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Impairment of endothelium-dependent relaxation: an early marker for atherosclerosis in the rabbit

Laal Jayakody, [1]Tissa Kappagoda, Manohara P.J. Senaratne & Alan B.R. Thomson

Department of Medicine, Surgical Medical Research Institute, University of Alberta, Edmonton, Alberta T6G 2N8, Canada

1 Cholesterol feeding of rabbits impairs the endothelium-dependent relaxation (EDR) evoked by acetylcholine (ACh) in the aorta. The experiments described in this paper were undertaken to examine the influence of age upon this phenomenon.

2 Rabbits aged 8 weeks and 46 weeks were fed a diet containing 2% cholesterol and other lipids for 4 weeks. Age-matched control animals were fed a standard rabbit diet. The concentrations of cholesterol and triglycerides in plasma were measured and the extent of atherosclerosis was estimated by staining the aortae with Sudan Red. Light and electron microscopy were undertaken also.

3 Rings of aorta were prepared for recording isometric tension. They were contracted with noradrenaline (NA) and EDR elicited by adding ACh.

4 The young rabbits showed weight gain, hypercholesterolaemia, prominent Sudan Red staining, together with scanning and transmission electron microscopic (SEM and TEM) features of cholesterol-induced atherosclerosis. The older animals showed significant weight loss and hypercholesterolaemia. The aortae of these animals showed no significant sudanophilia or light microscopic features of atherosclerosis. The SEM appearances were similar to the young animals fed cholesterol.

5 EDR to ACh was significantly impaired in both groups of cholesterol-fed rabbits. The maximal relaxations to ACh in young control and cholesterol-fed rabbits were $46.4 \pm 2.9\%$ and $24.0 \pm 4.3\%$ (mean $\pm$ s.e. mean, $n = 8$, $P < 0.05$) of the contractile response to NA ($1\,\mu mol\,l^{-1}$). The corresponding results in the age control and cholesterol-fed rabbits were $31.8 \pm 3.9\%$ and $9.1 \pm 1.5\%$ ($n = 9$, $P < 0.05$).

6 The young rabbits were far more susceptible to cholesterol-induced atherosclerosis than older animals and these changes were accompanied by loss of EDR. In the older animals the loss of the latter property was not accompanied by a significant degree of atherosclerosis although hypercholesterolaemia was present.

Introduction

In 1980 Furchgott & Zawadzki demonstrated that acetylcholine (ACh) caused a relaxation in rings of rabbit aorta pre-contracted with noradrenaline (NA). This phenomenon could be demonstrated only in the presence of an intact endothelium. Following these initial studies, endothelium-dependent relaxation (EDR) to a number of pharmacological agents has been demonstrated in different blood vessels in a variety of species including man (Vanhoutte & Rimele, 1982–83; Cherry *et al.*, 1982; Kalsner, 1985). 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 (Furchgott, 1983).

Increasing the dietary intake of cholesterol in rabbits produces hyperlipidaemia and eventually a form of atherosclerosis (Duff *et al.*, 1957). In this model of experimental atherosclerosis, damage to endothelial cells is a consistent feature (Klimov *et al.*, 1981) and these changes could be expected to interfere with endothelium-mediated vasodilator

[1] Author for correspondence at: 2C244 Walter MacKenzie Centre, University of Alberta, Edmonton, Alberta T6G 2R7, Canada.

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responses. Recent investigations from this laboratory as well as others, have provided evidence which suggests that the EDR to ACh is impaired in aortae from rabbits fed a diet containing excess cholesterol (Verbeuren *et al.*, 1986; Jayakody *et al.*, 1987). Previous studies have demonstrated that older rabbits were less likely to develop hypercholesterolaemia and atherosclerosis when fed with diet supplemented with cholesterol (Ignatowski, 1909; West *et al.*, 1982). However, the influence of age on the impairment of the EDR produced by a high cholesterol diet has not been investigated.

The study was undertaken to test the hypothesis that the age of an animal influences the effect of cholesterol feeding on EDR. The experiments were done to characterize the EDR to ACh in aortic rings of two groups of rabbits 8 and 46 weeks of age (at the start of study) following feeding a diet containing 2% cholesterol for 4 weeks.

Methods

Male, New Zealand White rabbits were used in the study. At the time of weaning (age approximately 8 weeks, weight 1.5–2.5 kg) the animals were divided into two batches. The first batch was 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, Masterfeeds Division, Maple Leaf Mills Ltd, London, Ontario, Canada) and the experimental group was fed a diet supplemented with 2% cholesterol and other lipids (5799C-9 Rabbit Purified Diet, Ralston Purina Co, Richmond, Indiana, U.S.A.). The detailed composition of the diets is given in Table 1. More animals than the required numbers were assigned to the two groups to compensate for possible 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 second batch of animals remained on the standard rabbit diet for 38 weeks. At this time (age of animals: approximately 46 weeks) the batch was assigned randomly to control and experimental groups. The control and experimental groups were fed the standard rabbit diet and the diet supplemented with 2% cholesterol and lipids respectively.

Animals from both the control and experimental groups were killed after 4 weeks on their respective diets. Each animal was anaesthetized by injecting pentobarbitone sodium ($25\,mg\,kg^{-1}$) 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 2000 r.p.m. for 10 min and the serum stored at −70°C until analysed.

