Br. J. Pharmacol. (1994), **113**, 190–194

Abolition of flow-dependent EDRF release before that evoked by agonists in hypercholesterolaemic rabbits

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1 We have used a pulsatile cascade bioassay system to investigate the effects of dietary-induced hypercholesterolaemia on EDRF release evoked by acetylcholine and by the oscillatory and time-averaged components of flow, in isolated segments of rabbit abdominal aorta.

2 Flow pulsatility (frequency range 0.1–10 Hz) was studied with constant flow (9 ml min^{-1}) at a pulse pressure amplitude of 2 mmHg. Frequency-related EDRF release, maximal at 6 Hz, was slightly attenuated after 4 weeks and abolished after 8 weeks of cholesterol feeding.

3 Time-averaged shear stress was manipulated with dextran (1–4% w/v, 80000 mol. wt.), to increase perfusate viscosity. EDRF release induced by increased perfusate viscosity was unaffected after 4 weeks but abolished after 8 weeks of cholesterol feeding.

4 Endothelium-dependent relaxations to acetylcholine (0.1–10 μM) were not influenced after 4 weeks and only partially attenuated (by 60% of the maximal response, EC_{50} unchanged at 6.45 ± 0.04 vs. 6.4 ± 0.1 μM) after 8 weeks of cholesterol feeding.

5 Blood cholesterol levels were significantly ($P<0.001$) increased after 4 weeks (26 ± 3.6 vs 2.6 ± 0.6 mmol l^{-1}) and 8 weeks (56.2 ± 3.8 vs 1.3 ± 0.1 mmol l^{-1}) of cholesterol feeding but after 8 weeks plasma L-arginine levels were not significantly different from the age-matched controls (0.2 ± 0.05 vs. 0.19 ± 0.04 mmol l^{-1}).

6 We conclude that hypercholesterolaemia impairs flow-related (pulsatile- and time-averaged shear-induced) EDRF release earlier than acetylcholine-induced relaxation in rabbit aorta. This is consistent with the view that different transduction mechanisms mediate EDRF release in response to agonists and flow.

Keywords: Rabbit aorta; pulsatile flow; hypercholesterolaemia; endothelium-derived relaxing factor (EDRF)

Introduction

Atherosclerosis is a major inflammatory disorder of the arterial wall that is characterized by recruitment of monocytes and their transformation into lipid-rich foam cells, smooth muscle proliferation, intimal thickening and deposition of extracellular matrix to form fibrous plaques. It has been proposed that injury to the endothelium is the initiating event in atherogenesis and progression of the disease occurs as a result of an excessive inflammatory-fibroproliferative response to this insult (Ross, 1993). A major risk factor in this disease process is hypercholesterolaemia which is associated with raised levels of low density lipoprotein (LDL), the principal carrier of cholesterol in the blood. Recently, oxidized LDL (OxLDL), a modified form of LDL, has been implicated both as a key factor in endothelial injury and a potential promoter of atheroma formation (Ross, 1993; Witztum, 1993).

A reduced level of EDRF activity could promote the atherogenesis by making the arterial wall a more thrombogenic and adherent surface for platelets and monocytes and by enhancing smooth muscle proliferation (Moncada *et al.*, 1988; Garg & Hassid, 1990; Bath *et al.*, 1991). Indeed, regions of blood vessel prone to atherosclerosis are associated with areas of low longitudinal endothelial shear stress and thus presumably a low degree of mechanical stimulus for EDRF synthesis (Ku *et al.*, 1985). An early manifestation of hypercholesterolaemia is an impairment in endothelium-dependent responses to specific agonists in both animals and human subjects (Verbeuren *et al.*, 1986; Bossaller *et al.*, 1987; Forstermann *et al.*, 1988; Flavahan, 1992). This dysfunction displays regional differences in conduit arteries, with the most severe impairment occurring at bifurcations and in the proximal aorta (Ragazzi *et al.*, 1989, McLenachan *et al.*, 1990) and is not a consequence of decreased vascular smooth muscle responsiveness since relaxations to exogenous donors of nitric oxide are unaffected (Bossaller *et al.*, 1987; Flavahan, 1992). The abnormality also extends to the microcirculation, so that the effects of hypercholesterolaemia cannot simply be attributed to a diffusion barrier to EDRF resulting from intimal thickening or plaque formation as resistance vessels do not show these pathological features (Yamamoto *et al.*, 1988; Kuo *et al.*, 1992).

