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Responses to endothelium-dependent agonists in subcutaneous arteries excised from hypercholesterolaemic men

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1 Vasomotor function of the vascular endothelium was examined in human subcutaneous arteries excised from 8 hypercholesterolaemic and 7 normolipidaemic subjects.

2 Left gluteal skin biopsies were performed under local anaesthesia. Subcutaneous arteries were isolated and two vessels from each subject mounted in separate myographs. A 20 ml fasting blood sample was taken at the time of the biopsy.

3 Hypercholesterolaemic subjects had either never been treated with lipid lowering therapy or therapy had been stopped at least two weeks before the study ($n=2$). At the time of the study total plasma cholesterol levels (control: 4.6 ± 0.3 vs hypercholesterolaemic: 8.3 ± 0.6 mmol l^{-1}: $P<0.01$) were significantly elevated in hypercholesterolaemic subjects when compared with controls.

4 Full concentration-response curves to the vasoconstrictor noradrenaline and the vasodilators acetylcholine and substance P were constructed. A single point concentration-response to sodium nitroprusside (10 μM) was also obtained. Dilator responses were obtained in vessels pre-constricted with a submaximal concentration of noradrenaline. Vessels were then incubated for 30 min with either L- or D-arginine (10 μM) and the concentration-response curves to the three dilator agonists repeated in the presence of the amino acid.

5 Maximum relaxation responses to acetylcholine (control vs hypercholesterolaemic: $83.3\pm6.1\%$ vs $47.4\pm13.5\%$; $P<0.05$), but not to substance P or sodium nitroprusside, were dampened in the hypercholesterolaemic group when compared with controls.

6 Neither incubation with L-arginine nor D-arginine had any effect on maximum relaxation responses to acetylcholine in either the control group (pre L-arginine vs plus L-arginine: 83.3 ± 6.1 vs $82.3\pm5.5\%$, pre D-arginine vs plus D-arginine: 98.9 ± 1.2 vs $98.2\pm1.1\%$) or the hypercholesterolaemic group (pre L-arginine vs plus L-arginine: 47.4 ± 13.5 vs $55.3\pm14.3\%$, pre D-arginine vs plus D-argenine: 43.3 ± 13.6 vs $65.4\pm12.3\%$).

7 When results from the two study groups were pooled, the strongest predictor of maximum relaxation obtained to acetylcholine was apolipoprotein A_1 ($r=0.67$; $P=0.001$).

8 In conclusion, relaxation responses mediated by the endothelium-dependent agonist acetylcholine, but not by substance P, are impaired in hypercholesterolaemic patients. L-Arginine did not improve the impaired relaxation responses to acetylcholine. We suggest that impaired endothelium-dependent relaxation is specific to acetylcholine and not to an abnormal L-arginine-nitric oxide pathway in subcutaneous arteries excised from this study group.

Keywords: Hypercholesterolaemia; nitric oxide; arginine; resistance arteries; acetylcholine; substance P

Introduction

The vascular endothelium plays an integral role in modulating vascular tone through production and release of various vasodilators and vasoconstrictors. Nitric oxide, a potent vasodilator, is synthesized within the endothelium and released tonically as well as in response to various mechanical and chemical stimuli such as acetylcholine (Moncada *et al*., 1991). Abnormal endothelium function has been observed in various cardiovascular disease states including hypercholesterolaemia (Creager *et al*., 1990; Drexler & Zeiher, 1991; Chowienczyk *et al*., 1992). In these clinical studies, endothelium dysfunction has been characterized as an impaired response to acetylcholine, whilst response to endothelium independent vasodilators such as sodium nitroprusside remained intact.

