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Vasorelaxant and antiaggregatory properties of the endothelium: a comparative study in normocholesterolaemic and hereditary and dietary hypercholesterolaemic rabbits

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1 A comparison of the effects of dietary and genetically-induced hypercholesterolaemia on the vasodilator and antiaggregatory capacity of the endothelium was made in rabbit isolated subclavian artery rings.

2 Dietary-induced hypercholesterolaemia in NZW rabbits decreased the maximum relaxation to carbachol (0.01 – 10 μM) and calcimycin (0.01 – 0.1 μM) in vessel rings precontracted with 5-hydroxytryptamine (5-HT), 0.1 μM), when compared to responses observed in rings obtained from control normocholesterolaemic NZW rabbits. The relaxant responses to SIN-1 (3-(4-morpholinyl)-sydnonimine hydrochloride) were attenuated but were not significantly different from controls. In Froxfield genetically hypercholesterolaemic (FHH) rabbits, the maximum relaxations to carbachol, calcimycin and SIN-1 were all reduced significantly.

3 Neither genetic nor dietary-induced hypercholesterolaemia modified the contractile responses of vessel rings to either KCl (10 – 100 mM) or 5-HT (0.01 – 10 μM).

4 Endothelium-dependent inhibition of collagen-induced platelet aggregation in whole blood was demonstrated by stimulation of a vessel ring, incorporated into the blood sample, with carbachol (10 μM, final blood concentration). This effect was inhibited by N^G-nitro-L-arginine (L-NOARG, 100 μM). SIN-1 (10 μM, final blood concentration) also decreased whole blood platelet aggregation, but only in the presence of an unstimulated vessel ring, and this was unaffected by L-NOARG. Superoxide dismutase (150 u ml^{-1}) did not influence the inhibition of aggregation by either a carbachol-stimulated vessel ring or by SIN-1.

5 Carbachol-stimulated artery rings from FHH rabbits inhibited platelet aggregation to a similar extent to that seen with rings from control normocholesterolaemic rabbits. Rings from hypercholesterolaemic NZW rabbits, however, did not significantly inhibit platelet aggregation when stimulated with carbachol. SIN-1 inhibited platelet aggregation in the presence of rings from either group of hypercholesterolaemic rabbits.

6 Hypercholesterolaemia induced by dietary modification induces changes in endothelial function which are characteristically different from those seen in genetically hypercholesterolaemic rabbits. It appears that dietary-induced hypercholesterolaemia primarily decreases NO release from the endothelium, while in genetically-induced hypercholesterolaemic vessel rings NO is released but there is a decreased responsiveness of the vascular smooth muscle cells to NO. This may reflect differences in the age and severity of the atherosclerotic lesions in the two groups of rabbits.

Keywords: Hypercholesterolaemia; endothelial function; NO release; endothelial inhibition of platelet aggregation

Introduction

Atherosclerosis is an occlusive disease which develops in response to various vascular insults such as hypercholesterolaemia (Ross *et al.*, 1993). Hypercholesterolaemia may be dietary or genetically determined and it is principally the lipids and lipoproteins associated with this condition that cause endothelial cell injury with the eventual formation of atherosclerotic lesions. Cholesterol and lipid-induced dysfunction of the vascular endothelium could potentially have important cardiovascular consequences in view of the actions of endothelium-derived NO to relax vascular smooth muscle (Moncada *et al.*, 1988) and inhibit platelet aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987) and adhesion (Radomski *et al.*, 1987; Sneddon & Vane, 1988).

It has been demonstrated in aortae from cholesterol-fed rabbits (Jayakody *et al.*, 1985; Verbeuren *et al.*, 1986; Ragazzi *et al.*, 1989b) and from the Watanabe Heritable Hypercholesterolaemic (WHH) rabbit (Ragazzi *et al.*, 1989a; Kolodgie *et al.*, 1990; Chinellato *et al.*, 1991) that there is impaired endothelium-mediated vasodilatation to agents such as acetylcholine. In the heritable hypercholesterolaemic rabbit this impaired vasodilatation appears to be due, at least in part, to the loss of endothelial cells (Kolodgie *et al.*, 1990). In the dietary-induced model of hypercholesterolaemia, possible mechanisms which may account for the loss of endothelial function include a decrease in the ability to synthesize endothelium-dependent relaxation factors or an impaired diffusion of relaxant substances produced by the endothelium (reviewed in Vanhoutte, 1991; Tagawa *et al.*, 1991). In animals with acute dietary-induced hypercholesterolaemia an increased destruction of NO by cholesterol also seems to be important (Minor *et al.*, 1990). Cholesterol-induced dysfunction of endothelial NO release leads to enhanced vasoconstrictor responsiveness (Cohen *et al.*, 1988; Osborne *et al.*, 1989; Zeiher *et al.*, 1991) and increased aggregation and adhesion of platelets (Stamler *et al.*, 1989; Radomski *et al.*, 1990). Hypercholesterolaemia can also have a direct action to modulate platelet function (reviewed in Aviram & Brooks, 1987).