Flow-dependent dilatation is compromised in human conduit arteries by hypercholesterolaemia (Cox *et al.*, 1989) and flow-related EDRF activity is attenuated in resistance arteries from the porcine coronary circulation in an experimental model of atheroma (Kuo *et al.*, 1993). Recently Randall *et al.* (1993) have demonstrated that a 4 week dietary supplement of 1% cholesterol has little effect on agonist-dependent EDRF release but severely depresses the EDRF-dependent opening of pre-existing collateral vessels following arterial ligation in the rabbit ear. However, after 8 weeks dietary supplementation both collateral perfusion and agonist-induced responses are significantly impaired. These observations may reflect a greater susceptibility of basal and flow-induced EDRF activity to the deleterious effects of hypercholesterolaemia than agonist-evoked responses and represent a further example of differences in the transduction mechanisms for flow- and agonist-induced EDRF release (Griffith *et al.*, 1987; Macarthur *et al.*, 1993; Hutcheson & Griffith, 1994). In the present study, a pulsatile flow cascade bioassay system was used to compare the effect of 4 and 8 weeks dietary-induced hypercholesterolaemia on flow- (both

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the oscillatory and time-averaged components) and agonist-induced EDRF release.

Methods

Feeding protocol

Male New Zealand White rabbits matched by age and weight (2–2.5 kg) were divided into two groups, one being fed a cholesterol-supplemented (1%) diet (Special Diet Services, Witham, Essex), the other kept on standard chow.

Experimental protocol

Half the rabbits in each group were killed by cervical dislocation after 4 weeks, the remainder after 8 weeks. Abdominal aortae were removed and placed into pre-gassed (95% O_2–5% CO_2, pH 7.4) Holman's Solution of the following composition (mM): NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5 glucose 11, sucrose 10, containing indomethacin (10 μM). N^G-nitro-L-arginine methyl ester (L-NAME) was used as a specific inhibitor of EDRF formation from L-arginine. Plasma L-arginine levels were determined by gas chromatography as described by Williams *et al.* (1993).

The bioassay system used to quantify EDRF release in response to changes in flow pulsatility has been previously described (Hutcheson & Griffith, 1991). Briefly, segments (3–5 cm) of endothelium-intact abdominal aorta from either the cholesterol or control group (the donor) were placed horizontally in an organ chamber filled with oxygenated buffer at 37°C. The preparations were perfused at a mean flow rate of 9 ml min^{-1} by a Watson-Marlow peristaltic pump (Type 503U), and an air-filled compliance chamber was connected immediately proximal to the infusion cannula. EDRF activity in the effluent from the donor vessel was assayed by relaxation of a preconstricted (phenylephrine; 300 nM) ring of endothelium-denuded thoracic aorta (the recipient) which was positioned directly below the organ bath outlet. Its tension was measured by an isometric force transducer (Dynamometer UFI) and the transit time between the donor and recipient was ca. 2 s. The recipient ring was taken from young control rabbits to eliminate the effects of aging on its ability to respond to EDRF.