Table 1 The compositions of the control diet and the experimental diet

	Control diet (%)	*Experimental diet* (%)
16:0 Palmitic acid	17.5	25.0
18:0 Stearic acid	2.5	12.6
18:1 Oleic acid	16.8	44.4
18:2 Linoleic acid	43.7	10.3
18:3 Linolenic acid	16.4	0.5
Others	3.1	7.2

All analyses done in triplicate.
Control diet (w/w): proteins 17.9%, lipids 4% (cholesterol 0.06%), carbohydrates, minerals, vitamins approximately 78.1%; Experimental diet (w/w): proteins 18.4%, lipids 24.0% (cholesterol 2%), carbohydrates, minerals, vitamins approximately 57.6%. The percentages of individual fatty acids within the lipids are given above.

Tissue bath studies

After anaesthetizing the animals, the thoracic aortae were removed and excess connective tissue excised. The specimens were cut into rings approximately 5 mm long from the proximal descending thoracic aorta. Special care was taken to avoid contact with the luminal surface of the rings in order to preserve the endothelium. 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 back and forth for 20 s on a filter paper wetted with Krebs-bicarbonate buffer (Senaratne & Kappagoda, 1984). The rings were suspended in tissue baths of 22 ml capacity containing Krebs-bicarbonate buffer solution at a pH of 7.4. The solution was maintained at 37°C with the aid of a heater/circulator (Model No. E15, Haake Mess-Technik, Karlsruhe, Federal Republic of Germany) and continuously aerated with a gas mixture containing 95% O_2:5% CO_2. The rings were mounted on two stainless steel triangular clips, the lower clip being attached to a moveable support and the upper clip to a force displacement transducer (Model No. FT 03C, Grass Instrument Co., Quincy, Ma., U.S.A.). Before experimentation the rings were stretched to an optimum basal tension of 8.0 g. [The optimum basal tension of 8.0 g was established on the basis of length-active tension curves carried out using a fixed concentration of NA ($0.1\,\mu mol\,l^{-1}$) in preliminary experiments.] The preparations were left in the tissue bath for a period of 90 min for equili-

bration before the experimental protocol was begun. The fluid in the tissue bath was changed every 30 min during this period. The distal portions of the descending thoracic aorta and the abdominal aorta from 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 being assigned randomly to one of the following procedures: (1) a control with no additional manipulation; (2) incubation with indomethacin (1 μmol l^{-1}); (3) incubation with atropine (10 nmol l^{-1}); (4) mechanical removal of endothelium. Each drug was permitted to equilibrate in the tissue bath for 30 min before the start of the experiment.

At the end of the 90 min period of equilibration the preparations were contracted by adding NA (1 μmol l^{-1}). After the contraction had reached a stable plateau, a cumulative concentration-effect curve to ACh was obtained (1 nmol l^{-1} to 100 μmol l^{-1}).

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

Histology (See Jayakody *et al.*, 1987 for details of methods)

(i) *Light microscopy:* The tissues were fixed in 10% buffered formalin for 24 h and stained with haematoxylin and eosin by methods described previously (Jayakody *et al.*, 1987).

(ii) *Scanning electron microscopy (SEM):* At the end of the experiments the rabbit aortic rings were fixed initially with 2.5% gluteraldehyde, post-fixed with 1% osmium tetroxide and prepared for scanning electron microscopy (Model 505, Philips, Eindhoven, The Netherlands). The methods used were modifications of those described by Glauert (1975) and Dawes (1981).

(iii) *Transmission electron microscopy (TEM):* The tissues were fixed in 2.5% gluteraldehyde, post fixed in 1% osmium tetroxide, dehydrated through alcohols and embedded in araldite CY212 (Hayat, 1981). Thin sections were counter-stained with uranyl acetate and lead citrate before viewing in a transmission electron microscope (Philips 410, Philips, Eindhoven, The Netherlands).

Sudan Red staining The specimens were rinsed in 70% ethanol (1–2 min) and immersed in Sudan Red at room temperature for 15 min with intermittent agitation. Next the tissues were transferred to 80% ethanol for 20 min, washed in running water for 1 h and stored in 10% buffered formalin (Holman *et al.*, 1958). The extent of atherosclerosis shown by the sudanophilia in each animal was visually graded on a scale of 0 to 4 as modified from methods described by Duff & McMillan (1949), and Kritchevsky *et al.* (1961). The levels of grading are: 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%.

Cholesterol and triglycerides estimation

The cholesterol and triglycerides estimations in the serum were carried out by use of an automated system (Multistat III, Instrumentation Laboratories, Lexington, Ky, U.S.A.) which incorporated the methods of Allain *et al.* (1974) and Pinter *et al.* (1967) respectively. Tissue cholesterol estimations were performed by the method of Morin (1976).

Drugs

The pharmacological agents used were: acetylcholine chloride, atropine sulphate, calcium disodium ethylenediaminetetraacetic acid ($CaNa_2EDTA$), noradrenaline bitartarate, indomethacin (Sigma Chemical Co., St Louis, Mo, U.S.A.). The Krebs-bicarbonate buffer solution used was of the following composition (mmol l^{-1}): NaCl 116.0, KCl 5.4, $CaCl_2$ 1.2, $NaHCO_3$ 22.0, NaH_2PO_4 1.2, glucose 10.1, $MgCl_2.6H_2O$ 1.2, $CaNa_2EDTA$ 0.023. 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 an equimolar sodium carbonate solution.

