Endothelium-Dependent Relaxation in Experimental Atherosclerosis in the Rabbit

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The effect of feeding a diet supplemented with lipids and containing 2% cholesterol on the endothelium-dependent relaxation of rabbit aorta to acetylcholine was assessed. The effect of feeding a standard rabbit diet after an initial period of 2% cholesterol feeding was assessed also. Age-matched male, New Zealand white rabbits were fed either a 2% cholesterol diet or a standard rabbit diet. The animals were anesthetized with pentobarbitone sodium (25 mg/kg) and killed either at the beginning of the study (0 weeks) or at 4, 8, or 10 weeks. The animals in the reversal study were fed the 2% cholesterol diet for 6 weeks and killed after an additional 14 and 32 weeks on standard diet. The extent of atherosclerosis in the aorta was assessed by Sudan Red staining, estimation of tissue cholesterol, and light and electron microscopy. The relaxation response to acetylcholine was measured in rings of the thoracic aorta following precontraction with norepinephrine (−6.0 log mol/l). The relaxation was significantly impaired in aortas from rabbits fed the 2% cholesterol diet compared to aortas from animals fed the standard diet. The impairment of relaxation was apparent as early as 4 weeks after the start of the 2% cholesterol diet and remained impaired over the next 6 weeks. No improvement in endothelium-dependent relaxation was seen in rabbits on the reversal diet for 14 and 32 weeks. Thus, endothelium-dependent relaxation is attenuated in animals fed a 2% cholesterol diet, and the loss of relaxation persists for at least 32 weeks after the animals are returned to a standard diet.

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Furchgott and Zawadzki demonstrated that acetylcholine (ACh) produced relaxation in rings of rabbit aorta that had been precontracted with norepinephrine (NE). This relaxation was shown to be dependent on the presence of an intact endothelium. Following these initial studies, endothelium-dependent relaxations to a number of pharmacological agents have been demonstrated in different blood vessels in a variety of species (for review see Vanhoutte and Rimele). Experiments in superfused preparations have suggested that this relaxation is mediated by one or more unstable factors synthesized in the endothelial cells, which diffuse into the smooth muscle cell layer of the blood vessels.

Increasing the dietary intake of cholesterol in rabbits produces hyperlipidemia and eventually a form of atherosclerosis. In this model of experimental atherosclerosis, damage to endothelial cells is a consistent feature, and these changes could be expected to interfere with endothelium-mediated vasodilator responses. The form of atherosclerosis produced by continuous feeding of high cholesterol diets to rabbits has been shown to regress on removal of the cholesterol fraction from the diet. It is known also that with regression of the atherosclerotic lesions the morphology of the damaged endothelium appears to recover. It is not known whether this recovery is associated with restoration of its functions, including endothelium-dependent relaxation.

The experiments described in this investigation were designed to characterize the endothelium-dependent relaxations to ACh in rings of rabbit aorta following induction of atherosclerosis by feeding the animals with a high (2%) cholesterol diet. Further, the effect of subsequent removal of excess cholesterol from the diet on endothelium-dependent relaxation was investigated also. A short preliminary report of the initial parts of this study has been previously published.

Materials and Methods

Male, New Zealand white rabbits were used in the study. At the time of weaning (age 8–10 weeks, weight 1.5–2.5 kg), the animals were assigned randomly to control and experimental groups. The animals were numbered and housed individually under the same conditions. The control group was given a standard rabbit diet (Baby Rabbit Pellets, M-0662, Maple Leaf Mills Ltd., Masterfeeds Division, London, Ont., Canada) and the experimental group was fed a diet supplemented with lipids and cholesterol (cholesterol 2% w/w) (5799C-9 Rabbit Purified Diet, Ralston Purina Co., Richmond, Ind.). The latter diet will be referred to as the 2% cholesterol diet in the text. The detailed compositions of the diets are given in Appendix 2. More animals than the required numbers were assigned to the 2 groups to compensate for deaths during the study. The diets and water were given ad libitum to all animals. The food intake was monitored by weighing the residual amount of food at regular intervals.

The design of the study is shown in Figure 1. A group of 6 animals were killed at the beginning of the study. Subsequently, 6 animals from both the control and experimental groups were killed after 4, 8, and 10
weeks on their respective diets. Each animal was anesthetized by injecting pentobarbitone sodium (25 mg/kg) through the marginal ear vein. A midline thoracotomy was performed and the aorta removed for preparation of the rings. Before removal of the aorta, a sample of blood was obtained via a cardiac puncture for total cholesterol and triglycerides estimation. This blood was centrifuged at 2,000 rpm for 10 minutes and the serum preserved at —70° C for the analysis.

Reversal Study

After feeding on the 2% cholesterol diet for 6 weeks, some animals from the experimental group were transferred to the standard rabbit diet. These animals, along with age-matched controls, were observed and used in experimental protocols for an additional 14 and 32 weeks (total 38 weeks). Serum cholesterol and triglycerides were measured in these animals at 2-week intervals until the concentrations in two successive samples were within the range observed in the control animals. Blood samples for these estimations were obtained from the central ear artery.