The pulse frequency of perfusion was varied by employing silastic tubing (Watson-Marlow) of three different internal diameters (3.2 mm, 1.6 mm and 0.8 mm). The narrower the diameter of the tubing, the higher the frequency of the oscillatory flow required to perfuse the donor aorta at a mean flow rate of 9 ml min^{-1}. Technical details have been provided elsewhere (Hutcheson & Griffith, 1991). Flow rate was calibrated against pump speed for each diameter of tubing before each experiment. Maximum damping of the perfusion circuit was introduced by a 70 ml compliance chamber when studying pulse frequency-related effects (from 0.15 to 9.75 Hz), the amplitude of the pressure pulse then remaining at 2 mmHg. Dextran (80,000 mol wt; 1–4% w/v) was added to the perfusate to study changes in viscosity and thus time-averaged shear stress. Relaxations to the endothelium-dependent vasodilator, acetylcholine were also assessed. When constructing cumulative concentration-response curves to acetylcholine, atropine (3 μM) was superfused over the recipient ring to eliminate direct vasoconstriction. The presence or absence of endothelium was also confirmed histologically by *en face* silver staining of the donor vessel at the end of each experiment.

Drugs

Acetylcholine, phenylephrine, dextran 80, indomethacin, atropine and N^G-nitro-L-arginine methyl ester were obtained from Sigma Limited, Poole, Dorset. All drugs were dissolved in Holman buffer with the exception of indomethacin (5% w/v $NaHCO_3$ in distilled water).

Statistics

All data are given as mean ± s.e.mean, where n denotes the number of animals studied for each data point. Statistical analysis was assessed by Student's t test for paired and unpaired data as appropriate, $P<0.05$ being considered as significant. EC_{50} values for vasodilator responses were obtained from individual concentration-response curves as the concentration at which half maximal reduction in recipient tone occurred.

Results

Effects of cholesterol diet on plasma cholesterol and L-arginine levels

Plasma cholesterol levels in animals receiving a 1% cholesterol diet were 26 ± 3.6 mmol l^{-1} after 4 weeks, (n = 10) and 56.2 ± 3.8 mmol l^{-1} after 8 weeks (n = 10) both being significantly ($P<0.001$) elevated above their corresponding controls (2.6 ± 0.6 mmol l^{-1}, and 1.3 ± 0.1 mmol l^{-1}, n = 10 respectively). Plasma L-arginine levels were 0.2 ± 0.05 mmol l^{-1} after 8 weeks of cholesterol supplementa-

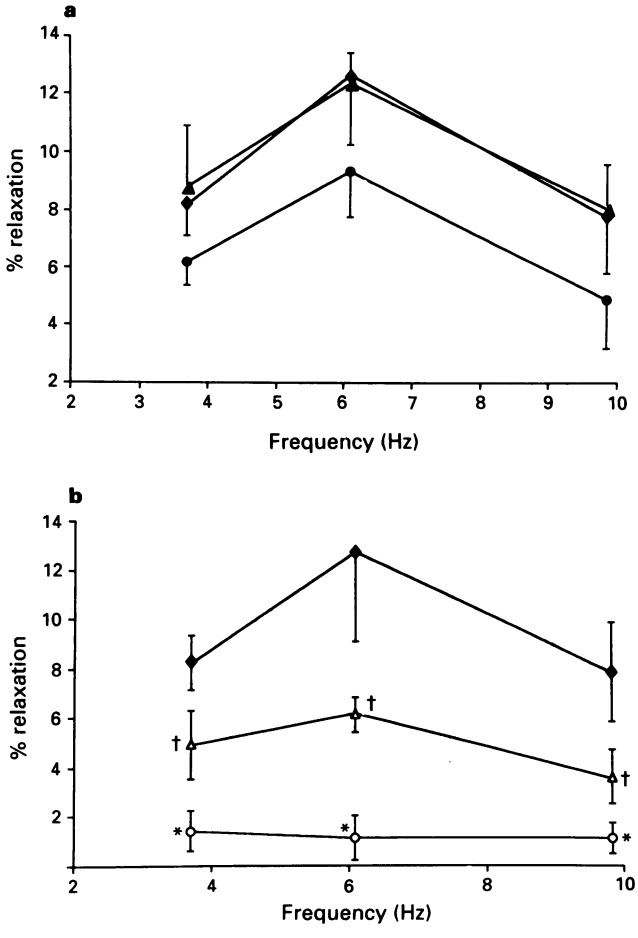

Figure 1 Effects of age and hypercholesterolaemia on frequency-related release of EDRF. This was significantly depressed after 8 but not 4 weeks in control animals. Hypercholesterolaemia abolished EDRF release after 8 weeks, but the slight reduction after 4 weeks was not statistically significant (†$P<0.05$ cf. 4 week control; *$P<0.05$ cf. 8 week control). (◆) Control 0–1 week; (▲) control 4 weeks; (△) control 8 weeks; (●) cholesterol-fed 4 weeks; (○) cholesterol-fed 8 weeks.

