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Investigation of endogenous nitric oxide vascular function in the carotid artery of cholesterol-fed rabbits

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1 The function of endogenous nitric oxide (NO) at the level of vascular smooth muscle, was assessed in a popular experimental model of accelerated atherosclerosis, the cholesterol-fed rabbit.

2 Endothelium-dependent vasorelaxation in response to acetylcholine (ACh, 1 μM) was significantly impaired in the carotid artery from rabbits maintained on a 1% (w/w) cholesterol diet for 8-10 weeks. Furthermore, the ability of an inhibitor of nitric oxide synthase (NOS), N^G-nitro-L-arginine methyl ester (L-NAME, 1-300 μM), to enhance the contractile reactivity to a submaximal concentration of noradrenaline (NA, 3 μM), was significantly attenuated in hypercholesterolaemia.

3 A significant linear correlation between the maximal contractile effect of L-NAME (300 μM) and maximal vasorelaxation to ACh (1 μM) was determined in the carotid artery from control rabbits. In contrast, no such linear correlation was found in the carotid artery from hypercholesterolaemic rabbits.

4 We conclude that there are lesions both in agonist-stimulated, endogenous NO-dependent vasorelaxation and in the regulation of vasoconstrictor reactivity by endogenous NO in the hypercholesterolaemic rabbit carotid artery. Furthermore, the normal linear relationship between the contractile effect of L-NAME and vasorelaxation to ACh is lost after cholesterol-feeding.

Keywords: Nitric oxide; endothelium; hypercholesterolaemia; rabbit carotid artery

Introduction

NO, now widely accepted as the endothelium-derived relaxing factor or EDRF of Furchgott & Zawadzki (1980) (Palmer *et al.*, 1987), is elaborated by the activity of either constitutive or inducible nitric oxide synthase (cNOS or iNOS) (Palmer *et al.*, 1988; Knowles & Moncada, 1994). NO, the endogenous nitrovasodilator, plays a major physiological role in the control of vascular tone (see Moncada *et al.*, 1991). This is mediated through both vasodilatation and a modulation of vascular reactivity and also via a modulation of vascular sympathetic transmission (Zanzinger *et al.*, 1994).

The modulation of vascular reactivity by endogenous NO may become excessive, as in septic shock (Guc *et al.*, 1992; Szabo *et al.*, 1993); while there is thought to be a deficiency in the vascular function of endogenous NO in a number of disease states, including hypertension, diabetes mellitus, ischaemia/reperfusion injury and atherosclerosis (Vallance *et al.*, 1992; Lefer & Lefer, 1993; Harrison, 1994). The site(s) of the functional lesion(s) at least in atherosclerosis, may conceivably concern defects in cNOS activity *per se* or receptor coupling to cNOS in addition to defects distal to NO synthesis/release (see Flavahan, 1992). In the cholesterol-fed rabbit, a popular experimental model of accelerated atherosclerosis, there are reports that agonist-stimulated, endogenous NO-dependent vasorelaxation is reduced (Osborne *et al.*, 1989; Matz *et al.*, 1994; Stewart-Lee *et al.*, 1994). However, less information is available concerning possible defects in the regulation of vascular reactivity by endogenous NO.

In order to extend investigations of functional endothelial integrity in atherosclerosis in the present study, the modulation of vasoconstrictor reactivity by endogenous NO was assessed by the ability of an inhibitor of NOS, N^G-nitro-L-arginine methyl ester (L-NAME) (Rees *et al.*, 1990), to enhance the contractile response to noradrenaline (NA) in isolated rings of the rabbit carotid artery after cholesterol-feeding. Endogenous NO has previously been shown to be an important modulator of contractile reactivity to α-adrenoceptor agonists in rabbit arterial smooth muscle (Du *et al.*, 1992; MacLean *et al.*, 1993). In addition, agonist-stimulated, endogenous NO-dependent vasorelaxation was assessed by use of ACh. Since the activity of cNOS can be postulated to be a common determinant in the vascular reactivity to both ACh and L-NAME, it was of further interest to assess the relationship between the contractile effect of L-NAME and vasorelaxation to ACh in the rabbit isolated carotid artery.