Tissue Bath Studies

After anesthetizing the animals, the thoracic aortas were removed and excess connective tissue excised. The specimens were cut into rings approximately 5-7 mm long from the proximal descending thoracic aorta. To preserve the endothelium, special care was taken to avoid contact with the luminal surface of the rings. The endothelium was removed deliberately in some rings by inserting the tip of a small forceps into the luminal surface of the ring and turning it back and forth for 20 seconds on a filter paper wetted with Krebs buffer.

The rings were suspended in tissue baths of 22-ml capacity containing Krebs-bicarbonate buffer solution at pH 7.4. The solution was maintained at 37° C with the aid of a heater/circulator (Haake Mess-Technik, Karlsruhe, Federal Republic of Germany, Model No. E 15) and continuously aerated with a gas mixture containing 95% O₂ and 5% CO₂. The rings were mounted on two stainless steel triangular clips. The lower clip was attached to a moveable support and the upper clip to a force displacement transducer (Grass Instrument Co., Quincy, Mass., Model No. FT 03C).

Before experimentation, the rings were stretched to an optimum basal tension of 8.0 g. This optimum basal tension was established on the basis of length-active tension curves carried out using a fixed concentration of —7.0 log mol/l NE in preliminary experiments. The preparations were left in the tissue bath for a period of 90 minutes for equilibration before the experimental protocol was begun. The fluid in the tissue bath was changed every 30 minutes during this period. The remaining pieces from the thoracic aorta and the abdominal aorta of each rabbit were used for estimation of total tissue cholesterol.

Experimental Protocol

The responses to ACh were examined in 4 rings from each animal with each ring assigned randomly to one of the following procedures: 1) a control with no additional manipulation; 2) incubation with indomethacin (—6.0 log mol/l); 3) incubation with atropine (—8.0 log mol/l); and 4) removal of endothelium. Each drug was left in its bath for 30 minutes to equilibrate. At the end of this 30-minute incubation period, all preparations were contracted by adding NE (—7.0 log mol/l). After the contraction had reached a plateau, a concentration-effect curve to ACh was obtained by adding the drug to the bath in a cumulative manner (—9.0 to —4.0 log mol/l). Indomethacin and atropine were present in the Krebs buffer throughout the experiment in the respective tissue baths.

On completion of the concentration-effect curves the tissue bath fluid was replaced with fresh Krebs buffer and the tension allowed to return to the baseline value with frequent replacement of the buffer. After a period of 45 minutes, the concentration-effect curve to acetylcholine was repeated in the control preparation with the tissue contracted by adding a lower concentration of NE (—6.0 log mol/l). The effect of nordihydroguaiaretic acid (NDGA) on the response to ACh was assessed by adding it to the tissue bath as a single dose (—4.4 log mol/l) when the relaxatory response to ACh was maximal (—6.0 or —5.5 log mol/l). In separate rings, the effect of quinacrine (—6.0 log mol/l) and hydroquinone (—4.0 log mol/l) on the en-
dothelium-dependent relaxation to ACh was assessed. These agents were also added to separate tissue baths as single doses when the relaxation response to ACh was maximal. Effects of quinacrine and hydroquinone were assessed only in the first group of 6 animals killed at the beginning of the study. The effect of sodium nitrite was assessed in rings with and without endothelium from rabbits at the 10-week stage of the study. As in the initial part of the protocol, the rings were precontracted with NE (—6.0 log mol/l) and sodium nitrite added at concentrations of —4.0 and —3.0 log mol/l.

At the end of each experiment some rings were prepared for histological studies (light microscopy, scanning, and transmission electron microscopy). The remaining rings were stained with Sudan Red for lipid studies (see below).

**Histological Studies**

**Light Microscopy.** The tissues were fixed in 10% buffered formalin for 24 hours. For lipid staining the specimens were washed in Millonig’s phosphate buffer for 1 hour and postfixed in 1% osmium tetroxide in Millonig’s buffer for 2 hours. The specimens were then transferred through a series of graded ethanol solutions (70, 80, 95, and 100% for 1 hour in each stage) and embedded in paraffin (Tissue Prep, 56°C).

For hematoxylin-eosin staining the tissues were transferred from 10% formalin to the alcohol dehydration stage (as above) and embedded in paraffin (Tissue Prep, 56°C). Sections were cut at 10 μm on a rotary microtome (American Optical Co., Belville, Ont., Canada, Model No. 820) and stained by the methods modified from those described by Humason and Cull

**Transmission Electron Microscopy.** The tissues were fixed initially with 2.5% glutaraldehyde in Millonig’s buffer (48 hours) followed by postfixation with 1% osmium tetroxide (45 minutes). The specimens were dehydrated using ethyl alcohol (15 minutes in 50, 70, 80, and 95% alcohol followed by three 10-minute periods in 100% alcohol) and then placed in a critical point dryer (seeVAC, Pittsburgh, Penn., LPD-100) for 5 minutes at 41°C and 1200 psi CO₂. The tissues were then mounted on aluminum stubs with silver glue, sputter coated with gold, and examined in a scanning electron microscope (SEM) (Philips, Eindhoven, The Netherlands, Model 505). These methods are modifications of those described by Glauert and Dawes.