tion ($n = 8$) and were not significantly different from the age-matched control value of 0.19 ± 0.04 mmol l^{-1} ($n = 7$).

Frequency-dependent responses

Increasing the pulse frequency of the perfusate through segments of abdominal aorta from rabbits receiving a normal diet evoked relaxation of the recipient ring with a peak response at 6 Hz (Figure 1). However, the amplitudes of the responses at 6 Hz were significantly ($P < 0.05$) smaller in the 8 week ($7.1 \pm 0.8\%$) compared to the 4 week ($12.2 \pm 1.1\%$) control group ($n = 8$ and 4 respectively, Figure 1). Aortae from rabbits receiving a high cholesterol diet for 4 weeks produced smaller frequency-dependent relaxations of $9.2 \pm 1.6\%$ relative to control, but this did not achieve statistical significance (Figure 1a). In contrast, frequency-dependent relaxations were absent in aortae from the 8 week cholesterol-supplemented group ($P < 0.05$; $n = 8$; Figure 1b). No frequency-dependent relaxations were observed in the 4 and 8 week control groups following pre-incubation of the donor with L-NAME ($n = 4$ in each case).

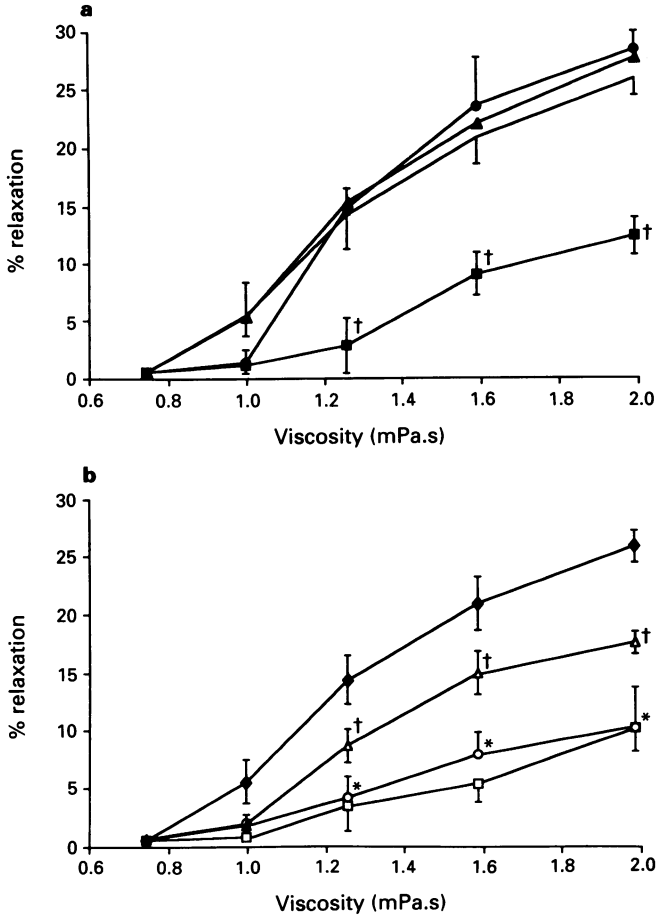

Figure 2 Viscosity-related EDRF release was abolished by 8 but not by 4 weeks of high cholesterol feeding. Control responses were again significantly depressed in the older animals. Note the small residual relaxation in the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) in both groups which represents a direct effect of dextran on the recipient. †$P < 0.05$ cf. 4 week control; (*$P < 0.05$ cf. 8 week control). (◆) Control 0–1 week; (▲) control 4 weeks; (△) control 8 weeks; (●) cholesterol-fed 4 weeks; (○) cholesterol-fed 8 weeks; (■) control 4 weeks + L-NAME; (□) control 8 weeks + L-NAME.

