Induction of a calcium-independent NO synthase by hypercholesterolaemia in the rabbit

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Endothelium-dependent and -independent relaxation of aortic ring preparations was assessed and nitric oxide (NO) synthase activity measured in the lung, and cerebellum of cholesterol-fed and normal rabbits. Endothelium-dependent relaxation of acetylcholine and ATP was depressed while that to the calcium ionophore, A23187, was unaltered in the cholesterol-fed group. Relaxation to sodium nitroprusside was however greater in aortae from the cholesterol-fed animals. Neither Ca^{2+} -dependent nor Ca^{2+} -independent NO synthase activity could be detected in aortae or hearts taken from either group of animals. Activity of both enzymes was unaltered in cerebellae from both groups of animals. Activity of the Ca^{2+} -independent enzyme was however significantly greater (ca. 2 fold) in lungs from the cholesterol-fed rabbits though the activity of the Ca^{2+} -dependent NO synthase was not significantly altered. This finding may account for the increased production of nitrogen oxides previously observed in this model of hypercholesterolaemia.

Keywords: Hypercholesterolaemia; vascular relaxation; NO synthase activity; Ca^{2+} -dependence

Introduction Reduced activity of endothelium-derived relaxing factor (EDRF), recently identified as nitric oxide (NO, Palmer *et al.*, 1987), has been demonstrated in both large and small arteries in various models of hypercholesterolaemia (for review see Henderson, 1991). The mechanism underlying this inhibition is unclear (for review see Flavahan, 1992). A recent study by Minor and colleagues (1990) showed in bioassay experiments a decreased dilator activity of aortic effluent from cholesterol-fed rabbits which was associated with increased production of nitrogen oxides as measured by chemiluminescence. This study therefore suggests that while there is increased NO production as a result of hypercholesterolaemia, the reduced EDRF activity results either from increased inactivation of NO, or an increased release of vasoconstrictors. In an attempt to account for increased NO production, we have compared the constitutive (Ca^{2+} -dependent) and non-constitutive (Ca^{2+} -independent) NO synthase enzyme activities in various tissues from rabbits fed on a normal or a high-cholesterol diet.

Methods *Cholesterol feeding* Male New Zealand white rabbits (2–2.5 kg) were randomly assigned to two groups. One group received a standard diet and the other a diet uniformly enriched with 1% cholesterol for 8 weeks.

Tissue preparation Animals were killed by cervical dislocation. Blood samples were taken via cardiac puncture into heparinised tubes, and plasma cholesterol levels assessed by a standard cholesterol oxidase method.

The thoracic aorta was removed into Krebs solution gassed with 95% O_2 /5% CO_2 at room temperature and divided in half. One half of the cleaned aorta, the heart, one lung and cerebellum were removed into liquid nitrogen, and stored at -70°C for the measurements of NO synthase activity.

The remaining aortic tissue was cut into 3 mm wide rings for isometric tension recording.

Measurement of relaxation responses The 3 mm wide endothelium-intact aortic rings were mounted in tissue baths as described previously (Chappell *et al.*, 1987) and precontracted to 50–70% of maximum with phenylephrine. Relaxation responses to cumulative addition of acetylcholine, ATP or sodium nitroprusside (SNP), or a single concentration of 3×10^{-7} M A23187 were measured and expressed as percentage relaxation of the phenylephrine-induced tone.

NO synthase assay The Ca^{2+} -dependent and -independent NO synthase activities present in the heart, lung, cerebellum and aorta were measured as previously described (Salter *et al.*, 1991). Briefly, tissues were homogenized in ice-cold lysis buffer containing protease inhibitors and centrifuged (4°C , 30,000 g, 10 min). An aliquot of the supernatant was added to the assay buffer containing L-[U- ^{14}C]-arginine, NO-synthase co-factors with or without EGTA and an NO-synthase inhibitor. The reaction was terminated by addition of AG 50 W-X8 exchange resin (sodium form). The [^{14}C]-citrulline content of the supernatant was measured by standard liquid scintillation counting techniques.

Materials Phenylephrine hydrochloride, acetylcholine chloride, ATP, sodium nitroprusside and A23187 were obtained from Sigma Chemical Co, Dorset, UK. All other chemicals were Analar grade.