**Scanning Electron Microscopy (SEM).** At the end of the experiments the rabbit aortic rings were fixed initially with 2.5% gluteraldehyde in Millonig’s buffer (48 hours) followed by postfixation with 1% osmium tetroxide (45 minutes). The specimens were dehydrated using ethyl alcohol (15 minutes in 50, 70, 80, and 95% alcohol followed by three 10-minute periods in 100% alcohol) and then placed in a critical point dryer (seeVAC, Pittsburgh, Penn., LPD-100) for 5 minutes at 41°C and 1200 psi CO₂. The tissues were then mounted on aluminum stubs with silver glue, sputter coated with gold, and examined in a scanning electron microscope (SEM) (Philips, Eindhoven, The Netherlands, Model 505). These methods are modifications of those described by Glauert and Dawes.

**Scanning Electron Microscopy (SEM).** The tissues were fixed initially with 2.5% gluteraldehyde in Millonig’s buffer (24—48 hours). They were then washed 3 times (15 minutes each) with Millonig’s buffer and postfixed for 1 hour in 1% osmium tetroxide in Millonig’s buffer. After washing for three 10-minute periods with double distilled water, the tissues were dehydrated through a graded series of ethanol solutions (as described under SEM). Samples were transferred from absolute alcohol into propylene oxide for three 10-minute periods and then into a 1:1 mixture of propylene oxide and araldite (Marivac Limited, Halifax, N.S., Canada, CY212) epoxy resin for 3—4 hours. They were then placed in pure resin in embedding blocks, left at room temperature overnight, and then at 60°C in an oven for 48 hours. Thin sections were cut on an ultramicrotome (C. Reichert Ag, Vienna, Austria, Reichert-Jung Ultracut E), and mounted on 300-mesh copper grids. The specimens were then counterstained with uranyl acetate and lead citrate and examined in a transmission electron microscope (TEM) at 80 kV (Philips, Eindhoven, The Netherlands, Model 410).

**Sudan Red Staining.**

The specimens were rinsed in 70% ethanol (1—2 minutes) and immersed in Sudan Red at room temperature for 15 minutes. The solution was agitated intermittently at this stage. Next, the tissues were transferred to 80% ethanol for 20 minutes and washed in running water for 1 hour. Tissues were stored in 10% buffered formalin. The extent of experimental cholesterol atherosclerosis (in the descending thoracic aorta) shown by the sudanophilia in each animal was visually graded on a scale of 0 to 4 as modified from methods described by Duff and McMillan and Kritchevsky et al: Grade 0, no lesions seen in aorta; Grade 1, lesions around orifices of intercostal arteries, surface involvement <5%; Grade 2, lesions between orifices of intercostal arteries in addition to Grade 1 lesions, total surface involvement 5—25%; Grade 3, confluent lesions present, total surface involvement 25—60%; Grade 4, confluent lesions present, total surface involvement >60%. Methods used in the preparation of solutions used in this section are given in Appendix 1.

**Cholesterol and Triglycerides Estimation.**

The cholesterol and triglycerides estimations in the serum were made using an automated system (Instrumentation Laboratories, Lexington, Ky., Multistat III) that incorporated the methods of Allain et al and Pinter et al, respectively. Tissue cholesterol estimations were performed using the method of Morin.

**Drugs.**

The pharmacological agents used were acetylicholine chloride, ascorbic acid, atropine sulfate, calcium disodium ethylenediaminetetraacetic acid (CaNa₂EDTA), norepinephrine bitartrate, sodium nitrite, nordihydroguaiaretic acid (NDGA), and indomethacin (Sigma Chemical Co., St. Louis, Mo.). The Krebs bicarbonate buffer solution used had the following mmol/l composition: NaCl 116.0, KCl 5.4, CaCl₂ 1.2, NaHCO₃ 22.0, NaH₂PO₄ 1.2, glucose 10.1, MgCl₂·6H₂O 1.2, CaNa₂EDTA 0.023, and ascorbic acid 1.1. Stock solutions of the drugs were prepared in distilled water. All concentrations are expressed as the final concentration in the tissue bath fluid. Indomethacin was dissolved in equimolar sodium carbonate. NDGA was dissolved in dimethyl sulfoxide (DMSO). Final bath concentration of DMSO did not exceed...
0.025 mol%. DMSO in this concentration had no effect on the rabbit aorta, on the contractile response to NE, or on the relaxation to ACh.

Statistical Analysis

In each protocol, the number of rings studied was also the number of rabbits used. The data are expressed as means ± standard errors of the mean. A p<0.05 was considered significant for all statistical analyses. Concentration-effect curves for ACh in control and experimental animals were compared by regression analysis followed by analysis of covariance. The body weights, serum cholesterol, and serum triglyceride levels in control and experimental groups were compared using the Student’s t test for paired data. The gradings of the sudanophilia in control and experimental groups were compared using the Wilcoxon rank sum test.