Statistical analysis

In each protocol the number of rings studied was also the number of rabbits used. The data are expressed as mean ± standard errors of the mean (s.e. mean). A P value less than 0.05 was considered as significant for all statistical analyses. Concentration-effect curves for ACh in control and experimental animals were compared by analysis of covariance (Snedecor & Cochran, 1980). The body

weights, serum cholesterol and serum triglyceride levels in control and experimental groups were compared by Student's t test for unpaired data. The gradings of the sudanophilia in control and experimental groups were compared by Wilcoxon's rank sum test.

Results

Twelve week old rabbits

The details relating to growth and serum lipids in these animals are given in Table 2. The animals in the experimental group had a significantly higher concentration of cholesterol and triglycerides in the blood at time of experimentation.

After 4 weeks of feeding with a 2% cholesterol diet, the experimental animals showed deposition of lipids throughout body organs, particularly in the liver. The aortae showed prominent yellow spots and streaks on the intimal surface. In some animals confluent yellow lesions covered a large area of the intimal surface. Such lesions were more common in the proximal parts of the thoracic aorta compared to the abdominal aorta. With Sudan Red staining these yellow areas assumed a bright red colour (Figure 1a). The extent of sudanophilia, although variable, was significantly greater in the aortae taken from experimental animals than in those control animals the majority of which showed no sudanophilic areas (Table 3, $P < 0.05$).

The rabbit aortic rings did not exhibit any spontaneous contractions in the basal state. Preparations from both control and cholesterol-fed animals contracted with the addition of NA reaching a plateau within 10–15 min. The mean tensions attained in the rings from control and experimental groups were not significantly different from each other (Table 3, $n = 8$, $P > 0.05$).

Control rings demonstrated a concentration-dependent relaxation to ACh (from 10 nmol l^{-1} to 1 μmol l^{-1} or 3.2 μmol l^{-1}). Higher concentrations of ACh led to a reversal of the relaxant response. Atropine (10 nmol l^{-1}) produced a parallel shift to the right of the concentration-effect curve to ACh whereas indomethacin (1 μmol l^{-1}) was without significant effect. No relaxation to ACh was seen in rings deliberately denuded of endothelium. Relaxation to ACh in the experimental group was significantly less than that observed in the control group ($n = 8$, $P < 0.05$, Figure 2).

Table 2 Body weight, total serum cholesterol and serum triglyceride levels in control and 2% cholesterol-fed rabbits

Young animals		
Start of study (age: 8 weeks) (n = 16)		
Body weight (kg)	2.1 ± 0.2	
Total serum cholesterol (mg%)	68.5 ± 5.2	
Serum triglycerides (mg%)	145.5 ± 10.4	
After 4 weeks feeding	*Control (n = 8)*	*2% Cholesterol (n = 8)*
Body weight (kg)	2.9 ± 0.3	2.9 ± 0.2
Total serum cholesterol (mg%)	76.6 ± 3.1	**1908.3 ± 149.1
Serum triglycerides (mg%)	164.6 ± 12.6	**407.3 ± 104.8
Older animals		
Start of study (age: 44–46 weeks) (n = 18)		
Body weight (kg)	4.3 ± 0.1	
Total serum cholesterol (mg%)	47.2 ± 2.9	
Serum triglycerides (mg%)	113.9 ± 7.2	
After 4 weeks feeding	*Control (n = 9)*	*2% Cholesterol (n = 9)*
Body weight (kg)	4.2 ± 0.1	*2.9 ± 0.1
Total serum cholesterol (mg%)	48.7 ± 6.0	**570.7 ± 164.7
Serum triglycerides (mg%)	121.0 ± 7.9	**312.0 ± 71.6

Values are means ± s.e. mean.
Difference from control significant at $^{*}P < 0.05$, $^{**}P < 0.01$.