Statistics Results are expressed as mean $\pm$ s.e.mean. The concentration-response curves were fitted to log-logistic curves by a least squares numerical minimization procedure. By comparing two curves simultaneously with first separate and then combined curve parameters, variance ratio tests were performed to detect differences in the asymptotes, intercepts and slopes. Other comparisons were made by use of Student's *t* test for unpaired data and considered significantly different when $P < 0.05$.

Results *Cholesterol feeding* Blood cholesterol levels were significantly increased in the cholesterol-fed rabbits (56.19 ± 3.81 mmol l^{-1} ; $n = 10$) compared to the normals (1.32 ± 0.13 mmol l^{-1} ; $n = 12$, $P < 0.01$).

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Relaxation responses Phenylephrine-induced tone was not significantly different in rings from control or cholesterol-fed animals for each drug studied (ACh: 4.9 ± 0.28 g and 5.3 ± 0.4 g; ATP: 5.5 ± 0.28 g and 5.3 ± 0.45 g; SNP: 4.0 ± 0.13 g and 4.6 ± 0.31 g; A23187: 4.5 ± 0.23 g and 4.8 ± 0.34 g respectively; $n = 12$ for ACh, ATP and SNP and 6 for A23187). The concentration-response curve for acetylcholine-induced relaxation in aortae from the cholesterol-fed rabbits ($ED_{50} = 5.1 \times 10^{-7}$ M) was significantly shifted to the right when compared to that of aortae from normal-fed rabbits ($ED_{50} = 4.7 \times 10^{-8}$ M, $P < 0.001$) (Figure 1a). Also, the peak relaxation to acetylcholine in aortae from cholesterol-fed rabbits ($39.4 \pm 7.0\%$) was significantly lower than those fed a normal diet ($54.9 \pm 2.9\%$; $P < 0.01$). The ATP concentration-response curve (Figure 1b) was similarly significantly shifted to the right in tissues from cholesterol-fed animals compared to normal animals $EC_{50} = 5.1 \times 10^{-5}$ M and 1.7×10^{-5} M respectively ($P < 0.001$). The peak relaxation achieved with ATP was not however significantly different between the two groups. Figure 1c by contrast shows that the concentration-response curve to SNP was significantly shifted to the left in aortae from the cholesterol-fed animals ($EC_{50} 2.3 \times 10^{-6}$ M) compared with control animals ($EC_{50} 6 \times 10^{-6}$ M; $P < 0.001$). Peak relaxation to nitroprusside was not however significantly altered by cholesterol feeding. Relaxation to A23187 (3×10^{-7} M) was similar in aortae from both groups of rabbits ($58.0 \pm 1\%$ ($n = 6$) in normals; $51.1 \pm 5.9\%$ ($n = 6$) in cholesterol-fed animals).

NO synthase activity Neither Ca^{2+} -dependent nor Ca^{2+} -independent NO synthase activity, could be detected in either the heart or aortic samples from either group of rabbits. Both enzymes were detectable in the cerebellar samples, but there was no significant differences between either the Ca^{2+} -dependent or -independent enzyme in the normal (1.68 ± 0.16 and 0.02 ± 0.01 fmol min $^{-1}$ μ g $^{-1}$ protein respectively) or the cholesterol-fed rabbits (1.72 ± 0.08 and 0.02 ± 0.01 fmol min $^{-1}$ μ g $^{-1}$ protein respectively). As can be seen from Figure 2 however, the Ca^{2+} -independent NO synthase activity expressed in the lung of the cholesterol-fed rabbits was significantly greater than in the lungs of control rabbits. The Ca^{2+} -dependent NO synthase was slightly (but not statistically significantly) lower in the lungs from the cholesterol-fed animals compared to normal animals.