Results

At the onset of the study, the rabbits weighed 2.1 ± 0.2 kg (n = 6). The control animals gained weight on the standard diet during the study and weighed 3.9 ± 0.5 kg at the end of 10 weeks. The experimental groups gained weight at a slower rate than the controls and these animals (n = 6) weighed 2.6 ± 0.1 kg at the end of 10 weeks. The body weights, serum cholesterol, and triglyceride concentrations in these animals are summarized in Table 1. The cholesterol and triglyceride levels in the serum in experimental animals were significantly elevated compared to the control animals at 4, 8, and 10 weeks of the study. The serum cholesterol levels showed considerable variation compared to the triglyceride levels, which showed a persistent upward trend throughout the 10 weeks of cholesterol feeding.

Distribution of Lesions in Aorta

After 4 weeks of cholesterol feeding, the experimental animals showed deposition of lipids throughout all the organs, particularly the liver. The aortas from this group showed prominent yellow spots and streaks on the intimal surface. Confluent yellow areas covering the whole intimal surface were seen in some areas and were more common in the proximal areas of the thoracic aorta. Sudan Red staining showed patchy areas of redness in all specimens of the cholesterol-fed group. An occasional aortic ring was stained red uniformly. At 8 and 10 weeks the lesions appeared to extend further down the descending aorta. However, considerable variation of the sudanophilia was seen in the specimens taken from rabbits fed for 4, 8, and 10 weeks with the 2% cholesterol diet. The extent of sudanophilia was not significantly different at these 3 stages of feeding (n = 6, p>0.05). These results are summarized in Table 2. A majority of the aortic specimens from control animals showed no sudanophilic areas, but an occasional fleck of redness was seen in a few.

Endothelium-Dependent Relaxation-Control State (0 Weeks)

The rabbit aortic rings did not exhibit spontaneous contractions in the basal state. Preparations from both control and cholesterol-fed animals contracted with the addition of NE, reaching a plateau in about 10–15 minutes. The mean tensions attained in the rings from animals at 4, 8, and 10 weeks in both control and experimental groups were not significantly different from each other (n = 6, p>0.05). These results are shown in Figure 2.

When ACh was added, the control rings demonstrated a concentration-dependent relaxation, beginning at an ACh concentration of −8.0 log mol/l and reaching a maximum about −6.0 to −5.5 log mol/l. In the group of 6 animals killed at the beginning of the study (0 weeks), the maximum relaxation to ACh was 48.8 ± 4.5% of the contractile response to −6.0 log mol/l NE. When a lower concentration of NE (−7.0 log mol/l) was used to precontract the vessel this relaxation increased to 70.5 ± 3.7%. A similar pattern of results (i.e., a greater relaxation when a lower concentration of NE was used to precontract the vessel) was seen throughout the study (Table 3). Indomethacin
Table 2. Grades of Sudanophilia and Cholesterol Contents of Aortas

<table>
<thead>
<tr>
<th>Stage of study</th>
<th>No. of animals</th>
<th>Visual grade</th>
<th>Tissue cholesterol content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nmol/mg protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mg/100 g wet wt</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0.2 ± 0.1</td>
<td>124 ± 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>112 ± 20</td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>6</td>
<td>2.6 ± 0.3</td>
<td>350 ± 62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>431 ± 71</td>
</tr>
<tr>
<td>8 weeks</td>
<td>6</td>
<td>2.4 ± 0.3</td>
<td>604 ± 66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>313 ± 90</td>
</tr>
<tr>
<td>10 weeks</td>
<td>6</td>
<td>2.6 ± 0.3</td>
<td>429 ± 80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>132 ± 40</td>
</tr>
<tr>
<td>20 weeks (reversal study)</td>
<td>4</td>
<td>3.6 ± 0.2</td>
<td>1913 ± 430</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1950 ± 500</td>
</tr>
<tr>
<td>38 weeks (reversal study)</td>
<td>5</td>
<td>2.8 ± 0.4</td>
<td>2084 ± 360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3470 ± 720</td>
</tr>
</tbody>
</table>

The visual grading of sudanophilia at 4, 8, 10, 20, and 32 weeks is different from control (p < 0.05, Wilcoxon rank sum test). The tissue cholesterol contents at different stages of the study are different from control (p < 0.05, t test for unpaired data).

Figure 2. Contractile responses to norepinephrine (−6.0 log mol/l) in control and atherosclerotic vessels at 4-, 8-, and 10-week stages (n = 6). Data for animals in reversal study are shown at 20 weeks (n = 4) and 38 weeks (n = 5). First group of control animals killed at beginning of study is shown as 0 weeks. Contractions at each stage were not different between control and atherosclerotic animals (p > 0.05).

(-6.0 log mol/l) had no significant effect on this relaxatory response to ACh.