The mechanism by which elevated cholesterol levels inhibit acetylcholine-mediated responses is unclear. Since lipid lowering therapy (Leung *et al*., 1993; Chin & Dart *et al*., 1994; O'Driscoll *et al*., 1997) and even a single session of plasma low density lipoprotein (LDL) apheresis (Tamai *et al*., 1997) successfully corrects impaired dilator responses to acetylcholine, it would appear that the presence of pathophysiological levels of LDL cholesterol is necessary for impaired relaxation. Although the acute effects of lipoproteins on endothelium-dependent relaxations are still controversial, this assumption is consistent with findings that lipoproteins inhibit endothelium-dependent vasorelaxation by inactivating nitric oxide after its release from endothelial cells (Galle *et al*., 1991; Chin *et al*., 1992). On the other hand, resistance vessels excised from hypercholesterolaemics and bathed in physiological buffer (ie in the absence of plasma cholesterol) still exhibit impaired acetylcholine-mediated responses (Goode & Heagerty, 1995). Since responses to sodium nitroprusside were also blunted and further, since both responses to acetylcholine and sodium nitroprusside were normalized following 3-months of lipid-lowering therapy, the inference is that reversible damage at the level of the smooth muscle cell rather than the endothelium had occurred.

In the current study, we further investigated the effects of hypercholesterolaemia on the L-arginine/nitric oxide pathway

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in subcutaneous arteries excised from gluteal biopsies. Responses to two endothelium-dependent agonists, acetylcholine and substance P, which act via different receptors to stimulate nitric oxide release, were examined as were responses to sodium nitroprusside, a directly acting smooth muscle dilator. In addition, the role of L-arginine, the precursor of nitric oxide was also studied in the light of previous experiments demonstrating the restorative effects of this amino acid on impaired responses to acetylcholine in vessels isolated from healthy people (Chin-Dusting *et al*., 1996), as well as on *in vivo* responses in hypercholesterolaemic subjects (Drexler *et al*., 1991; Creager *et al*., 1992).

Methods

Gluteal skin biopsies were obtained from 7 control subjects recruited by advertisement and 8 hypercholesterolaemic subjects recruited through the Alfred Lipid Clinic at the Alfred Hospital, Melbourne, Victoria. All participants were male and subjected to a stringent screening process, consisting of a complete medical history and physical examination and, with the exception of lipid levels in those with hypercholesterolaemia, all had normal findings from routine haematology and biochemical blood analyses. At the time of the initial screening, fasting total plasma cholesterol and triglycerides were measured. All subjects had fasting triglyceride levels less than 2.5 mmol l^{-1}. Total cholesterol was less than 5.5 mmol l^{-1} in the control subjects and greater than 6.0 mmol l^{-1} in the hypercholesterolaemic subjects. Inclusion into the study was dependent on the absence of the following factors: hypertension, smoking within 6 months, taking medication or vitamin supplements or a history of heart disease. Hypercholesterolaemic subjects already taking lipid lowering therapy ($n=2$) were asked to abstain for a minimum of two weeks before the day of biopsy. The study was approved by the Alfred Group of Hospitals Ethics Committee and written informed consent was obtained from all subjects.

Lipid analysis

On the day of the biopsy a 20 ml fasting blood sample was obtained. Plasma lipoproteins were isolated by use of discontinuous density ultracentrifugation. Analysis of plasma and lipoprotein fractions were conducted on a COBAS Bio centrifugal auto analyser (Roche). Analytical kits were purchased from Boehringer Mannheim for total cholesterol (237574), free cholesterol (310329), triglyceride without free glycerol (450032), phospholipid (691844), apolipoprotein A_1 (1 378 686) and apolipoprotein B (1378694). Calibrators (759350: Precipath 781827) and normal (Precinorm 1285874) control sera were also from Boehringer Mannheim. Kits for lipoprotein (a) were purchased from Incstar (Atlantic Antibodies ATA 86084).

LDL particles

Plasma samples were stored frozen at -80°C until the time of assay. LDL was isolated by a two step gradient ultracentrifugation method (Sattler *et al*., 1992). The density of plasma was increased to 1.25 g ml^{-1} by addition of KBr, 0.8 ml of which was overlaid with saline (density 1.006), sealed in a 2.0 ml ultracentrifuge tube (Beckman Instruments 344625) and centrifuged for 60 min in a TLA 100.2 rotor (Beckman Instruments 344625) at 100,000 r.p.m. and 20°C. A single LDL band was removed by aspiration with a small gauge needle and syringe and analysed for LDL particle size. This was performed within 3 days.

