr>
<td>K+PSS (N/m)</td>
<td>6.2±0.6 (24)</td>
<td>4.2±0.3 (24)</td>
</tr>
<tr>
<td>5-HT (%)</td>
<td>5.4±1.6 (24)</td>
<td>5.7±3.4 (24)</td>
</tr>
<tr>
<td>NE (%)</td>
<td>3.8±1.5 (24)</td>
<td>0.6±0.2 (24)</td>
</tr>
<tr>
<td><strong>Without endothelium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔTmax (N/m)</td>
<td>7.9±0.9 (10)</td>
<td>3.3±0.5 (9)†</td>
</tr>
<tr>
<td>5-HT (%)</td>
<td>10.7±2.4 (10)†</td>
<td>2.2±0.9 (9)</td>
</tr>
<tr>
<td>NE (%)</td>
<td>6.9±2.5 (10)</td>
<td>1.8±1.7 (8)</td>
</tr>
</tbody>
</table>

Values are geometric mean±SEM. (n), Number of vessel segments (one per animal).

The maximal response, ΔTmax, of rabbit proximal coronary (PC) and distal coronary (DC) arterial segments to 125 mmol/l potassium physiological salt solution (K+PSS) with 10−5 M prostaglandin F2α added and the relative maximal response (in percent of ΔTmax) to serotonin (5-hydroxytryptamine; 5-HT) and norepinephrine (NE). Responses of all endothelium-intact segments examined and responses of arterial segments after endothelial removal are included.

*Statistically significant difference between control and hypercholesterolemic rabbits (p<0.05, Student's t test for unpaired observations).

†Statistically significant difference between response of the vessel segment with intact endothelium and the same segment without endothelium (p<0.05, paired t test).
segments precontracted with U46619 to a larger degree (maximum relaxation of PC segments, 94.4±2.0%; n=6) than the same segments precontracted with 30 mmol/l potassium (maximum relaxation of PCs, 57.8±9.7%; n=6, p<0.01, paired t test; see Figures 3 and 4).

Study A: Rabbits fed a cholesterol-rich diet with vehicle

Study B: Rabbits fed a pure 1% cholesterol–enriched diet. In coronary arteries of the hypercholesterolemic rabbits, the response to ACh was reduced in PC segments precontracted either with 30 mmol/l potassium (Figure 3) or with U46619 (Figure 4) compared with control rabbits (Table 3). The whole area under the concentration–response curves (Figure 3) of PC and DC segments (study B) to ACh was significantly increased compared with control (p<0.01 and p<0.005, respectively; Figure 3). The maximum relaxation to ACh was attenuated in DC segments, while the sensitivity and pIC50 were not different from control segments (Table 2). There was variability in the endothelium-dependent response to ACh of the small coronary arteries of the hypercholesterolemic rabbits (Figure 6). ADP (10^{-9}-3×10^{-5} mol/l) evoked weak but significant relaxations of PC segments (maximum relaxation, 18±9.0%; n=4, p<0.05) from control rabbits and relaxed the DC segments to a larger degree (maximum relaxation, 47±6%; n=4, p<0.05 versus PC segments). PC segments from hypercholesterolemic rabbits did not relax to ADP (maximum relaxation, 5±3%; n=5, p>0.10), while relaxations to ADP of DC segments were not different from control rabbits (Table 3).

Similar to the results of study A, PC segments from hypercholesterolemic rabbits were more sensitive to SNP than those of control rabbits, although the segments relaxed to the same degree (Table 3). DC segments of cholesterol-fed rabbits relaxed like segments from control animals (Table 3).

Histological Studies of Coronary Segments

In all transverse sections of PC segments fixed in the myograph (n=13) or sections of the heart from hyper-
Increased Vasosconstriction to Serotonin in Large Atheromatous Proximal Coronary Arteries of Hypercholesterolemic Rabbits

All PCs of hypercholesterolemic rabbits examined in the present study exhibited an increased response to 5-HT. This agrees with earlier studies of large coronary arteries of hypercholesterolemic rabbits and the coronary circulation of atherosclerotic nonhuman primates. The abnormal response to 5-HT of the PCs could be associated with endothelial dysfunction, in the form of either impaired release of EDRF or release of a constricting factor from the endothelium. In normal PC segments, we found that endothelial removal increased the maximum response to 5-HT, which is consistent with responses in porcine and canine PCs. This suggests that the contractile response to 5-HT of PCs with intact endothelium might be reduced either by basal EDRF release or by 5-HT-like receptors mediating relaxation through the endothelium. The latter mechanism seems not to be important in rabbit arteries, because in vitro we found that 5-HT had no relaxing effect on arteries contracted in the presence of ketanserin. Furthermore, we found that the abnormal constriction of the PCs from hypercholesterolemic rabbits persisted after removal of the endothelium, indicating that it was not due to release of a constricting factor from the endothelium (Table 2). The increased maximum response to 5-HT seems to be receptor specific, as responses of the rabbit coronary vessels to NE remained unaffected by the presence of atheromatous lesions.