Platelet antiaggregatory activity of intact artery rings can be studied using whole blood aggregometry with the inclusion of an isolated artery (Bhardwaj *et al.*, 1988). However, no comparisons have been made of normal and atherosclerotic artery rings. Since inherited and dietary-induced hypercholesterolaemia may have different effects on the endothelium, the purpose of this study was to compare vascular reactivity of the

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rabbit subclavian artery and the influence of the endothelium on platelet aggregation in rabbit whole blood between dietary and heritable hypercholesterolaemic rabbits and normocholesterolaemic rabbits.

Methods

Three groups of male rabbits were studied. Group 1 consisted of New Zealand White (NZW) rabbits receiving a standard control diet. The second group consisted of NZW rabbits which received an atherogenic diet of 2% coconut oil and 1% cholesterol for 4 weeks before they were killed. The third group consisted of Froxfield Heritable Hypercholesterolaemic (FHH; Froxfield Farms, Alton, Hamps, UK) rabbits which also received a standard rabbit diet. All rabbits were fed *ad libitum*, consuming on average 125–150 g daily. Blood samples were taken at regular intervals for determination of plasma cholesterol.

Tissue preparation

All rabbits were terminally anaesthetized at 20 weeks of age with sodium pentobarbitone (30 mg kg^{-1}, i.v.) containing heparin (500 iu kg^{-1}) for removal of blood and tissue. After induction of deep anaesthesia the thorax was opened and 9.5 ml of blood was withdrawn from the pulmonary artery into syringes containing 0.5 ml of heparin (200 units). The animals were then killed with an overdose of the anaesthetic. The subclavian arteries were dissected free of fat and connective tissue, and ring preparations of 3–4 mm in length were cut. The presence of the vascular endothelium was confirmed in representative artery rings by silver nitrate staining. All experiments were performed under a project licence (No PPL6001129) issued under the U.K. Home Office Animals (Scientific Procedures) Act 1986.

Studies with isolated subclavian artery rings

Endothelium-intact vessel rings were suspended in an organ chamber on two intraluminal parallel wires, one of which was fixed, and the other attached to an isometric transducer. The rings were placed under an optimum resting force of 3g (Hadoke *et al.*, 1995). The organ chamber was filled with Krebs Henseleit solution (37°C) of composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2 and glucose 11.1, gassed with 95% O_2: 5% CO_2. The vessel rings were equilibrated at their optimum resting force for 30 min and then sensitized by contracting with 40 mM KCl until consistent responses were obtained. Vasoconstrictor and endothelium-dependent and -independent responses were then assessed by use of a previously determined protocol (Hadoke *et al.*, 1993).

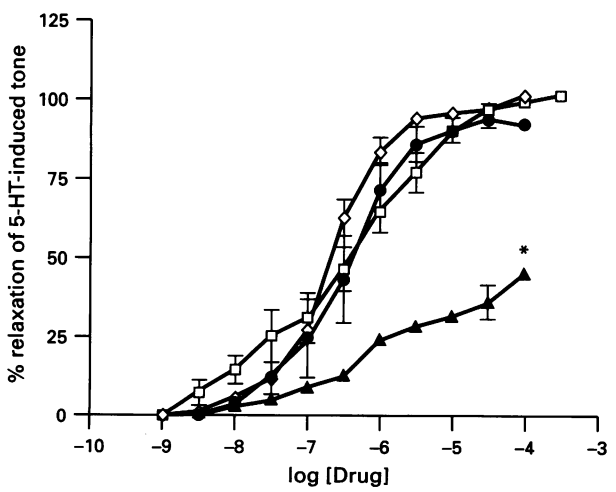

Figure 1 Relaxant responses to carbachol (◇) and SIN-1 (□) and of carbachol in the presence of L-NOARG (100 μM; ▲) and D-NOARG (100 μM; ●) in rabbit subclavian artery rings. Results are shown as mean ± s.e.mean of 4 experiments and are expressed as the percentage relaxation of 5-HT-induced tone. $^*P<0.05$ with respect to untreated carbachol-stimulated ring.

Cumulative concentration-response curves to KCl (10–100 mM) and 5-hydroxytryptamine (5-HT, 0.01 μM–10 μM) were constructed in parallel subclavian artery rings. Endothelium-independent relaxations to SIN-1 (3-(4-morpholinyl)-sydnonimine-hydrochloride, 0.01 μM–10 μM) were assessed in the vessel rings with intact endothelium and pre-contracted with 5-HT (0.1 μM). Cumulative concentration-response curves to the endothelium-dependent vasodilators carbachol (0.01–10 μM) and calcimycin (0.01–0.1 μM) were constructed in vessels pre-contracted with the EC_{85} of 5-HT (0.1 μM). In vessels from the normocholesterolaemic NZW rabbits vasorelaxation to carbachol was also studied in the presence of L-NOARG (N^G-nitro-L-arginine, 100 μM), a nitric oxide synthase inhibitor, and the inactive D-isomer, D-NOARG (N^G-nitro-D-arginine, 100 μM). These agents were added to the organ chamber 15 min or 30 min prior to contraction with 5-HT.