LDL particle diameters were determined on 3–13% non-denaturing gradient gels (Gradipore GS313); 10 μl of LDL in 10 μl of non-denaturing sample buffer (90 mM Tris base, 80 mM boric acid, 3 mM EDTA, 3 mM sodium azide, 0.1% bromophenol blue and 10% glycerol) was loaded onto the gradient gel, adjacent to which was loaded a lane with polystyrene latex microspheres 28 nm diameter (Duke 5003A) diluted 1 in 10 in non-denaturing sample buffer and sonicated at low power for 30 s, and a lane of high molecular weight standards (Pharmacia 17-0445-01). Electrophoresis was carried out in non-denaturing running buffer (90 mM Tris base, 80 mM boric acid, 3 mM EDTA, 3 mM sodium azide) for 16 h at 190 V with circulation at 10°C, gels were fixed with 10% sulphosalicyclic acid for 1 h, stained with 0.04% PAGE Blue G90 (Electran 44248) for 3 h and destained with 5% acetic acid overnight or until the background was clear. LDL peak sizes were estimated against a standard curve created from the markers of known diameter: latex 28 nm, thyroglobulin 17 nm, ferritin 12.2 nm and catalase 10.4 nm.

LDL particle sizes were quantified for each peak in each individual. As previously noted (Sherrard *et al*., 1996), some individuals had more than one peak. In these patients, the diameter of each peak was determined as well as the percentage contribution of each peak to the total area under all peaks. The weighted average LDL diameter was thus determined for each subject. Since there was no significant difference between the peak of the major LDL sphere and the weighted LDL diameter, and virtually identical results were obtained in all statistical analyses when either were used, data in which the weighted diameter was used are shown.

Buttock subcutaneous arteries

The method used for isolating subcutaneous arteries from gluteal biopsies has previously been described (Chin *et al*., 1994). Briefly, a left gluteal skin biopsy was performed under local anaesthesia (lignocaine 1%). The skin sample was placed in ice-cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5 EDTA 0.026, glucose 5.5) and subcutaneous arteries isolated by use of a dissecting microscope (Nikon 102, zoom lens) within half an hour of the biopsy. Arteries were threaded onto $2\times$ 40 μm diameter stainless steel wires and mounted as ring preparations (approximately 2 mm long) in an isometric myograph (Scientific Instruments, Australia). Vessels were bathed in Krebs solution and bubbled with carbogen (95% O_2, 5% CO_2) at a pH of 7.4 and kept at a constant temperature of 37°C.

After 30 min each vessel was passively stretched to an internal circumference equal to $0.9\times L_{100}$, where L_{100} is the internal circumference at the level of passive stretch equivalent to a transmural pressure of 100 mmHg. This was calculated by use of the length tension relationship obtained for each vessel (Mulvany & Warshaw, 1979). After a 30 min equilibration period the viability of each vessel was confirmed with a high concentration potassium solution (KPSS, K^+124 mM).

Full cumulative concentration-response curves were constructed to noradrenaline, acetylcholine and substance P. Response to a single concentration of sodium nitroprusside (10 μM) was also obtained. Responses to vasodilators were obtained in vessels pre-constricted to a steady level of force (approximately 70–80% maximal constriction) to noradrenaline. When the concentration-response curves to the agonists had been obtained, arteries were incubated with either L-arginine or D-arginine (10 μM) for 30 min and the concentra-

tion-response curves repeated in the continued presence of the arginine analogues.

Data analysis

Contractile responses were measured as *g* force. Relaxation responses were measured as a percentage of the maximum contractile response obtained to noradrenaline. Individual concentration-response curves to each agonist were fitted to a logistic equation of the form $E = MA^P/(A^PK^P)$ where E is the response, M is the maximum response, A is the concentration eliciting E, K is the concentration eliciting 50% of the maximum response (i.e. $-\log EC_{50}$) and P is the slope parameter (Nakashima *et al*., 1982). From this equation, the concentrations corresponding to 10–90% response ($-\log EC_{10}$ to $-\log EC_{90}$) were determined. The $-\log EC_{50}$ value provided a measurement of tissue sensitivity to the agonists investigated.