Viscosity-dependent responses

Dextran elicited concentration-dependent relaxations of the detector tissue when introduced into the aortic perfusate of both the 4 and 8 week control groups, although the absolute responses were significantly smaller ($P < 0.05$) in the older animals ($n = 4$ and 8 respectively; Figure 2). There was no significant loss of viscosity-related relaxations after 4 weeks of the cholesterol rich diet (Figure 2a) but those of the 8 week cholesterol-fed group were significantly ($P < 0.05$) reduced compared to their age-matched controls ($n = 4$, Figure 2b). The responses in the 4 week group were significantly attenuated by pre-incubation of the donor with L-NAME (Figure 2a), although a minor degree of relaxation to dextran was still evident which represents a direct effect of dextran on the recipient ring (Hutcheson & Griffith, 1994). In contast, L-NAME did not significantly decrease relaxation to dextran after 8 weeks of hypercholesterolaemia ($n = 4$, not shown). The effects of L-NAME in control vessels and those of 8 weeks of hypercholesterolaemia were similar in terms of their impairment of viscosity-related relaxations (Figure 2b).

Acetylcholine-induced responses

In the 4 week control group, acetylcholine evoked concentration-dependent, L-NAME-sensitive relaxations of the detector tissue in the cascade bioassay which were similar in terms of EC_{50} values and maximum reduction in tone to those obtained with donor aortae from 4 week cholesterol-fed rabbits (Table 1, Figure 3a). The maximum relaxation of control preparations from the 8 week group of animals was significantly lower than in the 4 week group (Table 1; Figure 3a and b), and was further depressed in animals fed a cholesterol-rich diet for 8 weeks (Table 1, Figure 3b).

Discussion

The object of the present series of experiments was to determine the degree to which EDRF release evoked by receptor stimulation, increased time-averaged shear stress and changes in the frequency of pulsatile flow is compromised in a model of dietary-induced hypercholesterolaemia. A previously described cascade bioassay system (Hutcheson & Griffith, 1991) was used to quantify EDRF release and the specificity of responses confirmed with L-NAME. The involvement of prostanoids was excluded by addition of indomethacin to the perfusate in all experiments.

Hypercholesterolaemia impaired EDRF release to both acetylcholine and flow, to an extent that was directly related to the duration of the dietary supplementation. After 8 weeks of hypercholesterolaemia, both viscosity- and frequency-

Table 1 Effect of age and cholesterol feeding on the EC_{50} values and maximum relaxations of the recipient ring to acetylcholine

	EC_{50} (−logM)	*Maximal response* (%)		EC_{50} (−logM)	*Maximal response* (%)
4-week group			*8-week group*		
Control	6.25 ± 0.06	57.2 ± 3.5 ($n = 4$)	Control	6.45 ± 0.04	34.5 ± 1.7 ($n = 4$)†
Cholesterol-fed	6.25 ± 0.02	57 ± 1 ($n = 4$)	Cholesterol-fed	6.1 ± 0.11	23.5 ± 7.7 ($n = 4$)*

Responses of endothelium-denuded aortic rings constricted by phenylephrine to EDRF released by aortae from rabbits fed a high-cholesterol diet for 4 and 8 weeks and that observed with the appropriate controls. Maximum responses were depressed both by age and dietary supplementation for 8 but not 4 weeks (†$P < 0.05$ cf. 4 week control: *$P < 0.05$ cf. 8 week control). EC_{50} values were similar in all groups.