Discussion

There are three major findings of the present in vitro investigation. First, large PCs were affected more than DCs of the same cholesterol-fed rabbits. PCs of hypercholesterolemic rabbits exhibited increased vasoconstrictor response to 5-HT, while DCs did not react to 5-HT. Endothelial dysfunction of the atherosclerotic PCs is reflected by impaired relaxation to ACh and reduced basal release of a factor, probably endothelium-derived relaxing factor (EDRF), inhibiting the relaxation to SNP. In contrast, the effect of endothelial function in DCs was confined to the agonist-stimulated endothelium-dependent relaxation. Second, the data suggest that even small modifications of the cholesterol-rich diet may influence the impairment of endothelial function in DCs. Third, endothelin-dependent relaxation of DCs seems to be more susceptible to the effects of exposure to high plasma cholesterol concentrations than do arteries in the systemic circulation of our cholesterol-fed rabbit model.29

FIGURE 4. Plot of endothelium-dependent relaxation to acetylcholine (ACh) of large segments from control (○) and hypercholesterolemic (●) rabbits contracted with the thromboxane mimetic U46619 (3×10−7 mol/l). Area under the ACh curve is significantly larger for arterial segments of hypercholesterolemic compared with control rabbits (p<0.001, n=5–6, Student’s t test for unpaired observations).

FIGURE 3. Plots of endothelium-dependent relaxation of potassium-contracted coronary artery segments to acetylcholine (ACh). Study A: Proximal coronary artery segments (panel a) and distal coronary arteries (panel b) from control rabbits (○) and rabbits fed a cholesterol-rich diet with the vehicle of ether (●). Areas under the curves to ACh (10−9–10−6 mol/l) are similar (p<0.10), but areas under the curves to ACh (10−4–10−3 mol/l) were significantly different for proximal coronary arteries (p<0.001, hypercholesterolemic vs. control); in distal coronary arteries, response was significantly affected at low ACh concentrations as indicated by asterisks (p<0.05, Student’s t test for unpaired observations). Study B: Proximal (panel c) and distal (panel d) coronary arteries from control rabbits (○) and rabbits fed a cholesterol-enriched diet with the vehicle of ether (●). Areas under ACh curves are significantly larger for large and small coronary arteries of hypercholesterolemic compared with control rabbits (p<0.01 and p<0.005, respectively; Student’s t test for unpaired observations). Results are expressed as mean±SEM of preparations from 12–13 rabbits.

Cholesterol-enriched diet with the vehicle of ether (●).
addition to being ascribed to endothelial dysfunction, the abnormal response to 5-HT may be due to an increased number of 5-HT receptors, as reported from radioligand studies of atherosclerotic large arteries,6 or increased efficacy of the 5-HT receptor excitation–contraction coupling process in the smooth muscle layer.

The present investigation indicates that hyperresponsiveness to 5-HT in PCs of the hypercholesterolemic rabbits probably is associated with the presence of atherosclerotic lesions. In pigs and dogs subjected to local endothelial denudation and concomitant long-term high-cholesterol feeding to induce atherosclerosis, administration of 5-HT resulted in locally enhanced coronary constriction,2-3 while the contractions to 5-HT in PCs of hypercholesterolemic rabbits and pigs with no detectable atherosclerotic lesions were not altered.4-12 Furthermore, in contrast to the PCs, the responses to 5-HT of unlesioned DCs of the hypercholesterolemic rabbits were not altered compared with control preparations in the present investigation. This suggests that high plasma cholesterol does not alter the 5-HT receptors of DCs. However, in atherosclerotic nonhuman primates, infused 5-HT produced a significant increase not only in large-artery but also in microvascular resistance in the coronary circulation.15 These differences may be due to development of distal collaterals hyperresponsive to 5-HT subsequent to atherosclerosis in the large arteries,19 to species differences, or to different methodology. Our results suggest that few contractile 5-HT receptors are present in DCs. First, we observed no increase in response to 5-HT after removal of the endothelium of DCs. Second, other studies suggest that 5-HT evokes much larger responses in PCs than in DCs.3,40 Also, human DCs do not contract to 5-HT in vitro.41

Thus, the increased response in the coronary circulation to 5-HT appears to be related to endothelial dysfunction, altered response at the vascular smooth muscle level, and the presence of atheromatous lesions in PCs.