When NDGA, quinacrine, or hydroquinone was added at the point of maximum relaxation to ACh (−6.0 to −5.5 log mol/l) each caused an almost immediate reversal of this relaxation. The residual relaxations after adding the three compounds were NDGA, 0.0 ± 0.0%; quinacrine, 14.9 ± 6.4%; and hydroquinone, 0.0 ± 0.0% (n = 6, p < 0.05 for each). No relaxation to ACh was seen in rings from which the endothelium was deliberately removed at the commencement of the experimental protocol (maximum relaxation 0.0 ± 0.0%, n = 6).

Endothelium-Dependent Relaxation After Cholesterol Feeding

The maximum relaxation to ACh in control animals at 4, 8, and 10 weeks was 45.4 ± 3.6%, 44.5 ± 5.1%, and 39.0 ± 5.8% of the contraction, respectively. The corresponding values in cholesterol-fed animals were 23.2 ± 5.8%, 20.1 ± 8.7%, and 18.5 ± 8.7%. Thus, endothelium-dependent relaxation was reduced by at least 50% after cholesterol feeding. The relaxation to ACh was significantly impaired in the cholesterol-fed animals compared to controls throughout this study (n = 6, p < 0.05). The results of the relaxation to ACh in the two groups of animals and the concentration-effect curves for ACh are given in Table 3 and Figure 3, respectively. The maximum relaxations to ACh in control animals at 4, 8, and 10 weeks were not significantly different (p > 0.05), and neither were the maximum relaxations in experimental animals at the three stages (p > 0.05). The relaxations when −7.0 log mol/l NE was used to precontract the vessels are also given in Table 3.

Indomethacin (−6.0 log mol/l) had no significant effect on the endothelium-dependent relaxation in both control and cholesterol-fed animals. Atropine (−8.0 log mol/l) produced a "shift to right" or completely inhibited the concentration-effect curve for ACh (n = 6, p < 0.05) in both control and cholesterol-fed animals. Addition of NDGA inhibited the relaxation seen with ACh (n = 6 for each group, p < 0.05).

Rings with and without endothelium from control and cholesterol-fed rabbits showed a relaxation response to sodium nitrite. No significant difference was seen in this relaxation to sodium nitrite in the 2 animal groups (n = 6, p > 0.05). These results are summarized in Figure 4. No relaxation to ACh was seen in rings devoid of endothelium from both control and experimental animals at 4, 8, and 10 weeks of study. At ACh concentrations over −5.0 log mol/l a contrac-
Table 3. Summary of Magnitudes of Maximum Relaxation to Acetylcholine (−5.5 log mol) Following Precontraction With Norepinephrine (NE) in Control and Experimental Animals

<table>
<thead>
<tr>
<th></th>
<th>Relaxation to acetylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−6.0 log mol/l NE used to precontract the aortic ring</td>
</tr>
<tr>
<td></td>
<td>Active tension (g)*</td>
</tr>
<tr>
<td>Control animals</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>Experimental animals</td>
<td>6.6 ± 0.7</td>
</tr>
</tbody>
</table>

*Contractile response to −6.0 log mol/l of NE.

Figure 3. Endothelium-dependent relaxation to acetylcholine during contractions to norepinephrine at different stages of 2% cholesterol feeding. Abscissa shows log mol/l concentration of acetylcholine, and ordinate shows relaxation expressed as percent of contraction to norepinephrine (−6.0 log mol/l). Responses at 4, 8, and 10 weeks of cholesterol feeding are different from control (p<0.05, n=6 for each).

Histological Studies

**Light microscopy.** Lesions of different grades of severity were seen. In the early stages (4 weeks), accumulations of lipid-laden cells resting on the subendothelial layers was a consistent feature. At 8- and 10-week stages, flat or slightly raised intimal lesions were seen. These raised intimal lesions (plaques) were more frequently seen in proximal areas than in the distal parts of the thoracic aorta. These plaques had variable combinations of lipid-laden cells and extracellular lipid. The cells were larger toward the base of the plaque. The endothelium was usually intact in most of the plaques, but a few lesions with surface ulceration were seen also. At the 8- and 10-week stages, the lipids occupied the interstices between the internal elastic lamina and between the bundles of muscle fibers in the inner parts of the tunica media. The fibers of the internal elastic lamina appeared fragmented in some areas. These features of a plaque in cross section are shown in Figure 5.

In aortic rings from control animals, the endothelium formed a continuous layer. The subendothelial layer extending to the internal elastic lamina was of uniform thickness and had collagen and elastic fibers. Accumulations of lipid-laden cells or plaques were not seen in these areas.