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Methods

General

Male New Zealand White rabbits were maintained for 8-10 weeks on a standard chow diet with or without supplementation with cholesterol (1% w/w). At the end of this period, animals were killed with an overdose of pentobarbitone sodium (120 mg kg^{-1} i.v.) and the left carotid artery carefully excised and divided into rings approximately 7 mm in length. Carotid rings were mounted under a resting tension of 2 g in 10 ml organ baths in physiological salt solution (PSS) gassed with carbogen and warmed to 37°C. The PSS had the following composition (in mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8, $CaCl_2$ 2.52. Indomethacin (5.6 μM) was included to abolish cyclo-oxygenase activity. Responses of ring preparations were recorded on a Grass model 79 polygraph with the use of Grass FT03C force transducers.

Experimental protocol

Rings were allowed to stabilize for 1 h, during which time the PSS was changed every 15 min. Rings were subsequently precontracted with a concentration of NA which elicited approximately 80% of the maximum response (10 μM) and maximal endothelium-dependent vasorelaxation to ACh (1 μM) assessed. Rings were then washed every 10 min for

30 min. To facilitate the vasoconstrictor effect of NOS inhibition with L-NAME (1–300 μM), rings were then precontracted with a concentration of NA (3 μM) which elicited approximately 50% of the maximal response. Control preparations in which vasorelaxation to ACh (1 μM) was less than 40% were excluded from the study.

Determination of plasma cholesterol

Total plasma cholesterol was measured by a commercially available enzymatic colorimetric test (Boehringer-Mannheim).

Drugs

Acetylcholine hydrochloride, noradrenaline bitartrate, indomethacin and N^G-nitro-L-arginine methyl ester were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The standard and cholesterol-supplemented (1% w/w) rabbit diets were obtained from Special Diet Services, Withan, Essex, U.K.

Statistical analysis

Data are expressed as mean ± s.e. mean unless otherwise stated. The differences between two means were evaluated by Student's unpaired, two-tailed *t* test. Correlations between the effects of L-NAME (10–300 μM) and ACh (1 μM) were investigated using Prism (GraphPad software Inc., U.S.A.). Significance was accepted at the 5% level.

Results

Rabbit plasma total cholesterol levels after 8–10 weeks were 0.8 ± 0.1 mM ($n=12$) and 44.4 ± 3.7 mM ($n=15$) in the control and cholesterol-fed groups, respectively ($P<0.01$). Contraction to NA was similar in the control group (10 μM NA: 2.7 ± 0.2 g; 3 μM NA: 2.1 ± 0.1 g, $n=12$) and cholesterol-fed group (10 μM NA: 3.0 ± 0.1 g; 3 μM NA: 2.3 ± 0.1 g, $n=15$) ($P>0.05$).

Endothelium-dependent relaxation to ACh (1 μM) in precontracted carotid rings was significantly reduced from 66.2 ± 2.4% ($n=12$) in the control group to 46.6 ± 3.4% ($n=15$) in the cholesterol-fed group ($P<0.01$) (Figure 1). In rings submaximally precontracted with NA (3 μM), L-NAME (1–300 μM) elicited a further rise in tone in a concentration-dependent manner with a pD_2 of 4.58 ± 0.09 ($n=12$) and 4.71 ± 0.05 ($n=15$) in control and cholesterol-fed groups, respectively ($P>0.05$) (Figure 2). Vasoconstriction to L-NAME (1–300 μM) in the presence of NA (3 μM), was significantly depressed after cholesterol-feeding as assessed by area under the curve (53.6 ± 7.7 in control group ($n=12$) versus 29.2 ± 4.7 in cholesterol-fed group ($n=15$), $P<0.01$). L-NAME at 300 μM enhanced vasoconstriction to NA (3 μM) by 46.1 ± 6.1% in the control group ($n=12$) and only 23.2 ± 3.7% in the cholesterol-fed group ($n=15$) ($P<0.01$). L-NAME (300 μM) did not elicit vasoconstriction *per se* in non-precontracted preparations ($n=3$, data not shown).

A significant linear correlation between maximal vasoconstrictor responses to L-NAME (300 μM) in the presence of NA (3 μM) and matched maximal vasorelaxation responses to ACh (1 μM), was determined in the control group ($r=0.6904$, $P<0.02$, $n=12$) (Figure 3). In contrast, no such linear correlation was found in the cholesterol-fed group ($r=0.3294$, $P>0.05$, $n=15$). Correlations of responses to L-NAME (10–100 μM) with matched responses to ACh (1 μM) provided similar findings (data not shown).