**Impaired Endothelial Function in Large Proximal Coronary Arteries of Hypercholesterolemic Rabbits**

The relaxation to Ach is endothelium dependent in both PCs and DCs (Figure 2) and may be mediated by the release of EDRF, endothelium-derived hyperpolarizing factor, release of PGs (prostacyclin or PGE2) from the endothelium, or direct electric coupling between endothelium and smooth muscle cells.42

In both parts of this study, the endothelium-dependent relaxation of the PC segments of hypercholesterolemic rabbits was reduced compared with control segments. This agrees with earlier studies showing im-
paired endothelium-dependent relaxation of large atherosclerotic coronary arteries from rabbits,\textsuperscript{5,28} pigs,\textsuperscript{13} and humans.\textsuperscript{10} Several mechanisms underlying abnormal endothelium-dependent responses of large atherosclerotic vessels have been proposed. First, hypercholesterolemia per se may alter endothelium-dependent response. The relaxation induced by ACh in angiographically normal human coronary arteries in vivo recently was shown to correlate inversely with the serum cholesterol concentration,\textsuperscript{43} and incubation with natural low density lipoprotein blocked endothelium-dependent relaxation in the normal rabbit aorta.\textsuperscript{44} Second, the impaired response to ACh might be caused by mechanical removal of the endothelium (Figure 2), but in the present study, the endothelial layer of all the coronary segments first mounted for functional studies and then examined by light microscopy was intact. Third, direct bioassay studies in hypercholesterolemic and atherosclerotic rabbit aortas do not support an impairment of signal transduction within the vascular endothelium but suggest that the impaired vasodilator activity of EDRF may result from loss of incorporation of nitric oxide into a more potent parent compound or accelerated degradation of EDRF.\textsuperscript{45} Large subendothelial thickenings do not in themselves appear to inhibit the EDRF-mediated relaxation,\textsuperscript{46} but one might speculate that foam cells and lipid inclusions in intimal lesions might contain modified lipoproteins\textsuperscript{47} capable of inhibiting endothelium-dependent cholinergic relaxation.\textsuperscript{48–50} Lastly, the reduced endothelium-dependent vasodilation may be due to a decreased response at the level of the vascular smooth muscle layer, but we found that SNP relaxed atheromatous coronary arteries to an even larger degree than control segments (Figure 5).

In contrast with these findings, earlier studies of humans have demonstrated a progressively decreased response to nitrovasodilators of large coronary arteries with severe atherosclerosis.\textsuperscript{9} These discrepancies could be explained by assuming an impairment of endothelial function as an initial phase in the development of atherosclerosis, as shown in our experimental rabbit model, whereas in advanced severe atherosclerosis in rabbits and humans, where the vascular smooth muscle is affected, the relaxations to several endothelium-independent vasodilators are diminished.\textsuperscript{5,9}

The endothelium-dependent relaxation in PC segments of study A was affected more than the DCs of the same hypercholesterolemic rabbits (Figure 3). Similarly, it has been reported that the relaxation to ACh and substance P of DCs from 5-month-cholesterol-fed rabbits with impaired relaxation to ACh of the proximal epicardial large arteries was unaffected compared with control rabbits.\textsuperscript{28} In both our studies and the study by Angus and colleagues,\textsuperscript{28} a concentric neointima filled with extracellular fat and foam cells formed in the PCs, while there was no evidence of atheromatous plaque formation or intimal thickening of the small arteries of