**Scanning electron microscopy (SEM).** The fatty spots recognized macroscopically and with the light microscope appeared in the SEM as elevated areas.
The endothelial cell borders could be traced to these elevations in most areas. In some areas, the endothelial cell borders were indistinct as they approached the elevations. The plaques were oval in most cases, with well-defined or irregular edges. The endothelium was usually intact, but an occasional lesion had surface ulceration. The cells appeared swollen, and in some areas thin filamentous projections or short projections with a broad base were regularly seen. Some cells with multiple short pseudopodia were seen also. Because the cells were swollen and globular they appeared to be separating from each other. In such areas, multiple strands or bridges running between the cells were noted. Some cells showed holes on their surface, and in some cases the entire cell surface was covered by one large hole or several small holes. The ulcers had irregular shapes with partly elevated margins. The fibrillar subendothelial lamina was seen in the floor of the ulcer. In most aortic rings from cholesterol-fed rabbits, approximately 80% of the surface appeared intact. There were no significant differences noted in the appearances of the different lesions (i.e., areas of abnormal endothelium, plaques, or ulcers) at 4-, 8-, and 10-week stages. Some areas of apparently normal-looking endothelium (i.e., the appearances seen in control animals) were seen in cholesterol-fed animals.

In the control animals, the endothelial surface was usually uniform and smooth. Longitudinal folds were seen in a few areas. Individual cells were identifiable with clear margins in most areas. These cells did not have the swollen appearance or the numerous surface projections that were seen in cholesterol-fed animals. The rings from which the endothelium was removed at the beginning of the experimental protocol, showed the fibers of the subendothelial layer. No endothelial cells were seen in these preparations. The SEM appearances of the aortic luminal surface from control and cholesterol-fed animals are shown in Figures 6 and 7.

Transmission electron microscopy (TEM). The most striking change in endothelial cells was vacuolation. Vacuoles of different sizes were seen spread throughout the endothelial cell cytoplasm. The subendothelial layer was widened and contained fragmented elastic fibers. In the subintimal smooth muscle layer, vacuolation was also conspicuous. The majority of these vacuoles were spherical in shape and did not appear to be limited by a membrane. Most vacuoles were empty while a few contained concentrically arranged irregular structures (Figure 8). The organelles (e.g., mitochondria) in the endothelial cells and the smooth muscles appeared prominent.

In control animals, the endothelial cells formed a continuous sheet lining the lumen. The cytoplasm contained a few vacuoles and numerous vesicles. The subendothelial layer was of uniform thickness and showed elongated smooth muscle cells with branching processes alternating with lamellae of elastic fibers.

Reversal Study

These animals were fed the 2% cholesterol diet for 6 weeks and then switched to the standard rabbit diet. They were monitored on this latter diet for an additional 14 and 32 weeks before being killed. The control animals were given the standard diet for the entire period. No significant differences in the body weights or cholesterol and triglyceride determinations were seen between these two groups (Table 1). Despite being on the standard diet for 14 and 32 weeks, the animals that had been fed the 2% cholesterol diet still showed deposition of lipids in the viscera at the time of sacrifice. In these animals, the visual grading of sudanophilia was significantly different from that in control animals (p < 0.05). Sudanophilia in these animals appeared to be greater than that seen in animals who were on the high cholesterol diet and killed at 4, 8, and 10 weeks (Table 2). The visual grading of sudanophilia in the animals of the reversal study (20-week state) was...
significantly more than that of the cholesterol-fed group at the 8-week stage of the study ($p<0.05$). However, this grading was not significantly different from the grading at 4- and 10-week stages of the study ($p>0.05$, Table 2).

Aortic rings from both control and experimental animals contracted to NE with no significant difference between the active tensions attained (Figure 2). When ACh was added the rings showed a relaxation response that was maximal at concentrations of $-6.0$ to $-5.5$ log mol/l. The maximum relaxation in control animals was $49.8 \pm 11.3\%$ and $34.6 \pm 3.9\%$ of the contraction to NE at 20 and 38 weeks, respectively. The relaxation in experimental animals was significantly impaired and amounted to $20.3 \pm 7.1\%$ and $14.5 \pm 3.8\%$ of the contraction, respectively ($p<0.05$, Table 3). Concentration-effect curves to ACh in control and experimental animals are given in Figures 9 and 10. Thus, the loss of endothelium-dependent relaxation as a result of feeding the 2% cholesterol diet persisted for 32 weeks after the animals were returned to the standard rabbit diet.

SEM of the aortic surface in these animals showed that the endothelium formed a continuous lining in most areas. Cell borders were more distinct and fewer surface projections were seen compared to animals who were still on the 2% cholesterol diet at the time of killing. Also, fewer holes were seen on cell surfaces in this group of animals (Figure 7). Under the TEM, a continuous sheet of endothelial cells was seen in most areas. The lipid vacuoles were less prominent compared to animals who were on cholesterol at the time of killing.

Six animals died in the group started on the 2% cholesterol diet. Three were jaundiced at the time of death and the other three were apparently in good condition. Experiments in three other animals were discontinued due to severe weight loss while on the high cholesterol diet. No deaths occurred in the reversal or control groups.