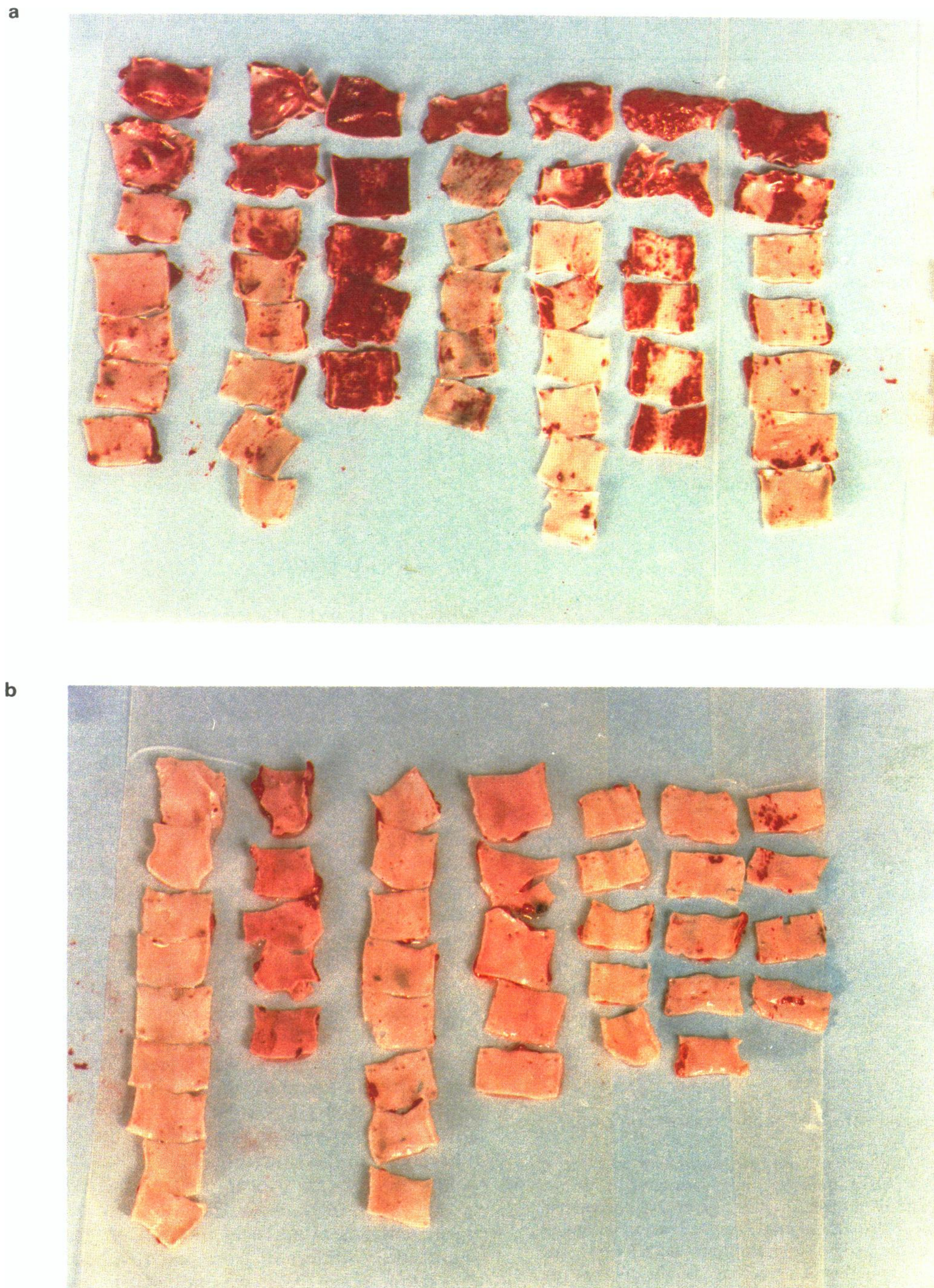

Figure 1 Segments of rabbit aortae showing luminal surfaces following staining with Sudan Red at the end of tissue bath experiments. Each vertical column represents aortic segments from one rabbit. Segments of rabbit aorta (4–6 cm) commencing from the region beyond the origin of the left subclavian artery are shown (the segments are arranged randomly i.e. not proximal to distal). (a) Specimens from young rabbits (12 weeks of age) given a 2% cholesterol diet for 4 weeks. Sudanophilia was prominent in this group of animals. (b) Specimens from older rabbits (50 weeks of age) given a 2% cholesterol diet for 4 weeks. Segments from only one of these animals (extreme right column) showed Sudan staining. The extent of sudanophilia seen in the young animals given a 2% cholesterol diet was significantly different from those of older animals given the same diet ($P < 0.01$, Wilcoxon's rank sum test).

Table 3 Summary of the results obtained from aortic rings from control and 2% cholesterol-fed rabbits of 12 and 50 weeks of age

	12 week old rabbits		*50 week old rabbits*	
	Control	*2% Cholesterol*	*Control*	*2% Cholesterol*
Number of animals	8	8	9	9
Visual grade of sudanophilia	0.1 ± 0.1	2.6 ± 0.3**	0.0 ± 0.0	0.2 ± 0.2
Tissue cholesterol content nmol mg^{-1} protein	114.2 ± 22.1	417.5 ± 75.4**	88.9 ± 11.4	181.2 ± 29.7**
Tissue cholesterol content mg 100 g^{-1} wet weight	112.0 ± 20.0	431.0 ± 71.0**	137.0 ± 10.0	251.0 ± 50.0**
Mean tension (g) produced by noradrenaline (1 μmol l^{-1})	7.5 ± 0.8	6.6 ± 0.7	8.9 ± 0.7	8.1 ± 0.4
Maximum relaxation to acetylcholine (3.2 μmol l^{-1}) as % contraction to noradrenaline	46.4 ± 2.9	24.0 ± 4.3%**	31.8 ± 3.9	9.1 ± 1.5%**

Values are means ± s.e. mean.
Difference from control significant at $^{**}P < 0.01$.

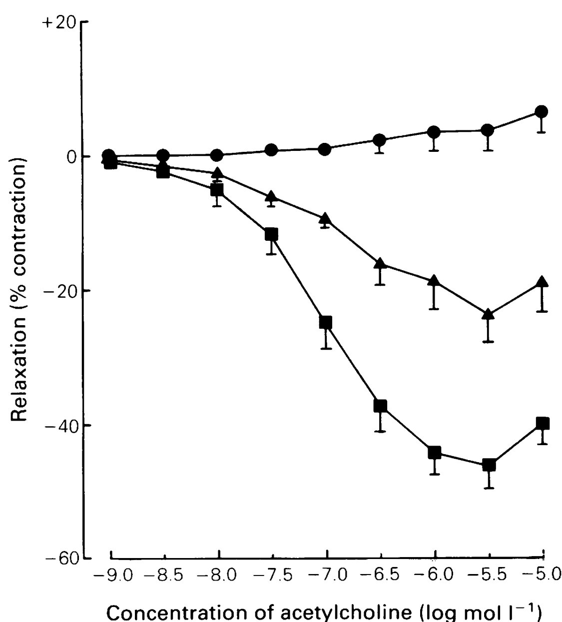

Figure 2 Cumulative concentration-effect curves to acetylcholine (ACh) in aortic rings from control rabbits and from rabbits of 8 weeks of age given 2% cholesterol diet for 4 weeks i.e. the animals were 12 weeks of age at the time of the study. The ordinate scale shows the relaxation expressed as a percentage of the contraction to noradrenaline (1 μmol l^{-1}). The control curve (■) is different from the curve obtained from cholesterol-fed animals (▲) ($P < 0.05$). The rings without endothelium (●) showed a contractile response at higher concentrations of ACh (indicated by a positive value on ordinate scale).