All data are presented as the mean ± s.e.mean. For comparison of maximum relaxation and $-\log EC_{50}$ values between control and hypercholesterolaemic subjects Student's unpaired *t* test was used. For comparison of responses in the absence and in the presence of either arginine analogue within the same subject group, Student's paired *t* test was employed. All statistics were executed by use of SigmaStat Statistical Software, (Jandel Scientific, California, U.S.A.), which examines data for normality before a parametric test is applied. Non-parametric data were examined by Mann Whitney rank sum test. The level of significance employed for all statistical analyses was $P < 0.05$.

Pearson product moment correlation was used to examine the relationship between each lipoprotein parameter and maximum relaxation attained to acetylcholine before incubation of either arginine analogue. Only maximum relaxation responses attained in vessels before incubation with L-arginine were examined. Analyses were performed by use of SigmaStat Statistical Software (Jandel Scientific, California, U.S.A.). Multiple linear regression analysis was performed with SPSS, Release 6.1 for Windows (Illinois, Chicago).

Drugs

Drugs used were: (−)-noradrenaline bitartrate, acetylcholine chloride, substance P, sodium nitroprusside dihydrate and L- and D-arginine (Sigma). Stocks were made up on the day of the study and diluted in Krebs solution. All drugs were stored on ice until ready for use.

Results

The control group did not differ from the hypercholesterolaemic group in age (control vs hypercholesterolaemic: 48.9 ± 1.4 yrs vs: 41.4 ± 3.4 yrs; $P > 0.05$) or mean arterial pressure (control vs hypercholesterolaemic: 92.4 ± 4.0 mmHg vs 92.6 ± 3.3 mmHg; $P > 0.05$).

Lipid profile

Hypercholesterolaemic subjects were characterized by marked elevation in total, free and esterified cholesterol concentration in plasma low density (LDL) and very low density (VLDL) lipoproteins (Table 1). In addition hypercholesterolaemic subjects had increased triglyceride and phospholipid concentrations and smaller LDL particle diameters. There were no differences in HDL composition between hypercholesterolaemia and normal subjects.

Table 1 Lipids measured in the lipoprotein fractions, VLDL, LDL and HDL in control and hypercholesterolaemic subjects

Lipid mmol l^{-1}	*Control*	*Hypercholesterolaemia*	P *value*
	Total plasma		
Total cholesterol	4.6 ± 0.3	8.3 ± 0.6*	0.0001
Free cholesterol	1.0 ± 0.1	1.8 ± 0.2*	0.002
Est. cholesterol	3.5 ± 0.3	6.5 ± 0.4*	< 0.0001
Triglycerides	1.2 ± 0.2	1.9 ± 0.2*	0.029
Phospholipids	2.6 ± 0.2	3.7 ± 0.2*	0.001
Apoprotein A_1 (mg dl^{-1})	127.2 ± 4.8	114.6 ± 8.8	0.250
Apoprotein B (mg dl^{-1})	90.9 ± 9.5	172.2 ± 24.5*	0.011
Lipoprotein (a) (mg dl^{-1})	7.2 ± 1.1	27.4 ± 7.2*#	0.009
	Very low density lipoprotein		
Total cholesterol	0.4 ± 0.1	0.7 ± 0.1*	0.040
Free cholesterol	0.1 ± 0.0	0.3 ± 0.1*	0.021
Est. cholesterol	0.3 ± 0.1	0.6 ± 0.1*	0.006
Triglycerides	1.1 ± 0.3	1.1 ± 0.2	0.953
Phospholipids	0.3 ± 0.1	0.4 ± 0.1	0.072
	Low density lipoprotein		
Total cholesterol	3.0 ± 0.4	6.3 ± 0.5*	< 0.001
Free cholesterol	0.6 ± 0.1	1.3 ± 0.1*	0.021
Est. cholesterol	5.0 ± 0.4	2.4 ± 0.3*	< 0.001
Triglycerides	0.2 ± 0.0	0.5 ± 0.1*	0.020
Phospholipids	1.2 ± 0.1	2.2 ± 0.2*	0.001
Particle size (μm)	24.7 ± 0.0	24.5 ± 0.1*	0.034
	High density lipoprotein		
Total cholesterol	1.1 ± 0.1	1.1 ± 0.1	0.937
Free cholesterol	0.2 ± 0.0	0.1 ± 0.0	0.287
Est. cholesterol	0.9 ± 0.1	1.0 ± 0.1	0.910
Triglycerides	0.2 ± 0.0	0.2 ± 0.0	0.181
Phospholipids	1.0 ± 0.1	1.0 ± 0.1	0.879

Data shown are means ± s.e.mean. Est. cholesterol: esterified cholesterol. *$P < 0.05$; #non-parametric statistics.