Carbachol and SIN-1 produced concentration-dependent relaxations in the rabbit subclavian artery rings pre-contracted with 5-HT. Pretreatment of the vessel rings for 15 min with L-NOARG (100 μM) was found to reduce significantly the carbachol-induced relaxations while incubation of the vessel rings with the inactive isomer D-NOARG for 15 min had no significant effect on the carbachol-induced relaxations (Figure 1). These results allowed the concentration and incubation time for L-NOARG to be determined for the aggregometry studies.

Platelet aggregometry studies in whole blood

Platelet aggregation in rabbit whole blood from each of the three groups was measured by electronic impedance aggregometry in a whole blood aggregometer (Chrono-log Corporation, Havertown, PA, U.S.A.). Aliquots of whole blood (0.5 ml) were transfered to a cuvette containing 0.5 ml of 0.9% NaCl and stirred continuously (1000 r.p.m.) with a magnetic stir bar. The blood was warmed at 37°C for 10 min and then transferred to the measuring chamber of the aggregometer where platelet aggregation was induced with collagen. Collagen caused aggregation of platelets with a maximal aggregatory response at 10 μg ml^{-1} and 65% of the maximal aggregatory response at 5 μg ml^{-1}. In order to detect both increases and decreases in the extent of platelet aggregation, a concentration of 5 μg ml^{-1} was employed for subsequent studies. The increase in electrical impedance, which illustrates the extent of platelet aggregation in ohms (Ω) resistance was measured 4 min after the addition of collagen.

To assess the antiaggregatory capacity of the vascular endothelium, a single subclavian artery ring (3–4 mm) was suspended by means of an S shaped wire in the cuvette containing the heparinized blood obtained from the same animal. Nitric oxide release from the vessel was triggered by the addition of carbachol (10 μM, final cuvette concentration) 30 s before the initiation of aggregation by the addition of collagen. This above procedure was repeated with vessels pre-incubated for 15 min with L-NOARG (100 μM). The effects of SIN-1 (10 μM, final cuvette concentration) on collagen-induced platelet aggregation were also studied in the presence and absence of a vessel ring. The effect of SIN-1 in the presence of an L-NOARG-treated vessel was also assessed. The susceptibility of the endothelium and SIN-1-derived NO to inactivation by oxygen free radicals was assessed in the normocholesterolaemic NZW rabbits by pre-incubating the anti-coagulated blood with superoxide dismutase (SOD, 150 u ml^{-1}, final cuvette concentration) in the presence of a subclavian artery ring.

To eliminate any contribution from prostanoids to the responses obtained, indomethacin (0.1 μM) was added to the blood samples and the subclavian artery rings utilised in these

aggregometry studies were pre-incubated for 10 min with indomethacin (0.1 μM). A time control study, whereby control responses to collagen were repeated after every 3 or 4 test responses, confirmed that maximal platelet aggregation at the start of each procedure was $24.43 \pm 1.22\Omega$ and $24.35 \pm 1.07\Omega$ on termination of the experimental procedure ($n=4$).

Cholesterol measurements

The plasma cholesterol content of blood sampled from the ear vein during the diet stage of the study was measured with a standard enzymatic (cholesterol esterase and cholesterol oxidase digestion followed by peroxidase treatment for colour change) colorimetric method (Siedel *et al.*, 1983).

Materials

The normal and hypercholesterolaemic diets used in the study were prepared by BS&S (Scotland) Ltd. Carbachol, calcimycin, 5-HT, indomethacin, SOD, D-NOARG and L-NOARG were all obtained from Sigma. Collagen was obtained from the Chrono-log Corporation. SIN-1 was generously provided by Dr P. Martorana, Hoechst-Marion-Roussel, Frankfurt, Germany.

Fresh solutions of SIN-1 were prepared on a daily basis and protected from light. Indomethacin was prepared daily in 10% $NaHCO_3$ and subsequently diluted in 0.9% NaCl. Calcimycin was prepared in 0.9% ethanol and subsequently diluted in H_2O.

Statistics

Results are shown as the mean $\pm$ s.e.mean where n refers to the number of rabbits. When analysing vasodilator responses in vessels precontracted with 5-HT, the responses are given as a percentage of the contractile tone where contraction to 5-HT is taken at 100%. In each experiment a parallel control ring allowed each point on the concentration-response curve to be corrected for any loss of 5-HT-induced tone that occurred as the experiment progressed. The following equation was employed:

$$T_{CL} = (T_1/T_0 \times 100) + (100 - (C_1/C_0 \times 100)$$

where T_{CL} = corrected test value (%); T_o = peak contraction of test value to 5-HT (g); T_1 = uncorrected test value (g); C_o = peak contraction of control vessel to 5-HT (g) and C_1 = control value corresponding to T_1(g).