Fifty week old rabbits

When rabbits were started on a 4 week diet containing 2% cholesterol at 46 weeks of age, their food intake decreased considerably, resulting in a significant loss of weight (Table 2). Serum cholesterol and triglyceride levels in experimental animals were significantly elevated compared to control animals (Table 2). However, serum cholesterol level in rabbits fed a 2% cholesterol diet starting at 46 weeks of age was significantly less than that in the rabbits fed this same diet starting at 8 weeks of age ($P < 0.05$).

After 4 weeks of feeding with a 2% cholesterol diet the animals showed fat deposition in the liver although less fat appeared to be present in subcutaneous tissue, mesentery and retro-peritoneal tissue than observed in the younger experimental animals (no quantitative data are available). The aortae from 8 to 9 older experimental animals did not show any evidence of fatty streaks or plaques. One animal demonstrated fatty streaks. Except for this animal the others did not show any evidence of sudanophilia (Figure 1b). The extent of sudanophilia in the aorta in the experimental group was not significantly different from the controls (Table 3, $P > 0.05$).

Preparations from both control and experimental groups contracted with the addition of NA. The mean tensions attained in the two groups were not significantly different from each other (Table 3, $P > 0.05$). When ACh was added the control rings demonstrated a concentration-dependent relaxation similar to that observed in the younger control animals.

Relaxation to ACh in the experimental group was significantly less than that observed in the control

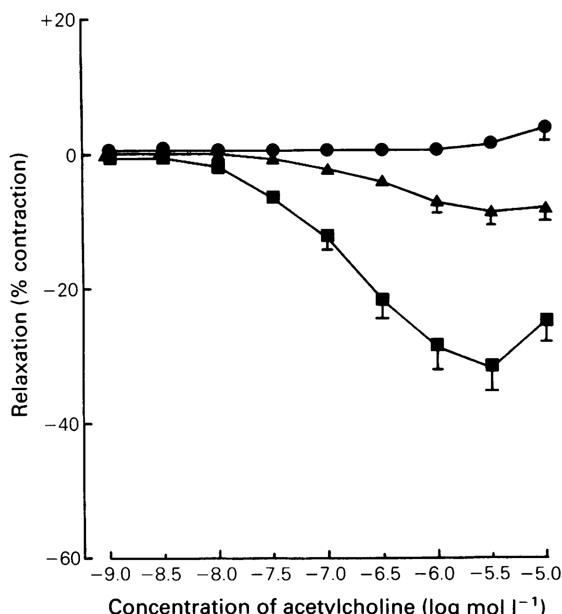

Figure 3 Cumulative concentration-effect curves to acetylcholine (ACh) in aortic rings from control rabbits and from rabbits of 46 weeks of age given 2% cholesterol diet for 4 weeks i.e. the animals were 50 weeks of age at the time of the study. The ordinate scale shows the relaxation expressed as a percentage of the contraction to noradrenaline ($1\,\mu mol\,l^{-1}$). The control curve (■) is different from the curve obtained from cholesterol-fed animals (▲) ($P < 0.05$). The rings without endothelium (●) showed a contractile response at higher concentrations of ACh (indicated by a positive value on ordinate scale).

group ($n = 9$, $P < 0.05$, Figure 3). No relaxation to ACh was seen in the rings without endothelium.

Histology

(i) *Light microscopy* In aortic rings from control animals (both 12 weeks and 50 weeks of age) the endothelium formed a continuous layer. The sub-endothelial layer extending up to the internal elastic lamina was of uniform thickness and had collagen and elastic fibres. Accumulations of lipid laden cells or plaques were not seen in these areas.

In the 12 week experimental animals, lipid laden cells were observed in the sub-endothelial layers of the vessel wall. In some animals, raised intimal lesions (plaques) were seen and these occurred more frequently in proximal areas than in distal areas of the thoracic aorta. Plaques had variable combinations of lipid laden cells (foam cells) and extracellular lipid deposits which were larger towards the base of the plaques. Aortic rings from the 50 week experimental animals did not show plaques on the intimal surface, lipid laden cells or extracellular lipid deposits in the sub-endothelial layer.

(ii) *Scanning electron microscopy* (*SEM*) In control animals (both 12 and 50 weeks of age) the endothelial cells formed a continuous sheet lining the lumen. The cells appeared uniform and smooth on scanning electron microscopy (Figure 4). Longitudinal folds were seen in a few areas. Individual cells were identifiable with clear margins in most areas. These cells did not appear to be oedematous and had no surface projections. Rings from which endothelium had been removed deliberately were devoid of endothelial cells on the intimal surface, revealing fibres of the sub-endothelial layer (Figure 4).

Scanning electron microscopy of the intimal surface of the aortic rings from the 12 week experimental animals demonstrated several abnormalities (Figure 4). The fatty spots recognized macroscopically and with light microscopy appeared raised on SEM. 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 shaped in most cases with irregular edges. The edges of the plaques were usually well-defined from the surrounding areas. In most aortic rings from cholesterol-fed rabbits, approximately 85% of the surface appeared intact. The endothelial cells in the aortic rings appeared oedematous and in some areas thin filamentous projections or short projections with a broad base were seen (see Figure 4). With the cells becoming swollen and globular they appeared to be separating from each other. In such areas multiple strands or bridges running between the cells were noted (not shown). 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 (not shown). Some areas of apparently normal looking endothelium (i.e. the appearances seen in control animals) were seen in cholesterol-fed animals. Scanning electron microscopy of aortic rings from the 50 week experimental animals also showed areas which appeared similar to those observed in the younger experimental animals.