Discussion

The finding that relaxation to ACh was abrogated in the rabbit carotid artery after cholesterol-feeding, is consistent with a previous report indicating a lesion in agonist-stimulated, endogenous NO vascular function in this model of atherosclerosis (Stewart-Lee *et al.*, 1994). In the present study, the reduced efficacy of NOS inhibition with L-NAME to enhance the contractile response to NA in the carotid artery after cholesterol-feeding, further documents the lesion in endogenous NO vascular function. Such a loss in endothelial function could promote the progression of atherosclerosis where injury to the endothelium is thought to be a critical initiating event (Schwartz *et al.*, 1991; Ross, 1993).

The vasoconstrictor effect of L-NAME indicates that endogenous NO exerts a tonic inhibitory regulation of vascular reactivity to NA in the precontracted intact carotid artery. A similar scenario has recently been reported in the rabbit aorta by Du *et al.* (1992) and in the rabbit pulmonary artery by MacLean *et al.* (1993). The EC_{50} value of approximately 19–26 μM for the vasoconstrictor effect of L-NAME, is in good agreement with its reported IC_{50} value as an inhibitor of cNOS

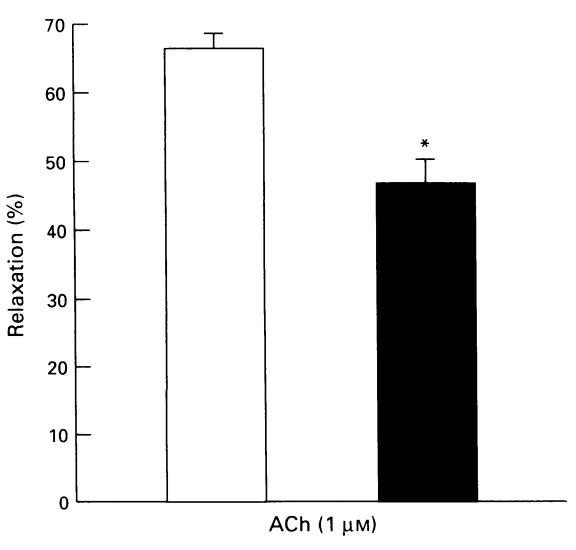

Figure 1 Relaxation in response to acetylcholine (ACh, 1 μM) of the noradrenaline (10 μM)-precontracted carotid artery from rabbits maintained for 8–10 weeks on standard chow (control, open column, $n=12$) or chow supplemented with 1% (w/w) cholesterol (shaded columns, $n=15$). *$P<0.05$.

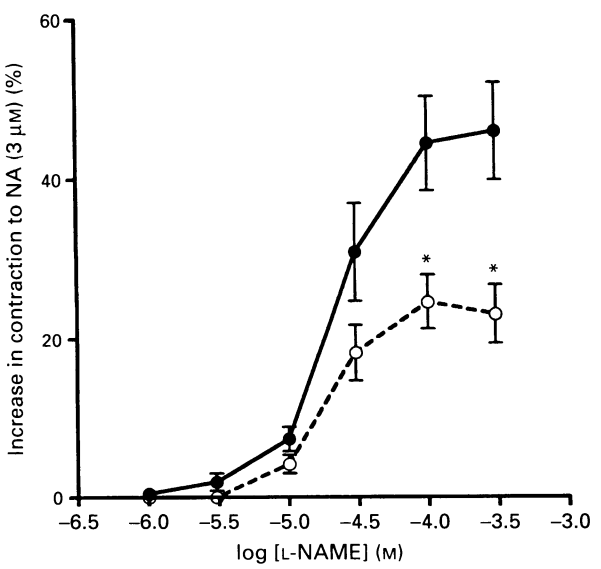

Figure 2 Vasoconstriction in response to N^G-nitro-L-arginine methyl ester (L-NAME, 1–300 μM) of the noradrenaline (NA, 3 μM)-precontracted carotid artery from rabbits maintained for 8–10 weeks on standard chow (control, ●, $n=12$) or chow supplemented with 1% (w/w) cholesterol (○, $n=15$). *$P<0.01$.