Myograph results

The smallest viable vessel was obtained from each subject. Where the length of the vessel was >4 mm, it was halved and one vessel incubated with L-arginine and the other with D-arginine. The internal diameter of each vessel at L_{100} did not differ in the controls (L-arginine: 382.9±62.3 μm; D-arginine: 374.9±39.4 μm) compared with the hypercholesterolaemic group (L-arginine: 350.7±35.4 μm; D-arginine: 396.2±26.2 μm). The one point contraction response to a single concentration of potassium (124 mM) obtained at the beginning of each experiment was not different between control and hypercholesterolaemic subjects.

Noradrenaline

Maximum contractile responses to noradrenaline did not differ between control and hyperlipidaemic subjects (control: 3.8±0.6 g vs H: 4.2±0.5 g). There was also no difference in the potency of noradrenaline on vessels from either group ($-\log EC_{50}$ M values, control: 6.5±0.2 vs hypercholesterolaemic: 7.3±0.3). Incubation with either L-arginine or D-arginine had no effect on maximum contractile responses (data not shown) or sensitivity in either group (Table 2).

Acetylcholine

Maximum relaxation responses mediated by acetylcholine were impaired in vessels excised from hypercholesterolaemic subjects when compared to control subjects (control: 83.3±6.1% vs H: 47.4±13.5%; $P<0.05$; Figure 1). Tissue sensitivity did not differ between groups (Table 2).

Incubation with L-arginine did not alter maximum relaxation responses to acetylcholine in either control vessels or hypercholesterolaemic vessels (Figure 2). Similarly incubation with D-arginine had no effect on maximal relaxation responses (data not shown). Neither L- nor D-arginine affected tissue sensitivity (Table 2).

Substance P

Neither maximum relaxation responses (Figure 1) nor $-\log EC_{50}$ (Table 2) values were significantly different in vessels excised from control subjects when compared to hypercholesterolaemia subjects.

Incubation with either L- or D-arginine did not alter tissue sensitivity (Table 2) or maximal relaxation responses (Figure 2) in either hypercholesterolaemic or control subjects.

Sodium nitroprusside

Relaxation responses mediated by sodium nitroprusside 10 μM did not differ between control and hypercholesterolaemic subjects. Incubation with either arginine analogue had no effect on these responses to sodium nitroprusside (data not shown).

Correlations of % maximal relaxation responses and subject lipid profiles

The extent of the maximal dilatation to acetylcholine showed strong (>0.6) negative correlations with plasma total, free and esterified cholesterol concentrations and a strong (>0.6) positive correlation with plasma apolipoprotein A_1. In addition, there were strong negative (>0.6) correlations with LDL total, free and esterified and VLDL esterified cholesterol concentration. There was a borderline ($P=0.053$) positive correlation between acetylcholine relaxation and LDL particle size.

Due to potential problems with multicollinearity, stepped multiple regression analysis was performed with the inclusion of apolipoprotein A_1 as an independent variable with the addition, in each analysis, of one other variable. In all analyses apolipoprotein A_1 was the only significant independent variable and gave $r=0.67$.

Discussion

The major finding from this study was that endothelium-dependent relaxation mediated by acetylcholine was significantly impaired, whilst relaxation mediated by substance P was preserved, in subcutaneous resistance arteries excised from hypercholesterolaemic subjects. Endothelium-independent relaxation mediated by sodium nitroprusside was not altered in these vessels and incubation with the nitric oxide precursor L-arginine had no effect on the dampened responses to

Table 2 $-\log EC_{50}$(M) values for noradrenaline, acetylcholine and substance P and response to 0.1 mM sodium nitroprusside (% dilatation) in subcutaneous resistance arteries isolated from control and hypercholesterolaemic subjects in the absence or presence of L-arginine or D-arginine