(iii) *Transmission electron microscopy* (*TEM*) In control animals the cytoplasm of the endothelial cells contained numerous vesicles (not evident on micrograph). The sub-endothelial layer was of uniform thickness and showed elongated smooth muscle cells with branching processes and lamellae of elastic fibres, in between these cells (Figure 5).

In the experimental animals of 12 weeks of age the endothelial cells showed vacuoles of different sizes spread throughout their cytoplasm. These appeared

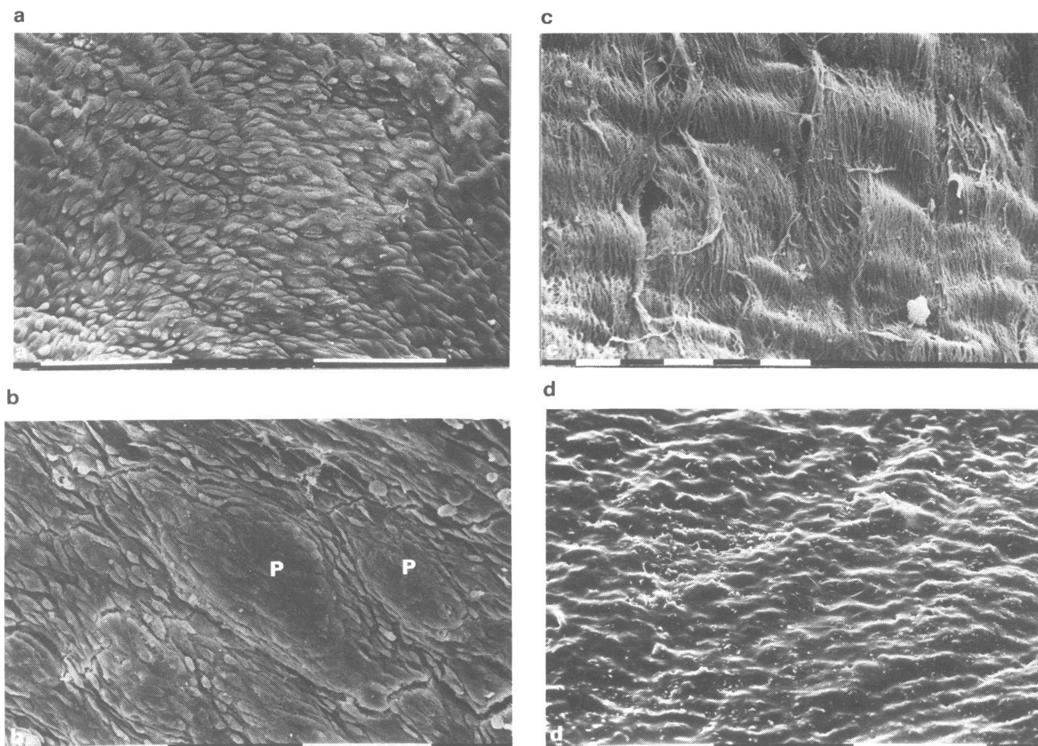

Figure 4 Scanning electron micrographs of luminal surfaces of rabbit thoracic aortae. The black and white bars at the bottom of each picture show the scales as defined below. (a) Endothelial cells on the intimal surface of a rabbit aortic ring from a control animal (12 weeks*) fixed and processed at the end of an experiment (×326; Bar = 0.1 mm). (b) Intimal surface of an aortic ring from young animal (12 weeks*) given 2% cholesterol for 4 weeks showing two small plaques (P). The cells in this preparation appear larger and swollen (especially in the top right hand corner) compared to controls, probably as a result of intracellular oedema (×312; Bar = 0.1 mm). (c) Intimal surface of a de-endothelialized ring taken from a control animal (50 weeks*) showing fibres in the sub-endothelial layer (×1150; Bar = 10 μm). (d) Intimal surface of an aortic ring from an older animal (50 weeks*) given 2% cholesterol for 4 weeks showing loss of definition of cell margins: Small filamentous projections from the cell surface are seen (×326; Bar = 0.1 mm).

* Refers to age of animal at time of study.

empty, probably as a result of extraction of lipids by reagents. The sub-endothelial layer was not of uniform thickness and contained foam cells. Vacuoles were found inside these foam cells as well as in the extracellular space. The vacuoles towards the bases of the plaques appeared larger than those at the apices. The majority of vacuoles were of ellipsoid profile, electron-translucent and did not appear to be limited by a membrane. Most of these vacuoles were 'empty' (probably the result of extraction of lipid contents by the reagents) while a few contained concentrically arranged irregular structures. Vacuoles were also seen interspersed between the smooth muscle cells of the tunica media. The organelles (e.g. mitochondria) in the endothelial and smooth muscle cells appeared more prominent than in controls. In some sections, membrane-bound areas of flocculent material (probably representing areas of cytoplasmic degeneration) were seen (Figure 5). These are similar to 'ghost bodies' described by other authors (Florentin *et al*., 1968).