Discussion The data confirm previous studies in aortae from cholesterol-fed rabbits showing impaired endothelium-dependent responses to acetylcholine and ATP, whereas responses to the calcium ionophore, A23187, and sodium nitroprusside are unaltered or enhanced. The observation that

Figure 2 Diagram showing mean ($\pm$ s.e.mean) Ca^{2+} -dependent (open columns) and -independent (hatched columns) NO synthase activity in lungs from rabbits fed a normal diet and a diet containing 1% cholesterol. (* $P < 0.05$ cf. control group; $n = 8-11$ observations).

the Ca^{2+} -independent NO synthase activity in the lungs of the cholesterol-treated animals was increased is a novel finding and if the findings can be extrapolated to the systemic vasculature may explain the observations by Minor and colleagues (1990) of an increased production of nitrogen oxides as a result of hypercholesterolaemia. The origin of the increased NO synthase in the lungs cannot be established with certainty however as lung tissue comprises many cell types. Blood vessels are one possible source, but other cell types like macrophages cannot be excluded.

The observation of reduced endothelium-dependent relaxation to ACh and ATP but unaltered relaxation to A23187 has been discussed elsewhere (Flavahan, 1992) and is unlikely to be due to altered receptor coupling between agonists and the endothelial cell. The fact that both NO synthase activity and nitrogen oxide production are increased in hypercholesterolaemia while endothelium-dependent relaxation is decreased, probably reflects increased destruction of NO, possible by free radicals. We were unable to detect any increase in activity of the Ca^{2+} -independent NO synthase in the heart or aortic tissue. The reason for this cannot be established with certainty though it is likely to be related to the sensitivity of the assay and also, at least in the case of the aorta, to the relatively small amount of tissue assayed. The source of Ca^{2+} -independent NO synthase activity in the lungs from control animals is not known.

The mechanism underlying the increase in inducible NO synthase activity is unknown. There is good evidence that cytokines are involved in the atherogenic process (Schwartz *et al.*, 1991) and are also known to induce the Ca^{2+} -independent NO synthase in endothelium and vascular smooth muscle (Busse & Mülsch, 1990; Lamas *et al.*, 1991). Evidence is also accumulating that oxidised low density lipoprotein may behave in a way similar to cytokines (for review see Rosenfeld, 1991), activating inflammatory and immune responses in cells in blood vessel walls and atherosclerotic plaques.

Interestingly no increase in the cerebellar activity of inducible NO synthase in the hypercholesterolaemia animals was found in the present study.

In conclusion, this study shows for the first time that hypercholesterolaemia in the rabbit increases expression of the Ca^{2+} -independent NO synthase enzyme in the lung and may account for increased production of nitrogen oxides observed previously in this model of hypercholesterolaemia.

Figure 1 Concentration-responses to relaxation induced by acetylcholine (a), ATP (b) and sodium nitroprusside (c) in aortic rings from rabbits fed a normal diet (○) and a diet containing 1% cholesterol (●). Each point is the mean and vertical bars indicate the s.e.mean of 12 observations.

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References

- BUSSE, R. & MÜLSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275**, 87–90.
- CHAPPELL, S.P., LEWIS, M.J. & HENDERSON, A.H. (1987). Effect of lipid feeding on endothelium-dependent relaxation in rabbit aortic preparations. *Cardiovasc. Res.*, **21**, 34–38.
- FLAVAHAN, N.A. (1992). Atherosclerosis or lipoprotein-induced endothelial dysfunction. Potential mechanisms underlying reduction in EDRF/nitric oxide activity. *Cir. Res.*, **85**, 1927–1938.
- HENDERSON, A.H. (1991). Endothelium in control. *Br. Heart J.*, **65**, 116–125.
- LAMAS, S., MICHEL, T., BRENNER, B.M. & MARSDEN, P.A. (1991). Nitric oxide synthesis in endothelial cells: evidence for a pathway inducible by TNF- α . *Am. J. Physiol.*, **261**, C634–C641.
- MINOR, R.L., MYERS, P.R., GUERRA, R., BATES, J.N. & HARRISON, D.G. (1990). Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J. Clin. Invest.*, **86**, 2109–2116.
- PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.
- ROSENFELD, M.E. (1991). Oxidised LDL affects multiple atherogenic cellular responses. *Circ.*, **83**, 2137–2140.
- SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution and changes in activity of Ca²⁺-dependent and Ca²⁺-independent nitric oxide synthases. *FEBS Lett.*, **291**, 145–149.
- SCHWARTZ, C.J., VALENTE, A.J., SPRAGUE, E.A., KELLEY, J.L. & NEREM, R.M. (1991). The pathogenesis of atherosclerosis: an overview. *Clin. Cardiol.*, **14**, 1–16.

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