	L-arginine		*D-arginine*	
	Absent	*Present*	*Absent*	*Present*
Noradrenaline				
Control	6.45±0.16	6.20±0.31	6.43±0.14	6.01±0.24
Hypercholesterolaemic	7.27±0.33	6.59±0.23	6.59±0.37	6.23±0.20
Acetylcholine				
Control	6.89±0.3	6.62±0.3	7.28±0.4	6.46±0.2
Hypercholesterolaemic	6.82±0.5	6.70±0.4	6.48±0.6	7.30±0.3
Substance P				
Control	9.78±0.4	9.4±0.2	9.46±0.6	9.46±0.2
Hypercholesterolaemic	10.01±0.4	9.07±0.3	9.27±0.2	9.18±0.3
Sodium nitroprusside				
Control	97.60±0.92	90.30±6.82	94.70±2.06	93.30±3.37
Hypercholesterolaemic	90.00±4.40	89.00±5.37	97.14±1.42	89.86±6.01

Results are given as mean±s.e.mean.

acetylcholine. Finally, we demonstrated that plasma levels of apolipoprotein A_1 were the strongest predictor of maximum relaxation mediated by acetylcholine in this preparation.

While the finding that L-arginine supplementation did not improve responses to acetylcholine has previously been observed in an *in vivo* study on forearm resistance arteries of patients with hypercolesterolaemia (Casino *et al*., 1994) and again in arteries isolated from hypercholesterolaemic rabbits (Bult *et al*., 1995), the findings from the present study differ from the one performed on similar arteries from hypercholesterolaemic subjects (Goode & Heagerty, 1995). Although both studies demonstrated a diminished response to acetylcholine, in the previous investigation these responses were improved by incubating the vessels with L-arginine. In addition, responses to sodium nitroprusside were significantly dampened. A probable difference between the two studies is the patient population studied. In the Goode and Haegerty study, hypercholesterolaemic subjects had plasma cholesterol levels that were 15–20% higher than the patients in the present study. In addition, blood pressure was significantly higher in the hypercholesterolaemic subjects in the Goode and Heagerty study (Goode & Heagerty, 1995) with systolic blood pressure recorded at 139 ± 4 mmHg compared with 123 ± 3 mmHg in controls ($P < 0.01$) and diastolic blood pressure at 84 ± 1 mmHg vs 75 ± 2 mmHg in controls ($P < 0.01$). Blood pressure recorded from subjects in the present study was not significantly different between groups. Thus, it may be that the differing results observed were due to the difference in the severity of the hypercholesterolaemia or the presence of other