**Discussion**

In the first group of 6 animals at 0 weeks of the study, the rings of rabbit aorta with intact endothelium showed a relaxation response to ACh. This relaxation was maximal at ACh concentrations of $-6.0$ to $-5.5$ log mol/l. As atropine ($-8.0$ log mol/l) produced a "shift to right" of the concentration-effect curve for ACh, it is likely that ACh elicits its effects by acting through muscarinic cholinergic receptors. The cyclooxygenase inhibitor indomethacin ($-6.0$ log mol/l) had no significant inhibitory effect on this ACh-induced relaxation. The lipoxygenase inhibitor and antioxidant NDGA ($-4.4$ log mol/l) antagonized the relaxation to ACh. Similarly, the phospholipase A2 inhibitor quinacrine ($-6.0$ log mol/l) and the free radical scavenger hydroquinone ($-4.0$ log mol/l) were able to inhibit the endothelium-dependent relaxation to...
FIGURE 6. Scanning electron microscopic appearances of endothelial surface of rabbit aorta. Upper left: Specimen from control animal on standard rabbit diet showing uniform cells with clear margins (bar = 0.1 mm; magnification ×326). Upper right: Specimen from control animal from which endothelium was mechanically removed at beginning of experiment. Criss-crossing fibers of subendothelial layer are seen (bar = 10 μm; magnification × 1,150). Lower left: Specimen from animal on 2% cholesterol diet for 8 weeks (bar = 10 μm, magnification × 1,310). Lower right: Specimen showing cells with irregular margins, projections with bulbous ends and microvilli from animal on 2% cholesterol diet for 10 weeks (bar = 0.1 mm, magnification × 312).

ACh. All features suggest that the relaxation responses observed in the present study are consistent with those described by Furchgott. The chemical nature of the endothelium-dependent relaxing factor is unknown, but the results of this study raise the possibility of the involvement of a lipid product from the lipoxygenase pathway.

The principal finding in this study was the impairment of endothelium-dependent relaxation to ACh in aortas of cholesterol-fed animals. As previously reported, the magnitude of the relaxation response observed in aortic rings is inversely related to the concentration of agonist used to contract the vessel. In the main protocol of the experiments described here, a fixed concentration of NE (−6.0 log mol/l) was used to contract all the blood vessels. Because the experiment involved comparing relaxations in aortic rings from control and cholesterol-fed animals, this procedure would minimize variation. Further, in the present study no significant difference was seen in the mean active tensions generated by NE in the aortic rings from control and cholesterol-fed animals. Thus, the impaired endothelium-dependent relaxation to ACh in the cholesterol-fed animals is probably a result of the atherosclerotic process itself.

Considerable changes are seen in the endothelium and subendothelial layers in experimental atherosclerosis. Scanning electron microscopy has revealed endothelial cells with holes on their surface as well as filamentous projections (microvilli) and pseudopodia extending from the cell membrane. Increased endocytosis with formation of vesicles along the plasma membrane and increased numbers of ribosomes have been described in these cells. Partial or complete opening of interendothelial spaces has been shown in other cells. All these changes coupled with subendothelial edema lead to destruction of endothelial cells. Most of these features were seen in the aortas taken from cholesterol-fed animals in the present study.

The impairment of endothelium-dependent relaxation seen in cholesterol-fed rabbits could be due to one factor or a combination of several factors.

**DESTRUCTION OF ISOLATED ENDOTHELIAL CELLS.** Electron microscopy revealed extensive morphologi-
Impaired ability to synthesize endothelium-dependent relaxation factor(s).

Transmission electron microscopy demonstrated structural changes in several organelles in endothelial cells. These include swollen mitochondria, distended vesicles, and degenerating nuclei. These changes could be expected to influence the enzyme systems in the cells leading to an impaired production of endothelium-dependent relaxation factor(s). Impairment of cellular functions could also be caused by an increase in intracellular calcium, which is usually associated with atherogenesis.

Impaired diffusion and enhanced degradation of relaxatory factor(s) during transit from endothelial cells to underlying smooth muscle. The subendothelial layers are thickened and edematous due to deposition of large amounts of lipids. Recent experiments have shown that endothelium-dependent relaxing factor(s) are unstable, with a half-life as short as 6 seconds. Hence the physical barriers to diffusion produced by the edematous tissue could lead to an impairment of endothelium-dependent relaxation. Such barriers could result in an enhanced degradation of relaxing factor(s).

Impairment of smooth muscle function in aorta due to changes in experimental atherosclerosis. Thickening of basement membrane, distended vesicles, and lipid vacuoles have been described in muscle cells in atheromatous aortas. However, no significant differences were seen in either the contractile responses to NE or the relaxation responses to sodium nitrite in control and experimental animals.

It is likely that one or several of these factors contribute to the impairment of endothelium-dependent relaxation to ACh, seen in atherosclerotic rabbit aorta. Recent studies from this laboratory suggest strongly that the synthesis/release of the relaxing factor is impaired after feeding a diet containing 2% cholesterol.

There are reports suggesting that aortas and coronary arteries exposed to endogenous hyperlipidemia exhibit an enhanced responsiveness (hyper-reactivity).
to ergonovine.\textsuperscript{29} However this hyper-reactivity appears to be mediated via serotonergic mechanisms. Yokoyama et al\textsuperscript{29} found no appreciable differences in the concentration-response relation for the $\alpha$-agonist phenylephrine in control and Watanabe hereditary hyperlipidemic (WHHL) rabbits. The relaxations achieved by sodium nitrite in both control and experimental animals did not differ significantly from each other. These findings, taken collectively, suggest that the impairment of endothelium-dependent relaxation seen in cholesterol-fed animals is caused by factors within the endothelium itself.