For isolated vessel studies, Student's t test was employed to determine the significance of vasodilator responses in the presence of either L-NOARG or D-NOARG. Comparisons of the vascular reactivity in the three groups of rabbits studied were carried out by oneway ANOVA followed by the Tukey comparison test. For the aggregometry studies, Student's one-sample t test was employed with the Bonferroni correction factor (where applicable) to determine significance in the presence of an isolated vessel.

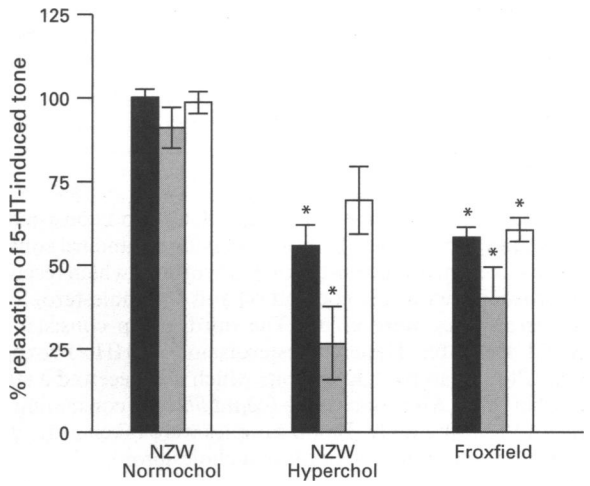

Figure 2 Relaxant responses to carbachol (solid columns), calcimycin (stippled columns) and SIN-1 (open columns) of rabbit subclavian artery rings from NZW normocholesterolaemic and heritable (Froxfield) and dietary NZW hypercholesterolaemic rabbits. Results are shown as mean $\pm$ s.e.mean of 5, 8 and 7 experiments respectively and are expressed as the maximum relaxation observed as a percentage of relaxation of 5-HT-induced tone. $^*P<0.05$ with respect to the corresponding agonist in the normocholesterolaemic group. In the dietary hypercholesterolaemic rabbits there is a significant difference between the relaxant responses to SIN-1 and calcimycin.

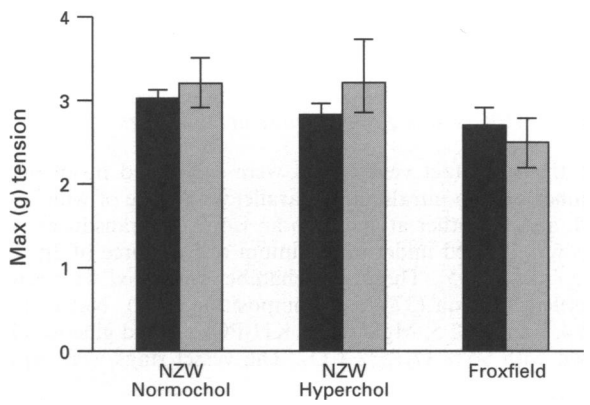

Figure 3 Maximum contractile responses of the rabbit subclavian artery rings from the NZW normocholesterolaemic and heritable (Froxfield) and dietary NZW hypercholesterolaemic rabbits to 5-HT (solid columns) and KCl (stippled columns). Results are shown as mean $\pm$ s.e.mean of 5, 8 and 7 experiments and are expressed as the maximum tension developed (g) in each concentration response-curve. There were no significant differences among the three groups of rabbits.

Results

Vascular reactivity in normo- and hypercholesterolaemic rabbits

In the normocholesterolaemic rabbits, carbachol, calcimycin and SIN-1 each produced almost complete relaxation of 5-HT-precontracted subclavian artery rings. Inspection of the concentration-response curves (data not shown) showed that both genetic and dietary hypercholesterolaemia resulted in changes in the maximum relaxation, but not in the EC_{50}, to the three relaxant agents. The maximum relaxation in response to the endothelium-dependent agonists, carbachol and calcimycin, was significantly reduced in both Froxfield and dietary hypercholesterolaemic rabbits compared to normocholesterolaemic controls (Figure 2). For SIN-1, the maximum relaxation was significantly reduced in the Froxfield heritable hypercholesterolaemic rabbits compared to controls, however in the dietary hypercholesterolaemic rabbits the attenuation of the maximum relaxation to SIN-1 did not achieve significance in comparison with the normocholesterolaemic rabbits (Figure 2). While there was no significant difference between the FFH and dietary hypercholesterolaemic rabbits with respect to the responses to SIN-1, there was a significant difference between the maximum relaxation to calcimycin compared with the maximum relaxation to SIN-1, within the dietary hypercholesterolaemic group (Figure 2).

In all three groups of rabbits 5-HT and KCl produced concentration-dependent contractions of the subclavian artery.

There was no significant difference in either the maximal contraction achieved (Figure 3) or in the EC_{50} (data not shown) to 5-HT or KCl.