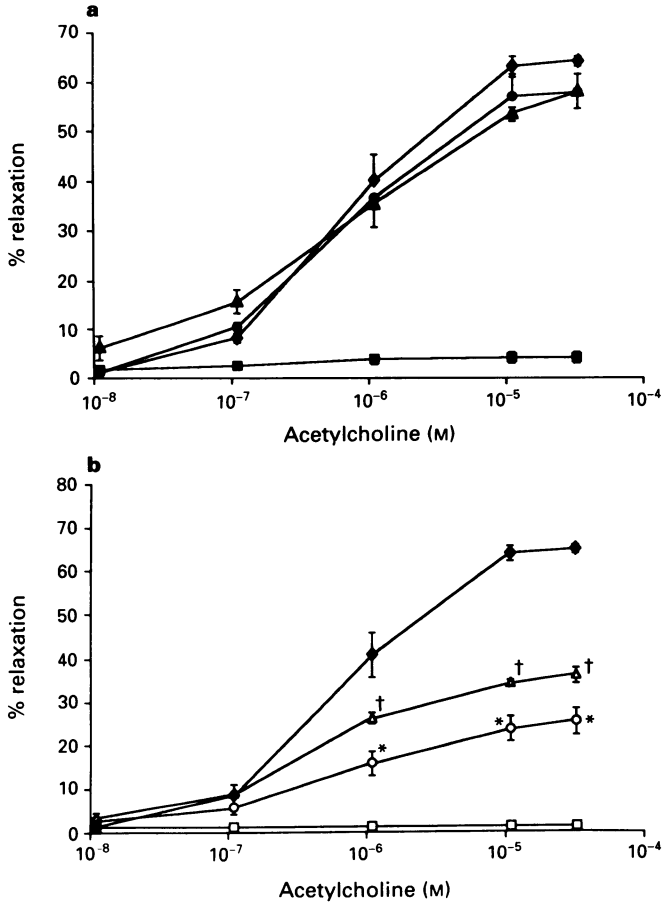

Figure 3 EDRF release evoked by acetylcholine was abolished by 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) and significantly depressed by 8 weeks of high cholesterol diet. As in Figures 1 and 2, control responses were significantly smaller in the older animals. (†$P<0.05$ cf. 4 week control; *$P<0.05$ cf. 8 week control). (◆) Control 0–1 week; (▲) control 4 weeks; (△) control 8 weeks; (●) cholesterol-fed 4 weeks; (○) cholesterol-fed 8 weeks; (■) control 4 weeks + L-NAME; (□) control 8 weeks + L-NAME.

related EDRF release were abolished (i.e. attenuated to the same extent as with 100 μM L-NAME) consistent with previous evidence that EDRF-mediated flow-dependent dilatation is impaired in hypercholesterolaemia (Kuo *et al.*, 1993; Randall *et al.*, 1993). The response to acetylcholine, however, was normal after 4 weeks of dietary supplementation and partially retained even after 8 weeks. This suggests that the response to acetylcholine is less susceptible to the deleterious effects of hypercholesterolaemia. It is possible that this may simply reflect varying strengths of stimulus on the same mechanistic pathway; however, it is also consistent with evidence from a number of groups that flow- and agonist-induced EDRF release involve distinct signal transduction mechanisms (Griffith *et al.*, 1987; Macarthur *et al.*, 1993; Hutcheson & Griffith, 1994). There was also a progressive decrease in both agonist- and flow-induced EDRF release over the 8 week period in the rabbits fed standard chow. This is consistent with previous reports of an age-related reduction in endothelium-dependent relaxation that does not simply reflect a reduced capacity of vascular smooth muscle to respond to nitrovasodilators (Shirasaki *et al.*, 1986).