The experimental animals of 50 weeks of age did not show the prominent changes of atherosclerosis which were seen in the younger (12 weeks) animals. Although the endothelial cells had abnormal vacuoles, the sub-endothelial changes were less conspicuous compared to the vacuolation in the younger experimental animals. Very few lipid-laden cells were seen in the sub-endothelial layer of these animals. The TEM appearances of the intima and adjacent media of the aortae from control and cholesterol-fed animals are shown in Figure 5.

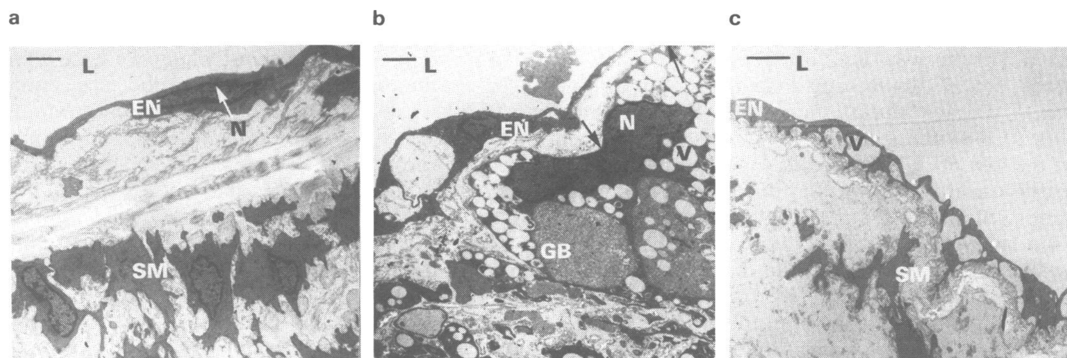

Figure 5 Transmission electron micrographs of sections from rabbit thoracic aortae. (a) Specimen from control animal (12 weeks*) showing an endothelial cell and smooth muscle cells of the media (× 5600; Bar = 2 μm). Cytoplasmic vesicles in the endothelial cell are not readily visible at this magnification. (b) Section through a plaque from a young animal (12 weeks*) given 2% cholesterol for 4 weeks showing a foam cell. Numerous lipid vacuoles are present in this cell and elsewhere in the sub-endothelial region. The foam cell (indicated between arrows) has two adjacent areas containing granular material which are probably 'ghost bodies' (GB; see text) (× 5600; Bar = 2 μm). (c) Section from an older animal (50 weeks*) given 2% cholesterol for 4 weeks showing vacuolation in endothelial cells (× 7200; Bar = 2 μm). In contrast to (b), there are no lipid vacuoles below the endothelial cells.
EN = endothelial cell, SM = smooth muscle cell, L = lumen, N = nucleus, V = vacuole.

* Refers to age of animal at time of study.

Discussion

The present study demonstrates an impairment of the endothelium-dependent relaxation to ACh in 12 week old rabbits made atherosclerotic by feeding a diet containing 2% cholesterol and other lipids. Furthermore, in 50 week old rabbits fed a similar diet, an impairment of the EDR was observed although the aortae did not show morphological features suggestive of classical atherosclerosis, i.e. pathological changes in the sub-endothelial layer with the formation of plaques. Since the characterization of the EDR by Furchgott & Zawadzki (1980) there has been considerable interest about its role in animals and man (Cherry *et al.*, 1982). EDR has been described in human blood vessels (Cherry *et al.*, 1982; Kalsner, 1985) with speculation that coronary artery spasm could be related to an impairment of the EDR by metabolites released from platelets (Shepherd & Vanhoutte, 1985).

Recent studies from this laboratory (Jayakody *et al.*, 1987) as well as from others (Habib *et al.*, 1984; Verbeuren *et al.*, 1986; Chappell *et al.*, 1987) have demonstrated an impairment of the EDR to ACh in rabbits made atherosclerotic by feeding a high cholesterol diet. These results were re-confirmed in the present study. This impairment of the endothelium-dependent relaxation in atherosclerosis could be due to one or more of the following factors:

(1) Impaired cholinoceptor activity: Bossaller *et al.* (1987) demonstrated that EDR to ACh is impaired in atherosclerotic human coronary arteries and strips of atherosclerotic rabbit aorta. However, the relaxations to histamine and substance P were preserved partially. The relaxations to A23187 were preserved completely at high concentrations of the ionophore ($1.0\,\mu mol\,l^{-1}$). As a result of these findings, Bossaller *et al.* (1987) concluded that the defect in atherosclerotic vessels was a partial selectivity for ACh at the muscarinic receptor level rather than an inability of endothelial cells to produce and release EDRF. However, this interpretation is not supported by observations from bioassays where it has been shown that the production/release of EDRF was impaired in atherosclerotic vessels (Sreeharan *et al.*, 1986; Goodwin *et al.*, 1987; Verbeuren *et al.*, 1987).
(2) Loss of endothelial cells by the atherosclerotic process: this factor is unlikely to account for the loss of EDR as greater than 75% of the endothelium was still physically intact in the aortic rings from atherosclerotic animals in the present study.
(3) Impaired ability to synthesize/release the EDRF due to functional/structural alterations in the endothelial cells.
(4) Impaired diffusion and/or enhanced degradation of relaxant factors during transit from endothelial cells to the underlying smooth muscle.
(5) Impairment of the ability of the smooth muscle cells of the atherosclerotic aorta to relax in response to the EDRF.