Platelet aggregation in the normo- and hypercholesterolaemic rabbits

Influence of endothelium-derived NO on platelet aggregation in normocholesterolaemic blood Table 1 summarizes the effect of stimulating endothelial NO release on platelet aggregation. Collagen alone (5 $\mu g\,ml^{-1}$) produced an aggregatory response of $25.1 \pm 1.9\Omega$ in whole blood obtained from the normocholesterolaemic rabbits. Carbachol, L-NOARG or indomethacin alone had no effect on collagen-induced platelet aggregation. In the presence of an unstimulated vessel ring suspended within the cuvette, collagen-induced platelet aggregation was similar to that seen in the absence of the ring. However, when the artery ring was suspended in the cuvette and stimulated with carbachol, collagen-induced platelet aggregation was significantly reduced compared to that observed in the presence of the unstimulated vessel (Table 1).

The effect of SIN-1 on platelet aggregation was assessed in both the absence and presence of an isolated artery ring. In the absence of an isolated artery ring the response to collagen in the presence of SIN-1 was similar to that observed with collagen alone (Table 1). However, in the presence of an unstimulated vessel ring, SIN-1 significantly reduced collagen-induced aggregation compared to that observed in the presence of the unstimulated vessel ring alone. Treatment of the blood sample and the isolated vessel ring with L-NOARG did not affect this reduction observed with SIN-1 (Table 1). Pre-incubation of the vessel with SOD, prior to the addition of either carbachol or SIN-1 did not potentiate the anti-aggregatory capacity of either of these drugs in the presence of the vessel ring. SOD did not affect platelet aggregation in either the absence or in the presence of an unstimulated vessel ring.

Influence of endothelium-derived NO on platelet aggregation in hypercholesterolaemic blood The responsiveness of the rabbit whole blood to 5 $\mu g\,ml^{-1}$ collagen was not significantly different among the three groups of rabbits. In the normocholesterolaemic and heritable-hypercholesterolaemic rabbits, the presence of the unstimulated vessel, treated either with or without L-NOARG, did not affect collagen-induced aggrega-

Table 1 The effect of endothelial stimulation and SIN-1 on collagen-induced (5 $\mu g\,ml^{-1}$) platelet aggregation in whole blood from normocholesterolaemic rabbits

Added to blood sample	n	*% Collagen response*
Carbachol (10 μM)	4	96.6±8.3
SIN-1 (10 M)	4	95.9±9.0
L-NOARG (100 μM)	4	99.9±6.2
Indomethacin (0.1 μM)	4	113.9±15.2
Vessel		
Unstimulated	4	97.2±2.1
+Carbachol (10 μM)	4	67.1±4.2**
+Carbachol+SOD (150 $u\,ml^{-1}$)	4	61.6±14.5**
+SIN-1 (10 μM)	4	58.9±8.2**
+SIN-1+L-NOARG (100 μM)	4	72.5±7.9**/NS
+SIN-1+SOD (150 $u\,ml^{-1}$)	4	79.4±13.8**/NS

Values are expressed as a percentage of the response to collagen alone. $^{**}P<0.01$ vs unstimulated vessel. NS = not significant vs vessel + Sin-1.

Table 2 The effect of an unstimulated vessel ring on collagen-induced (5 $\mu g\,ml^{-1}$) platelet aggregation in whole blood

		Impedence (Ω)		
	n	*Collagen control*	*Collagen+vessel*	*Collagen+vessel+L-NOARG*
Normocholesterolaemic	10	25.1±1.9	22.1±2.1	18.7±1.5
Dietaryhypercholesterolaemic	8	19.9±1.8	13.9±1.6*	12.4±1.2*
Heritablehypercholesterolaemic	7	24.9±2.1	25.9±2.7	21.3±2.3

$^{*}P<0.05$ compared to the normocholesterolaemic control.

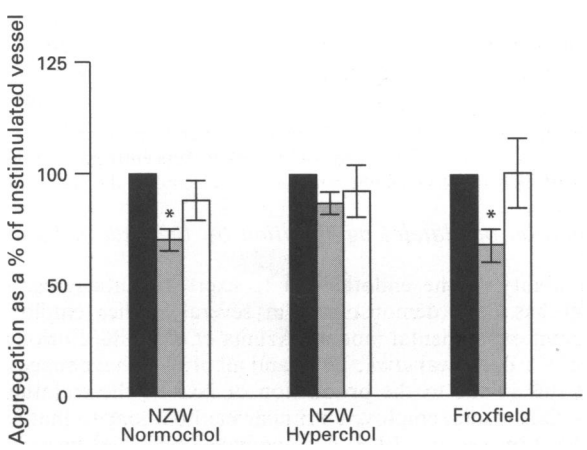

Figure 4 Collagen (5 $\mu g\,ml^{-1}$)-induced platelet aggregation in the presence of an isolated carbachol-stimulated rabbit subclavian artery ring treated with (open columns) and without (stippled columns) L-NOARG in the NZW normocholesterolaemic and heritable (Froxfield) and dietary NZW hypercholesterolaemic rabbits. Results are shown as mean±s.e.mean of 10, 8 and 7 experiments respectively and are expressed as a percentage of collagen-induced platelet aggregation in the presence of an unstimulated artery ring (solid columns). $^{*}P<0.05$ with respect to responses observed in the presence of the unstimulated vessel.