In the animals fed the standard rabbit diet after a 6-week exposure to a high cholesterol diet, the endothelial function remained impaired even after an additional 14 and 32 weeks. Though the serum cholesterol and triglyceride levels returned to control levels in these animals, the visual grade of sudanophilia was even higher compared to animals that were still on the 2% cholesterol diet at the time of killing. This finding is similar to that reported by Albrecht and Schuler.\textsuperscript{30} In addition, it was noted that the degree of sudanophilia was not correlated with the duration of cholesterol feeding in the earlier part of the study (Table 2). These
observations suggest that this technique of visual grading, though used commonly,\textsuperscript{16,31} is a relatively insensitive index of the severity and, particularly, the depth of the lesion. Under the SEM the cell surfaces appeared smooth and the margins were clearer compared to animals already on the 2% cholesterol diet at the time of killing.\textsuperscript{12} Atherosclerotic lesions from rabbits fed the 2% cholesterol diet for 2 months or more generally have been shown to regress, if at all, slowly over a period of up to 16 months.\textsuperscript{33} Hence, the failure of the endothelial function to recover after 14 and 32 weeks is not unexpected. Clearly, monitoring the effect of a therapeutic intervention on the plasma cholesterol concentration does not reflect the more important pool of cholesterol in the aorta. Furthermore, impairment of endothelium-dependent relaxation may occur in the presence of a normal plasma cholesterol concentration.

Thus, in summary, feeding rabbits a diet supplemented with lipids and containing 2% cholesterol produces atherosclerotic lesions in the aortas after 4 weeks. These changes are accompanied by a persistent loss of endothelium-dependent relaxation, and it is suggested that this phenomenon could be an early functional marker in the development of atherosclerosis.

Appendix 1

Details of Preparation of Solutions Used

The Millonig's buffer used for TEM studies was prepared in the following manner: 500 ml water was placed in a liter beaker with a large magnetic stirrer, and another 500 ml water was measured in a cylinder. Amounts of 16.8 g NaH$_2$PO$_4$·H$_2$O, 3.85 g NaOH, and 5.4 g glucose were weighed in separate cups and dissolved in the beaker. Then, 300 ml of water from the cylinder was added to the beaker and mixed. CaCl$_2$ (0.05 g) was dissolved in 3-5 ml of water in a small beaker. With a Pasteur pipette, the CaCl$_2$ solution was added dropwise to the chemicals in the beaker. The remaining 200 ml of water in the cylinder was added, and the buffer solution was kept at a pH 7.25-7.40 and stored in a refrigerator after thorough mixing. The water used was double distilled for electron microscopy work.

The uranyl acetate solution was prepared in the following way. Uranyl acetate was added to a dark bottle containing 100 ml water. Once the solution was totally saturated, 100 ml of absolute alcohol was added and stored at room temperature. The uranyl acetate solution was then allowed to settle for 3-4 days and was then purged.

The lead citrate solution was prepared by dissolving 3.52 g of sodium citrate (Na$_2$C$_6$H$_5$O$_7$·2H$_2$O) in 80 ml of water.
water. To this solution 2.66 g of lead nitrate was added. A white flocculation formed and was stirred with a magnetic stirrer for 30 minutes, and then 20 ml of 1 M NaOH was added to remove the flocculation. This solution was allowed to settle for 4–5 days and was stored at room temperature.

The Sudan Red solution was prepared by mixing 5.0 g Sudan Red with 500 ml of 70% ethyl alcohol and 500 ml of acetone. This solution was filtered 3 times before use.

Appendix 2

The compositions of the control diet and the experimental diet are as follows: Control diet (w/w): proteins 17.9%, lipids 4% (cholesterol 0.06%), carbohydrates, minerals, and vitamins approximately 78.1%. Experimental diet (w/w): proteins 18.4%, lipids 24.0% (cholesterol 2%), carbohydrates, minerals, and vitamins approximately 57.6%. The percents of individual fatty acids in the lipids are given below.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control diet (%)</th>
<th>Experimental diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0 Palmitic acid</td>
<td>17.5</td>
<td>25.0</td>
</tr>
<tr>
<td>18:0 Stearic acid</td>
<td>2.5</td>
<td>12.6</td>
</tr>
<tr>
<td>18:1 Oleic acid</td>
<td>16.8</td>
<td>44.4</td>
</tr>
<tr>
<td>18:2 Linoleic acid</td>
<td>43.7</td>
<td>10.3</td>
</tr>
<tr>
<td>18:3 Linolenic acid</td>
<td>16.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Others</td>
<td>3.1</td>
<td>7.2</td>
</tr>
</tbody>
</table>

All analyses were done in triplicate.

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**KEY WORDS** • rabbit aorta • endothelium-dependent relaxation • atherosclerosis • acetylcholine • norepinephrine
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