Recent studies (Sreeharan *et al.*, 1986) using superfused preparations demonstrated that a control

(non-atherosclerotic) recipient rabbit aortic ring relaxed significantly less when superfused via an atherosclerotic donor (approximately 50 mm long piece of thoracic aorta) than via a control donor (using ACh as the stimulus). These results indicated that the loss of endothelium-dependent relaxation in the atherosclerotic rabbit was, due at least in part, to an impaired ability to synthesize/release the EDRF.

In a study using the atherosclerotic rabbit aorta, Goodwin *et al.* (1987) found that EDR to both ACh and A23187 ($1.0\,\mathrm{pmol\,l^{-1}}$ to $0.1\,\mathrm{mmol\,l^{-1}}$) were impaired significantly compared to controls. Using a bioassay method the same authors showed that A23187 produced less relaxation when administered through the atherosclerotic aorta than the normal aorta. They concluded that EDRF production in response to both receptor-mediated and non-receptor-mediated agonists was reduced in atherosclerotic vessels. Impaired EDR in atherosclerotic vessels is at least in part related to impaired production of EDRF. Verbeuren *et al.* (1987) obtained results similar to those of Goodwin *et al.* (1987) with ACh.

Part of the findings and conclusions of Bossaller *et al.* (1987) could be explained on the basis that A23187 is approximately 10–30 times more potent than ACh in producing EDRF in rabbit aorta (Zawadzki *et al.*, 1980; Furchgott *et al.*, 1981). Against high levels of tone, the maximal relaxation produced by A23187 ($0.1\,\mu\mathrm{mol\,l^{-1}}$) was always greater than that produced by ACh (1.0 to $3.0\,\mu\mathrm{mol\,l^{-1}}$). In the presence of maximally effective concentrations of A23187 (0.1–$1.0\,\mu\mathrm{mol\,l^{-1}}$), ACh produced no additional relaxation in contracted arteries (Furchgott *et al.*, 1983). However, A23187 produced further relaxation following maximum relaxation with ACh. Thus, the absence of a difference between atherosclerotic and control vessels with high doses of A23187 may be related to the high potency of the compound overcoming a partial impairment of synthesis of EDRF.

Further evidence to support the conclusion of an impaired ability to synthesize/release the EDRF in the atherosclerotic rabbit aorta is evident in the present study. The aortae from 12 week old cholesterol-fed rabbits demonstrated changes compatible with experimental atherosclerosis in the rabbit. Thus, lipid deposits in the sub-endothelial layer leading to the formation of plaques were found in the present study. The morphological alterations in the sub-endothelial layer (through which the EDRF has to diffuse to reach the smooth muscle cells) has been suggested as a factor responsible for the impairment of EDR in the atherosclerotic aorta. However, scanning and transmission electron microscopy have revealed ultrastructural changes in the endothelial cells (responsible for generation of the relaxant factor) which could result in alteration of function. Thus, the endothelial cells were found to be oedematous with holes and surface projections on their surface. Further, large vacuoles were found in the endothelial cells on transmission electron microscopy. These findings are similar to those described by others (e.g. Duff *et al.*, 1957; Klimov *et al.*, 1981).

In the older (50 week) cholesterol-fed rabbits, the aortae did not show any evidence of atherosclerosis on macroscopic examination, Sudan Red staining or light microscopy. Thus the aortae did not take up any Sudan Red and no evidence of atherosclerotic plaques were found on light microscopy. Further, except in one animal, the light microscopic appearances of the vessel walls were not different from those of the controls. The aortic wall from one animal demonstrated lipid laden cells in the intima. In contrast to the above findings, scanning and transmission electron microscopy of the endothelium in 50 week experimental animals revealed abnormalities similar to those observed in the 12 week experimental animals. Thus, the endothelium of the aorta appeared to be structurally abnormal in these animals. The impairment of the EDR in these aortic rings suggests an impairment in the synthesis/release of the relaxation factor from the endothelial cells. An impairment in diffusion and/or an increased degradation of the relaxation factor during transit from the endothelial cells to the smooth muscle appears less likely in view of the paucity of demonstrable changes in the sub-endothelial layer.

A further observation in the present study is the greater susceptibility of young rabbits for developing hypercholesterolaemia and atherosclerosis (when fed a diet supplemented with cholesterol) compared with older rabbits. This effect has been shown previously by West *et al.* (1982) and Ignatowsky (1909). However, in the older animals, evidence of ultrastructural abnormalities in the aortic endothelium appeared before the development of changes suggestive of classical atherosclerosis. Further, these ultrastructural abnormalities were accompanied by an impairment of the EDR to ACh.

The findings in the present study are complicated by the fact that the older experimental rabbits demonstrated a significant loss of weight during the period of high cholesterol feeding. The effect could be attributed to a reduced intake of food (and cholesterol) during the period of the study. In addition, it has been shown that older rabbits have a reduced absorption of cholesterol (Thomson, 1981). The effect of the loss of weight on the morphological changes observed in the endothelium remains unclear at present. Hypercholesterolaemia has been shown to occur with weight loss in rabbits (Swaner & Connor, 1975). Thus, the loss of weight observed in the present study may have contributed to the loss

of EDR in the present study. However, this impairment of the EDR appears to be due to an impairment of the synthesis and/or release of the EDRF in the endothelial cells as no significant structural abnormalities were identified in the sub-endothelial layer and the smooth muscle cells in the media. Thus in summary, the present study has demonstrated that cholesterol feeding impairs EDR in both young (8 weeks) and older (46 weeks) rabbits. However, in the older animals, there is a dissociation of the impairment of EDR from the changes of classical atherosclerosis in the rabbit aorta.

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