**FIGURE 5.** Response curves to sodium nitroprusside (SNP) of rabbit coronary arterial segments contracted with 30 mmol/l potassium physiological salt solution. Response to SNP of proximal (panel a) and distal (panel b) coronary segments with intact endothelium (——) from control rabbits and the response to SNP after removal of the endothelium (—) are shown. Endothelium-denuded proximal and distal segments are more sensitive to the relaxation of SNP compared with endothelium-intact segments, as indicated by significantly smaller areas under the curve (p<0.01 and p<0.005, respectively; n=9–10, Student’s t test for paired observations). This suggests that a factor inhibiting the response to SNP is released from the endothelium of intact segments. Proximal (panel c) and distal (panel d) coronary arteries with intact endothelium from control (○) and hypercholesterolemic (●) rabbits in both studies (A and B). The area under the curve for proximal coronary segments is significantly less for hypercholesterolemic compared with control rabbits (p<0.001, Student’s t test for unpaired observations), suggesting impairment of the release of the inhibiting factor in proximal but not distal arteries of hypercholesterolemic rabbits. Each point is mean±SEM of 24–25 rabbits. Response to SNP of proximal (panel e) and distal (panel f) endothelium-intact coronary segments from hypercholesterolemic rabbits and after removal of the endothelium (—) is shown. Response of proximal segments to SNP is unaltered by endothelial removal, whereas endothelium-denuded distal segments were more sensitive compared with endothelium-intact segments (p<0.05, Student’s t test for paired observations, n=5–7).
Coronary Arteries of Cholesterol-Fed Rabbits

(a) ACh-9

5mN

W

SmN

U46619

ICPSS U46619

-Smln

the cholesterol-fed rabbits. Atheromatous lesions of large coronary arteries may be a sufficient barrier to trap and destroy EDRF released from an obviously intact endothelium.

Impaired Release From the Endothelium of a Factor Inhibiting Relaxations to Sodium Nitroprusside in Coronary Segments

In PC and DC segments of normal rabbits, endothelial removal augmented relaxations induced by SNP, which might indicate that the endothelium releases a factor, probably basally released EDRF, that inhibits the direct relaxing effect of SNP on the smooth muscle cells (Figure 5). Mechanical removal of the endothelium could have damaged the smooth muscle layer, but this is an unlikely explanation, as the contractions to potassium (taken as an indicator of the contractile activity of the vessels) were not significantly different in the large segments before and after endothelial removal. Furthermore, other studies have obtained similar results in the aorta and small mesenteric arteries of the rat.

The relaxations to SNP of the endothelium-intact atherosclerotic PCs were enhanced compared with control preparations, and a decreased preconstricting tone as a cause of this increased sensitivity is unlikely because the response to other vasodilators such as isoproterenol was not changed. Endothelial removal did not augment the response to SNP of the atherosclerotic PC segments, the relaxation to SNP of the endothelium-denuded segments of hypercholesterolemic and control rabbits not being different. This fact suggests that the basally released EDRF inhibiting the SNP-induced relaxations is reduced in rabbit atheromatous large coronary arteries. Interestingly, arteries of hypertensive animals also exhibit enhanced response to SNP.

In contrast to the effect on PCs, SNP relaxed the DC segments of hypercholesterolemic rabbits similar to control segments, in agreement with other studies of the response to nitrovasodilators in small coronary arteries from cholesterol-fed animals. Endothelial removal in segments of treated animals enhanced the relaxation to SNP as it did in control animals (Figure 5), thus indicating that the basally released EDRF inhibiting the relaxation of the small coronary arteries to SNP was not affected by the hypercholesterolemia.

Our study indicates that endothelial dysfunction of the atherosclerotic large PCs is reflected in two ways. First, the agonist-stimulated release to ACh of a relaxing factor is impaired, and second, the basal release of EDRF inhibiting the relaxation of SNP is reduced. In contrast, the effect of endothelial function in the small coronary arteries is probably confined to the agonist-stimulated endothelium-dependent relaxation.

Evidence for Diet-Dependent Effect of Endothelium-Mediated Relaxation in Coronary Resistance Arteries of Hypercholesterolemic Rabbits