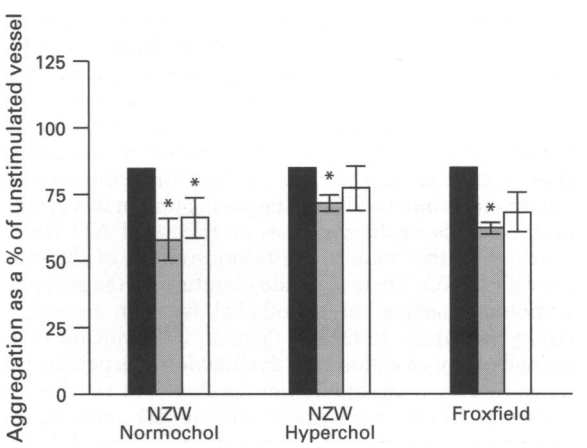

Figure 5 Collagen (5 $\mu g\,ml^{-1}$)-induced platelet aggregation in the presence of an isolated SIN-1 stimulated rabbit subclavian artery ring treated with (open columns) and without (stippled columns) L-NOARG in the NZW normocholesterolaemic and heritable (Froxfield) and dietary NZW hypercholesterolaemic rabbits. Results are shown as mean±s.e.mean of 10, 8 and 7 experiments respectively and are expressed as a percentage of collagen-induced platelet aggregation in the presence of an unstimulated artery ring (solid columns). $^{*}P<0.05$ with respect to responses observed in the presence of the unstimulated vessel.

tion (Table 2). However, with rings from rabbits with dietary hypercholesterolaemia there was an apparent reduction in platelet aggregation in the presence of the unstimulated vessel. Close examination of the vessels upon removal from the blood sample revealed that large platelet aggregates had adhered to the luminal surface of the vessel. L-NOARG had no effect on aggregation in the presence of unstimulated vessel rings in any of the groups (Table 2).

Carbachol-stimulated vessel rings from both the normocholesterolaemic and FHH rabbits caused a reduction in platelet aggregation when compared with the unstimulated vessel. This effect was prevented by L-NOARG (Figure 4). However, in contrast, subclavian artery rings from rabbits with dietary hypercholesterolaemia did not significantly inhibit platelet aggregation when stimulated with carbachol (Figure 4). SIN-1, in the presence of an isolated artery ring from all three groups of rabbits significantly reduced collagen-induced platelet aggregation compared with the response observed with the unstimulated vessel. The antiaggregatory action of SIN-1 did not appear to be affected when vessels from either group were pretreated with L-NOARG (Figure 5).

Cholesterol measurements

The dietary induced hypercholesterolaemic rabbits had a plasma cholesterol concentration of 29.4 ± 4.7 mmol l^{-1} ($n=8$) whilst that of the heritable hypercholesterolaemic rabbits was 14.9 ± 1.0 mmol l^{-1} ($n=7$). These values were significantly different from each other; however, plasma cholesterol in normocholesterolaemic NZW rabbits is 2.6 to 3.6 mmol l^{-1} (Hadoke *et al.*, 1995).

Discussion

The aim of this study was to perform a comparative analysis of endothelial function in normocholesterolaemic and dietary-induced hypercholesterolaemic New Zealand White (NZW) rabbits and Froxfield heritable hypercholesterolaemic (FHH) rabbits. To this end we studied both endothelial-dependent relaxation in isolated subclavian artery rings and the ability of endothelial stimulation to inhibit collagen-induced platelet aggregation in whole blood.

Endothelium-dependent relaxation

The main finding from the experiments designed to assess the influence of hypercholesterolaemia on endothelium-dependent relaxation was an attenuated relaxation response to both carbachol and calcimycin, suggesting a reduced production of nitric oxide by the endothelial cells or reduced responsiveness of the smooth muscle cells to NO. In the dietary hypercholesterolaemic rabbits the reduction in the maximum relaxation to calcimycin was significantly greater than the reduction in the maximum relaxation to SIN-1, suggesting that in this group the primary defect is in the synthesis or release of NO from the endothelium, rather than in the responsiveness of the smooth muscle cells to NO. There is a wide literature on the influence of hypercholesterolaemia on endothelial function (recently reviewed by Flavahan, 1992) and there is clear evidence that the nature and extent of endothelial dysfunction is dependent upon the stage of atherosclerotic lesion development (Verbeuren *et al.*, 1986). It has been suggested that in the early stages of atherosclerosis there may be dysfunction of *Pertussis* toxin-sensitive G-proteins linked to receptors such as the muscarinic cholinoceptor (Shimokawa *et al.*, 1991; Hadoke *et al.*, 1995) resulting in a reduction in the relaxant response to cholinoceptor agonists. Under these circumstances the endothelium would still be able to release NO in response to non-receptor-mediated stimulation, such as with calcimycin. However, as the disease progresses it has been suggested that either a decrease in the availability of L-arginine as a substrate for NO production (Cooke *et al.*, 1991), or an accelerated degradation of NO (Minor *et al.*, 1990) may occur. Either or both of these would result in an attenuated response to non-receptor stimulated NO release, as seen in the present experiments.