The endothelium of atherosclerotic arteries becomes refractory to acetylcholine, ATP and 5-hydroxytryptamine, whereas responses to bradykinin and the receptor-independent calcium ionophore A23187 are preserved until a relatively late stage of the disease when there may be non-specific effects (Bossaller *et al.*, 1987; Shimokawa *et al.*, 1991; Flavahan, 1992). A similar pattern of impaired endothelial function follows administration of pertussis toxin to normal vessels, suggesting that hypercholesterolaemia selectively inhibits a pertussis toxin-sensitive G_i protein and/or its coupling to phospholipase C (Shimokawa *et al.*, 1991; Flavahan, 1992). Both the EDRF response to acute changes in shear and its upregulation by prolonged increases in flow are also reportedly sensitive to inhibition by pertussis toxin, which may reflect direct G_i protein–K_{Ca} channel coupling (Miller & Burnett, 1992; Ohno *et al.*, 1993). Interestingly, the endothelium which regenerates following balloon angioplasty exhibits prolonged functional impairment to agonists at the level of G_i protein subtype (Shimokawa *et al.*, 1990), but flow-dependent dilatation reportedly returns to normal after one week (Hayashi *et al.*, 1988). This raises the possibility that G_i proteins may be coupled to more than one transduction pathway.

Oxidized LDL may contribute to the dysfunction in hypercholesterolaemia as it is a potent and rapidly acting inhibitor of endothelium-dependent relaxation (Jacobs *et al.*, 1990; Flavahan, 1992), that may chemically inactivate EDRF (Jacobs *et al.*, 1990) and directly inhibit NO synthase (Mitchell *et al.*, 1992). It also contains lysophosphatidylcholine (LPC) which can impair endothelium-dependent relaxation (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990). This may result from transient activation of protein kinase C (Kugiyama *et al.*, 1992) which attenuates agonist-mediated endothelium-dependent relaxation by inhibiting a G_i protein (Flavahan *et al.*, 1991), depletion of internal Ca^{2+} stores (Inoue *et al.*, 1992), and direct disruption of receptor-G protein coupling (Flavahan, 1993). Lysophospholipids are also capable of rapid incorporation into the endothelial plasma membrane (Kugiyama *et al.*, 1990) potentially affecting transmembrane ion transport kinetics (Karli *et al.*, 1979). Such an action could specifically impair the flow response since in rabbit aorta, EDRF release induced by flow but not agonists involves the activation of K_{Ca} and K_{ATP} channels (Hutcheson & Griffith, 1994).

There is accumulating evidence that *in vivo* administration of L-arginine can improve impaired EDRF activity in conduit vessels from both animals and man, although this has not been a universal finding *in vitro* (Cooke *et al.*, 1991; Drexler & Zeiher, 1991; Mugge & Harrison, 1991). Complete restoration of 5-HT, histamine and ADP responses has nevertheless been reported in isolated coronary resistance arterioles from hypercholesterolaemic pigs (Kuo *et al.*, 1993). It has been suggested that the availability of L-arginine for NOS becomes a rate-limiting step for NO synthesis in hypercholesterolaemia, even though pathways exist to recycle L-citrulline to L-arginine at the expense of other amino acids (Mitchell *et al.*, 1990). However, plasma L-arginine levels were found to be normal in our hypercholesterolaemic rabbits. Similarly, enhanced formation of vasoconstrictor prostaglandins, which has also been proposed as an explanation for reduced endothelium-dependent relaxation in hypercholesterolaemic pigs (Shimokawa & Vanhoutte, 1989), cannot account for the impairment of flow- and agonist-induced reponses in the present study since the cyclo-oxygenase inhibitor indomethacin was present throughout.

In conclusion, this study confirms previous findings that short term hypercholesterolaemia impairs agonist- and flow-induced EDRF release in rabbit aorta. Both the pulsatile- and shear-related components of flow-induced EDRF release are abolished at an early stage in the disease process whereas acetylcholine-induced relaxations are still present reinforcing the suggestion that basal/flow-induced EDRF release is more susceptible to the deleterious actions of hypercholesterolaemia in conduit vessels. This greater susceptibility also provides further evidence that the mechanisms of pulsatile flow-induced EDRF release differ from those of agonist-induced release in this artery type.

This work was funded by a grant from the British Heart Foundation. The authors are grateful to Mrs R. Maylin for secretarial assistance and thank Professor G.M. Roberts of the University of Wales College of Medicine for his support and interest.

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(Received February 14, 1994
Revised April 19, 1994
Accepted April 27, 1994)