In the first part of the study (study A), the endothelium-dependent relaxation to ACh was only affected at
low concentrations of ACh, while in the second part (study B), the area under the concentration–response curves was significantly altered compared with that of the control group (Figures 2, 3, and 6). The only difference between the two parts of the study is the manner in which we diluted the cholesterol in the standard diet composition. There was no difference in total plasma cholesterol or triacylglycerol concentration at the time the animals were killed, but the arterial system in the cholesterol-fed rabbits of study A was exposed to a higher plasma cholesterol concentration than that of study B during the feeding period (Table 1). Similar to our study, others found that addition to the diet of cholesterol in an evaporable solvent without the use of additional fat resulted in a more atherogenic hypercholesterolemia than that produced when oils were used for the addition of cholesterol, although the plasma cholesterol level was higher in the group that received the additional fat.\(^{53}\) Hypertriglyceridemia is accompanied by larger lipoprotein particles that reduce the influx of lipoprotein into the arterial wall,\(^{54}\) and in study A of the present investigation, there was a more rapid rise in triacylglycerol compared with that in study B (Table 1). We have previously suggested that this might explain why even small diet modifications influence the impairment of endothelial function in small arteries. However, this is an unlikely explanation, as the triacylglycerol to cholesterol ratio was the same in studies A and B. Furthermore, others found that the distribution of cholesterol between the plasma lipoproteins in the cholesterol-fed rabbits fed additional fat was similar to the rabbits fed a diet where cholesterol was added by use of ether.\(^{53}\) Although speculative, the use of the vehicles in the present studies may have affected the levels of lipid peroxides present in the hypercholesterolemic rabbits, which have been reported to reduce PG and EDRF release in large arter
dies.\(^{49,50,55}\) Our results give some evidence that even small diet modifications might influence the effect on endothelium-dependent relaxation in hypercholesterolemic rabbits and might explain the contrasting results between studies that have reported either unaltered\(^{25,26}\) or impaired\(^{25,26}\) endothelium-dependent relaxation of DCs. However, it should be emphasized that studies A and B were performed consecutively, and examination of the tables shows some differences in the control values obtained in each study. Therefore, confirmation of this apparent effect of diet on the vascular effects of hypercholesterolemia will require a direct comparison of the diets in parallel studies.

Are Impaired Endothelium-Dependent Relaxations in Small Distal Coronary Arteries Due to Extension of Atherosclerosis?

We did not find any alterations in the vessel wall of the small coronary arteries of the hypercholesterolemic compared with control animals, and an atheromatous intimal barrier in the large coronary arteries cannot explain the reduced relaxation to ACh of the DCs of hypercholesterolemic rabbits in study B. Observations in other studies, however, suggest that a blunted endothelium-dependent response of small coronary arteries from hypercholesterolemic animals could be an early manifestation of atherosclerosis, as in porcine large coronary arteries.\(^{12,13}\) First, Osborne and colleagues\(^{26}\) showed with electron microscopy that some small coronary arteries with impaired relaxation to ACh from hypercholesterolemic rabbits did not show intimal proliferation, although fatty streaks were present at bifurcation points and occasionally in the linear unbranched segments of the coronary arteries. Furthermore, transmission electron microscopy of primate small coronary arteries with impaired endothelium-dependent response showed foam cells and vacuoles, representing lipid droplets within the endothelium.\(^{27}\) Second, these submicroscopic alterations found in other studies were shown to precede the development of atheromatous lesions.\(^{56}\) Third, atheroma-like lesions have been reported to develop in intramural small DCs of 8–12-week-cholesterol-fed rabbits.\(^{57–60}\) However, in the latter-mentioned studies, the atheroma-like lesions were located primarily in the intramural small arteries and probably had a different genesis compared with large arteries, as calcium antagonists suppressed atherogenesis in the aorta but not in the intramural coronary arteries.\(^{59}\) In our rabbit model, as in humans, the main branches of the coronary arteries become severely involved before the more distal, intramural parts are affected.\(^{58,61}\) Thus, atherosclerosis seems to extend into the distal coronary circulation, and the impaired endothelium-dependent response of DCs might be of importance in response to changing blood flow, as has been suggested earlier from clinical studies.\(^{11}\) Recently, we reported that the ACh-induced relaxation of cerebral, femoral, and mesenteric small arteries in vitro was not affected by feeding rabbits a cholesterol-rich diet,\(^{29}\) and the present results show that endothelium-dependent relaxations of small coronary arteries are more susceptible to the effects of exposure to high plasma cholesterol concentrations than are arteries in the systemic circulation of our cholesterol-fed rabbit model. In summary, the present investigation shows that the major changes after induced hypercholesterolemia take place in the PCs, producing extensive atheromatous lesions associated with hyperreactivity of the vascular smooth muscle layer and endothelial dysfunction. In contrast, no atheromatous lesions were found and the functional changes were confined to the agonist-stimulated endothelium-dependent relaxation in the small DCs of the hypercholesterolemic rabbits. Even small modifications of the cholesterol-rich diet appear to influence the impairment of endothelial function in DCs, although further investigation is necessary to confirm this.

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