In the later stages of lesion development it has been reported that the sensitivity of the vascular smooth muscle cells to NO is reduced (Verbeuren *et al.*, 1990; Galle *et al.*, 1990). In the FHH rabbits there was a marked reduction of the relaxant response to the directly acting vasodilator, SIN-1. A similar result was seen in the study by Kolodgie *et al.* (1990) who reported an attenuation of responses to nitroglycerin in severely atherosclerotic arteries. This suggests that in the FHH rabbits used in the present experiments, the deterioration in smooth muscle function was advanced. In the NZW rabbits with dietary modification there was an attenuated response to SIN-1, but this was not significantly different from either the normocholesterolaemic controls or the FHH rabbits, presumably due to a wider spread of data in this group. The fact that some rabbits given dietary modification demonstrated a greater reduction in SIN-1 responses than others suggests that the extent of lesion formation in this group was more variable than in the FHH rabbits. This is supported by the fact that the variability in the plasma cholesterol concentrations was much greater in the dietary group than in the Froxfield rabbits. In a comparative study of lesions in Watanabe Heritable Hypercholesterolaemic (WHHL) and dietary-induced hypercholesterolaemic rabbits (Rosenfeld & Ross, 1990) no difference in lesion size was found when WHHL rabbits of a certain age were compared with fat-fed rabbits given a high cholesterol diet for the same period of time (i.e. 8 weeks old vs 8 weeks feeding). In our study, although the rabbits were age-matched the dietary cholesterol group were only on the diet for 4 weeks, thus we could perhaps expect that lesions of different severity were present in the two groups. While gross histological examination (using Sudan red staining) of vessels from both groups of hypercholesterolaemic rabbits demonstrated similar plaque cover in the vessels, we have previously shown that in NZW rabbits of the same age and subjected to the same dietary changes the lesions are rich in foam cells and macrophages (Hadoke *et al.*, 1995), whereas in the FHH rabbits used in this study the plaques were similar to those observed in human subjects, that is they had features typical of atheromatous and fibrous plaques (with and without calcification) and no foam cells present (Greenlees *et al.*, unpublished observations).

The lack of vasorelaxant responses seen in both the hypercholesterolaemic groups cannot be explained by an increase in vasoconstrictor tone to 5-HT since there was no difference in the contractile responses to 5-HT (or KCl) in either group when compared to control normocholesterolaemic vessels. In the study by Kolodgie *et al.* (1990) a hypersensitivity to the vasoconstrictor action of histamine was evident at all stages of atherosclerotic lesion development. However, an increase in the vasoconstrictor response to 5-HT was observed only in vessels with mild atherosclerosis.

Inhibition of platelet aggregation by the endothelium

The ability of the endothelium to exert an antiaggregatory effect has been demonstrated in several studies employing different experimental models (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Bhardwaj *et al.*, 1988) and all of these have suggested that this is due to the production of NO by the endothelial cells. Our studies employed a similar methodology to that used by Bhardwaj *et al.* (1988) and we have confirmed in normocholesterolaemic rabbits that the antiaggregatory effect of the endothelium can be readily demonstrated in whole blood by stimulating a vessel ring included in the blood sample with carbachol. This effect can be attributed to nitric oxide generation from the endothelium, since it was reversed by addition of the nitric oxide synthase inhibitor, L-NOARG. Furthermore, all of the experiments were performed in the presence of indomethacin to remove any contribution from antiaggregatory prostanoids such as prostacyclin.

Hypercholesterolaemia itself, either hereditary or induced by diet, did not influence collagen-induced platelet aggregation in the absence of the vessel, suggesting that hypercholesterolaemia *per se* does not increase platelet responsiveness to this stimulant. There is again a wide literature on the influence of hypercholesterolaemia on platelet function. For example, platelet activity (i.e. release, adhesion and aggregation) has been shown to be enhanced in hypercholesterolaemic patients (reviewed in Aviram & Brooks, 1987). Furthermore, Dalal *et al.* (1990) have shown that platelets from rabbits fed a 1% cholesterol diet for 4 weeks (comparable to the present study) showed a hypersensitivity of the release reaction to low (5 $\mu g\ ml^{-1}$) but not high ($>10\ \mu g\ ml^{-1}$) concentrations of collagen, although they did not measure aggregation itself. This effect was not seen in rabbits given 0.5% cholesterol. On the other hand, Barrett & Butler (1983) were unable to show any change in collagen-induced platelet aggregation in platelet-rich plasma obtained from rabbits given 1% cholesterol for 4 weeks, although an increased sensitivity to the aggregatory response to collagen has been observed, but only at concentrations below 1.2 $\mu g\ ml^{-1}$ (Löbel & Schrör, 1989).