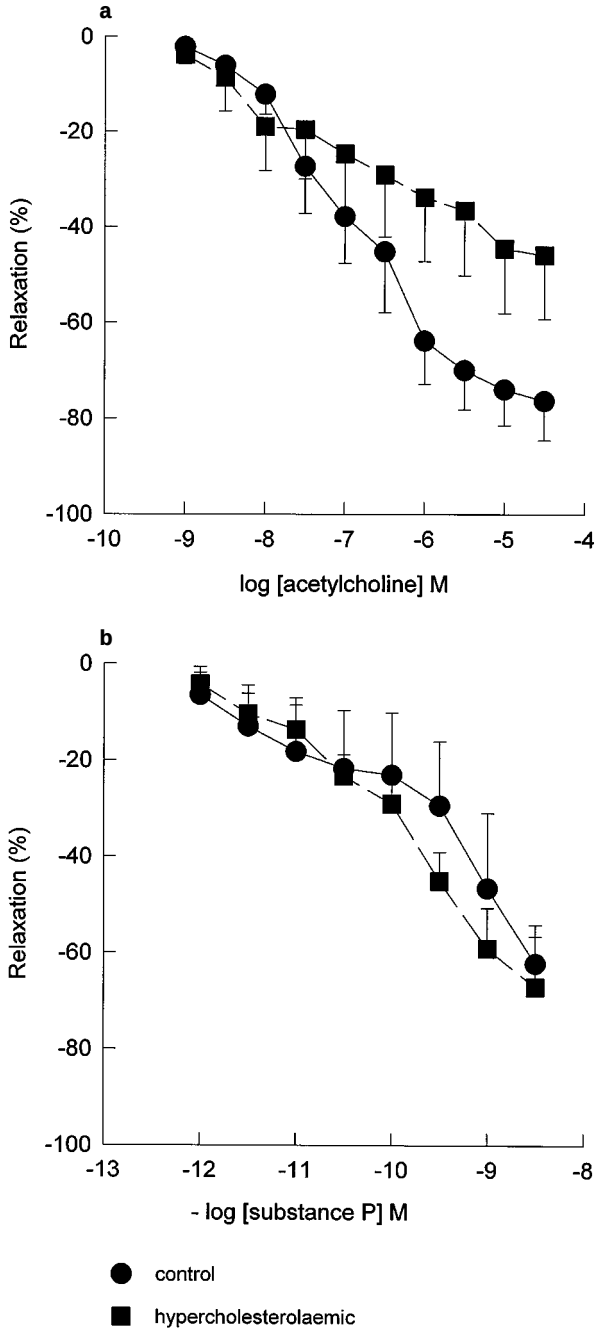

Figure 1 Concentration-relaxation curves obtained to acetylcholine (a) and substance P (b) in subcutaneous resistance arteries excised from control subjects ($n = 7$) and in patients with hypercholesterolaemia ($n = 8$). Each point represents the mean and vertical lines indicate s.e.mean.

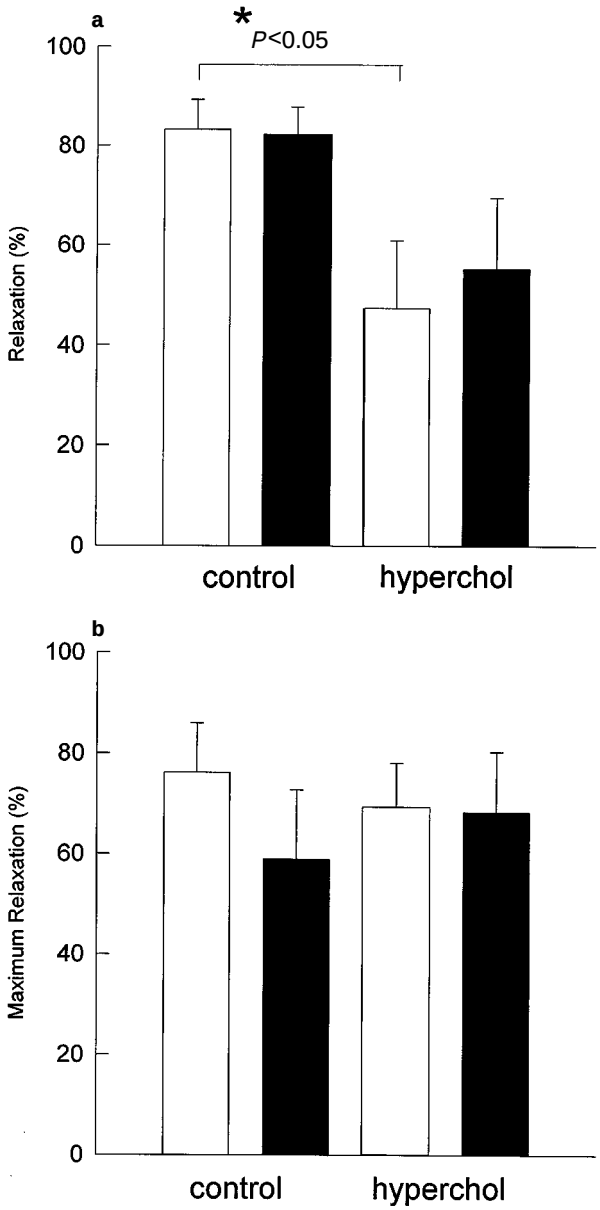

Figure 2 Shows the lack of effect of L-arginine 10 μM (open columns: pre L-arginine; solid columns: post L-arginine) on mean maximum relaxation responses ($\pm$s.e.mean) to acetylcholine (a) and substance P (b) in subcutaneous resistance arteries excised from control subjects ($n = 7$) and in patients with hypercholesterolaemia ($n = 8$).