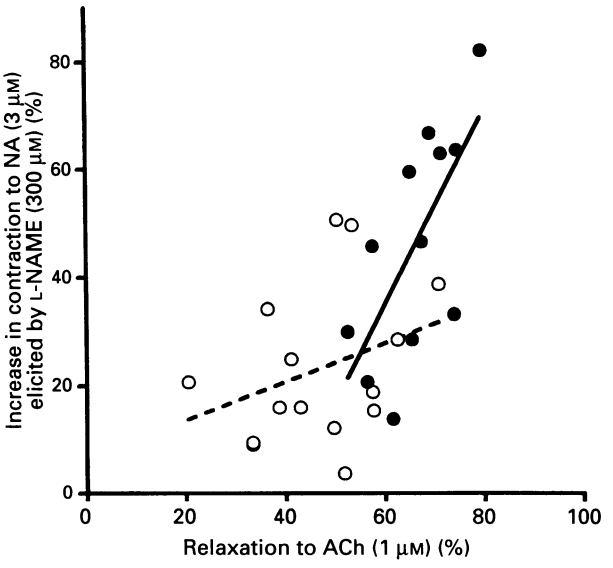

Figure 3 Correlation of vasoconstrictor responses to N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) with matched vasorelaxation responses to acetylcholine (ACh, 1 μM) in individual noradrenaline (NA, 3 μM)-precontracted carotid arteries from rabbits maintained for 8–10 weeks on standard chow (control, ●, $r=0.6904$, $P<0.02$, $n=12$) or chow supplemented with 1% (w/w) cholesterol (○, $r=0.3294$, $P>0.05$, $n=15$).

(Mitchell *et al.*, 1993). Experiments in both the rat isolated aorta and rabbit isolated carotid artery, indicated that precontraction of the tissue was obligatory for vasoconstriction to L-NAME (data not shown). These findings suggest that a manifestation of the vasodilator influence of NO, which is abrogated by L-NAME, requires the presence of contractile tone.

It is clear from intra-carotid comparisons, that the contractile response to NA exhibits a significantly smaller maximal increase following NOS inhibition after cholesterol-feeding. This could point to reduced basal NO vascular function in hypercholesterolaemia. The nature of a postulated defect in basal NO vascular function is still unclear, with conflicting reports concerning changes in the level of vascular basal NO release in hypercholesterolaemia (Minor *et al.*, 1990; Lefer & Ma, 1994; Woditsch & Schrör, 1994). However, these differences may reflect heterogeneous levels of vascular oxidant stress (Mugge *et al.*, 1991). Oxygen free radicals such as the superoxide anion, may modulate the activity of endogenous NO in hypercholesterolaemic rabbits (Harrison & Ohara, 1995; Matz *et al.*, 1994). Interestingly, it would appear that the expression of iNOS reported in hypercholesterolaemic rabbit vascular smooth muscle (Verbeuren *et al.*, 1993; Pommerantz *et al.*, 1993), is not able to overcome such defects in endogenous NO vascular function. Alternatively, given reports that NA may stimulate NO release from the endothelium (Gray & Marshall, 1992; MacLean *et al.*, 1993; Kaneko & Sunano, 1993; Rajanayagam & Rand, 1993; Arribas *et al.*, 1994) it cannot be excluded that the limited contractile effect of L-NAME after cholesterol-feeding may, at least in part, further reflect defects in agonist-stimulated, endogenous NO vascular function. It has been suggested by Stewart-Lee *et al.* (1994), that such a defect may occur at the level of receptor coupling involving endothelial G-proteins (see Tsutsui *et al.*, 1994).

The determination of a significant linear correlation between vasoconstriction to L-NAME and vasorelaxation to ACh is intuitively reasonable, since both L-NAME and ACh are postulated to act at the common level of cNOS. Vasorelaxation in response to ACh (1 μM) was therefore found to be a predictor of vasoconstriction in response to L-NAME (300 μM) in the presence of NA (3 μM) in the carotid artery of control rabbits. However, interestingly, no such association could be determined in hypercholesterolaemic rabbits. This suggests that the mechanisms of vasorelaxation and vasoconstriction for ACh and L-NAME, respectively, diverge after cholesterol-feeding. It is tempting to speculate from this result, that the residual vasorelaxation of the hypercholesterolaemic rabbit carotid artery in response to ACh (1 μM) is less dependent on NOS. This notion receives some credence from a recent report by Najibi *et al.* (1994), that an NO-independent pathway may predominate in the vasorelaxation elicited by ACh in the hypercholesterolaemic rabbit carotid artery.

In conclusion, vasorelaxation to ACh was defective in the carotid artery of rabbits fed a 1% (w/w) cholesterol diet for 8–10 weeks. Furthermore, NOS inhibition with L-NAME unmasked a weaker inhibitory regulation by endogenous NO of contractility to NA. Taken together, the evidence points to lesions both in vasodilatation to agonist-stimulated, endogenous NO and in the regulation of vasoconstrictor reactivity by endogenous NO in hypercholesterolaemia. Finally, a linear association between the vascular effects of ACh and L-NAME, is lost in the hypercholesterolaemic rabbit carotid artery.

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