In vessel rings from the FHH rabbits, stimulation with carbachol also exerted an antiaggregatory effect which was prevented by addition of L-NOARG. Thus it appears that NO release from the endothelium does indeed occur in vessels from these animals, at least in the luminal direction. In contrast, in the dietary hypercholesterolaemic group, the ability of a carbachol-stimulated vessel ring to inhibit platelet aggregation was not apparent. The lack of effect of dietary-induced hypercholesterolaemia on platelet aggregation described above rules out an enhanced platelet aggregation as an explanation for this finding. However, the antiaggregatory capacity of the endothelium was hard to assess in this group since the unstimulated vessels were found to decrease the extent of platelet aggregation. Subsequent histological examination of these vessel rings revealed the presence of large platelet aggregates on the luminal surface, presumably due to stimulation of platelet adhesion/aggregation by the vascular lesions on the vessel, which would reduce the amount of aggregation recorded by the electrode and thus over-estimate any inhibition of platelet aggregation by the endothelium. Thus the small amount of inhibition of aggregation observed in the dietary modification group may in fact be due to this shift in baseline and not a real response.

SIN-1 is a potent inhibitor of aggregation in washed platelet preparations but is ineffective in whole blood (Salvemini *et al.*, 1990). Interestingly, however, we found that SIN-1 was able to exert an antiaggregatory effect when an unstimulated vessel ring was present in the blood sample. This could suggest that activation of SIN-1 is enhanced by contact with either the endothelium or some other part of the vessel. Indeed, there is evidence that while initial studies suggested NO release from SIN-1 to be spontaneous, it has been shown that oxidation is required, at least in part, for the release of the NO moiety from its chemical structure (Bohn & Schönafinger, 1989). If this takes place at the surface of the endothelial cell then the present study shows that this process was not influenced by either dietary-induced or heritable hypercholesterolaemia. Alternatively, it is possible that the presence of a blood vessel prevents the interaction between SIN-1 and haemoglobin.

The antiaggregatory effect of SIN-1 was not enhanced by SOD, which would scavenge superoxide anions responsible for the breakdown of NO to peroxynitrite ion ($OONO^-$). This might suggest that antiaggregatory effect of SIN-1 is completely independent of NO generation. However, SOD also failed to potentiate the antiaggregatory effect of carbachol-stimulated vessel rings. Superoxide anions enhance platelet adhesion and aggregation (Salvemini *et al.*, 1989), while SOD potentiates the antiaggregatory capacity of an acetylcholine-stimulated vessel ring co-incubated with washed platelets, showing the potential of superoxide to break down NO (Furlong *et al.*, 1987). Furthermore, the vascular release of superoxide is enhanced in hypercholesterolaemic rabbits (Mügge *et al.*, 1994). However, in the present study using whole blood, the lack of effect of SOD may well reflect the fact that haemaglobin is more important for inactivation of NO under physiological conditions. A similar lack of effect of SOD was reported by Bhardwaj *et al.* (1988) in human whole blood stimulated with ADP. An alternative explanation is that platelets also produce superoxide and contain SOD (Marcus, 1979; Joseph *et al.*, 1987) and it is feasible that this endogenous form of SOD is already actively scavenging superoxide anions to such an extent that administration of exogenous SOD will not have any significant effect on the aggregatory response under physiological conditions.

In summary, we have demonstrated that both heritable and dietary-induced hypercholesterolaemia results in endothelial dysfunction. In the heritable hypercholesterolaemic rabbits there is clearly a defect in endothelium-dependent relaxation, but not in endothelium-dependent inhibition of aggregation by NO. The attenuated vasodilator response to carbachol, calcimycin and SIN-1 in these rabbits could be due to a reduced sensitivity of the responsiveness of the vascular smooth muscle cells to NO, or indeed to an increased physical barrier to NO diffusion in the form of the atherosclerotic lesion. Conversely, in dietary-induced hypercholesterolaemia, the defect in the vasodilator and antiaggregatory capacity of the endothelium is characterized by a decreased responsiveness to carbachol and calcimycin, with only a partial suppression of the vascular response to SIN-1. Since the data in this group were more variable, this suggests that these rabbits had lesions of a more variable severity. Notwithstanding this, the dysfunction seen in rabbits given dietary modification resembles changes previously reported to occur in developing lesions in both animal models (Verbeuren *et al.*, 1986; Kolodgie *et al.*, 1990) and human subjects (Zeiher *et al.*, 1991), whereas the dysfunction in heritable hypercholesterolaemic rabbits is more akin to changes seen in an advanced stage of the disease. This is despite the fact that the cholesterol levels in the dietary-modification group were significantly higher than in the heritable hypercholesterolaemic group. From an experimental point of veiw, these two models of hypercholesterolaemia may be useful in assessing both the pathophysiology and potential drug effects on endothelial function at different stages in the atherosclerotic process.

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