risk factors. The hypothesis that the severity of the abnormality may be dependent on the severity of the disease was originally raised by Flavahan (1992), who concluded in his review, based on animal models of hypercholesterolaemia, that endothelium dysfunction observed in the early stages of the atherosclerotic process was limited to the pertussis toxin-sensitive, G_i protein-dependent pathway. A fundamental difference between the actions of acetylcholine and substance P is that the latter compound is pertussis toxin-insensitive (Macdonald *et al*., 1996) and this may be why only the pertussis toxin-sensitive responses to acetylcholine were affected at the moderate levels of hypercholesteroaemia seen in the present study. An important implication from the finding that dampened acetylcholine responses are still apparent in vessels removed from circulating plasma levels of lipids and placed in a physiological buffer free of lipoprotein is that the abnormality is not dependent on the continued presence of circulating lipoproteins. This would argue again the suggestion that lipoproteins inhibit endothelium-dependent vasorelaxation by inactivation of nitric oxide after its release from endothelial cells (Galle *et al*., 1991; Chin *et al*., 1992) and, instead, is consistent with the suggestion that the pathophysiological plasma levels of LDL modulate G_i coupling probably by down regulating the α_{i2} subunit of the protein (Liao, 1994). It is possible that the effect on G_i-coupling is still apparent upon removal from the plasma medium.

The relative contribution of nitric oxide, endothelium-derived hyperpolarizing factor and prostaglandins to the relaxations induced by acetylcholine and substance P in human buttock subcutaneous arteries has not been previously examined, nor was it investigated in the present study. The role of EDHF in particular may be of significant relevance given the recent finding that the contribution of EDHF to endothelium-dependent relaxations is significantly larger in human microvessels than in large arteries and that hypercholesterolaemia significantly impairs these relaxations (Urakami-Harasawa *et al*., 1997). Another explanation for the differences observed for the effect of hypercholesteroaemia on the two endothelium-dependent compounds is that the impaired responses in hypercholesterolaemic arteries reflect a muscarinic defect rather than an abnormality of endothelium function *per se*. This has previously been suggested for both human (Bossaller *et al*., 1987; Crossman *et al*., 1989) and rabbit (Bossaller *et al*., 1987) atherosclerotic arteries.

To investigate further the contribution of the lipoprotein profile to the dampened response to acetylcholine, univariate followed by multivariate analyses were undertaken. A positive correlation was observed between the concentration of apolipoprotein A_1 in plasma and maximum relaxation obtained to acetylcholine, despite there being no significant difference with this parameter between the two groups. Since each high density lipoprotein (HDL) contains one apolipoprotein A_1 molecule, the positive correlation also applies to HDL levels. The anti-atherogenic effect of HDL has been documented by the Framingham study (Castelli *et al*., 1986), and it may be that HDL exerts its protective action by an effect on endothelium function. The corollary to this is the finding of significant negative correlations between total and LDL plasma cholesterol (free and esterified) and maximal responses to acetylcholine. Univariate analysis revealed that all LDL lipid components correlated inversely, with the exception of LDL particle size which tended towards a positive correlation with relaxation. While it has been shown that multiple, small, dense LDL particles are associated with other cardiovascular risk factors (Sherrard *et al*., 1996), this is the first time a possible association between LDL particle size and abnormal endothelium function has been suggested.

In summary, we have demonstrated that subcutaneous arteries isolated from patients with uncomplicated hypercholesterolaemia display a dampened response to acetylcholine, while responses to substance P and sodium nitroprusside are unaffected. In these vessels the nitric oxide precursor, L-arginine, had little influence. Taken together with the findings from the Goode and Heagerty (1995) study, our findings are supportive of the hypothesis that the severity of the abnormality may be dependent on the severity of the disease (Flavahan, 1992). We speculate from this that a dampened response to acetylcholine, in the face of preserved responses to other endothelium-dependent compounds, is the first indication of endothelium abnormality and may be of predictive value for disease progression, ie that a dampened response to the pharmacological marker acetylcholine is the first preclinical sign of an abnormal endothelium function in people with high levels of cholesterol, and that it has predictive value for more extensive endothelial dysfunction in patients with severe hypercholesterolaemia.

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