Paradoxical increase in nitric oxide synthase activity in hypercholesterolaemic rats with impaired renal function and decreased activity of nitric oxide

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Abstract
Background. We have shown that acute exposure of oxidized low-density lipoprotein (Ox-LDL) induces vasoconstriction in renal vessels and reduces glomerular filtration rate (GFR) in an isolated perfused rat kidney model by decreasing the activity of nitric oxide (NO). L-arginine has a protective role against Ox-LDL-induced vasoconstriction. Micropuncture studies have demonstrated that short-term diet-induced hypercholesterolaemia is associated with decreased GFR and renal blood flow and increased glomerular capillary pressure. This may be mediated by decreased activity of NO.

Methods. Rats were made hypercholesterolaemic by supplementing the standard chow with 4% cholesterol and 1% sodium cholate. A group of rats on hypercholesterolaemic diet also received L-arginine in the drinking water. After 4 and 6 weeks, blood samples and 24-h urine samples were collected for the measurement of biochemical parameters. After 6 weeks, all rats were subjected to isolated perfusion of kidneys at a constant pressure of 100 mmHg. During isolated perfusion, the unused contralateral kidney was taken for morphological studies and for assessing the activity of nitric oxide synthase enzyme by β-NADPH diaphorase histochemistry.

Results. Rats fed a high-cholesterol diet had LDL levels 3–6 times greater than the rats fed standard chow. Rats that received L-arginine in the drinking water had serum L-arginine levels 5–6 times greater than control rats. At 6 weeks, creatinine clearance was significantly lower in the rats on the high-cholesterol diet compared to the rats on standard chow and rats on high-cholesterol diet plus L-arginine. Twenty-four-hour urinary total nitrate and nitrite excretion in the hypercholesterolaemic rats was 1.5–2 times greater than that of control rats. Twenty-four-hour urinary cGMP excretion was significantly lower in the rats on a high-cholesterol diet, but in the rats on high-cholesterol diet and L-arginine, 24-h urinary cGMP excretion was not significantly different from that of control rats. During isolated perfusion of kidneys, renal perfuse flow was found to be significantly reduced in the kidneys taken from the rats on a high-fat diet compared to controls. L-arginine supplementation in the drinking water almost completely reversed the effect of a high-fat diet. Inulin clearance was also significantly reduced in kidneys on a high-fat diet in contrast to controls but not in kidneys on high fat-diet and L-arginine. Basal cGMP excretion in urine was significantly lower in the kidneys taken from the rats on a high-fat diet compared to controls. L-arginine supplementation restored the basal cGMP excretion in these kidneys. NO synthase (NOS) enzyme activity as assessed by NADPH diaphorase activity showed that kidney sections taken from the rats on a high-fat diet showed more intense staining, indicating increased activity compared to the kidney sections taken from the rats on a normal diet.

Conclusion. Though activity of NO is diminished in hypercholesterolaemic rats with impaired renal function, there is a paradoxical increase in NO production and NOS activity. L-arginine reverses the effects of a high-fat diet.

Keywords: cGMP; hypercholesterolaemia; isolated kidney perfusion; L-arginine; nitric oxide synthase; renal impairment

Introduction
Abnormalities of lipid metabolism are almost always present in renal disease. Characteristic patterns of abnormality occur in the nephrotic syndrome, end-stage renal failure, dialysis patients, and after renal transplantation [1]. Studies on experimental renal insufficiency clearly indicate that hyperlipidaemia
exerts a nephrotoxic effect and accelerates glomerulonephritis [2]. Diet-induced hypercholesterolaemia has been shown to cause renal injury in rats, rabbits, and guinea-pigs [3]. Indeed, lipid-lowering agents ameliorated the amount of renal injury in rats with reduced renal mass and also in the obese Zucker rat model with endogenous hyperlipidaemia [4]. However, the exact mechanism by which hyperlipidaemia induces renal injury remains unclear. We have recently shown that acute exposure to oxidized low-density lipoprotein (Ox-LDL) induces vasoconstriction and reduces glomerular filtration rate (GFR) in isolated perfused rat kidney (IPRK) by decreasing the activity of nitric oxide (NO) [5]. We have also demonstrated that l-arginine protects the kidney from Ox-LDL-induced vasoconstriction [5].

A number of studies have found that short-term experimentally induced hypercholesterolaemia caused abnormal vasoactivity of the arteries, studied either in vitro or in vivo in the absence of overt atherosclerotic lesions and is, therefore, functional in nature [6,7]. Its importance is based on the assumption that early biochemical processes herald the development of atheromatous plaque. Nevertheless it is thought that atherosclerosis and glomerulosclerosis may have some common lipoprotein-mediated pathogenic mechanism [8]. It is now widely accepted that NO is an important mediator of renal blood flow and basal renal vascular tone [9]. Therefore we investigated whether short-term diet-induced hypercholesterolaemia produces similar haemodynamic changes in the kidney, as observed during acute exposure of Ox-LDL in IPRK. The role of NO in the alteration of renal function in hypercholesterolaemic rats was also investigated by assessing the activity of NO. Whether or not decreased activity of NO is associated with decreased NO synthesis was also studied. The protective role of l-arginine was investigated by dietary supplementation of l-arginine to the hyperlipidaemic rats.

Subjects and methods

Preparing hypercholesterolaemic rats

Male Sprague-Dawley rats (CBU, Royal Free Hospital) weighing 250–300 g were employed for these studies. They were housed 2–3 per cage at an ambient temperature of 21°C, humidity of 45%, and light/dark cycles of 12 h. The rats were divided into four groups.

Group I. Normal controls: six rats were allowed free access to standard animal chow and tap water.

Group II. Cholesterol-supplemented diet: six rats were fed standard chow supplemented with 4% (wt/wt) cholesterol and 1% (wt/wt) sodium cholate. The standard chow contained 22.4% protein and 0.61% phosphorus, while the high cholesterol chow contained 21.0% protein and 0.59% phosphorus. They had free access to tap water.

Group III. Cholesterol-supplemented diet and l-arginine in water: six rats were fed the same cholesterol-supplemented diet as group II but the tap water was supplemented with 1.5% l-arginine.

Group IV. Normal diet and l-arginine in water: six rats were fed standard chow but tap water was supplemented with 1.5% l-arginine as the group III.

All the rats were fed ad libitum. After 4 and 6 weeks, rats were placed in metabolic cages and 24-h urine samples were obtained to measure urinary cGMP, nitrate and nitrite, urinary creatinine, and albumin excretion. Urine for estimation of cGMP was collected in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (0.1 mmol/l), and immediately frozen at −20°C until assayed. Urinary cGMP was assayed using a commercially available cGMP assay kit (Amersham Life Science). Total concentration of nitrate and nitrate (stable end-products of NO) in urine was measured spectrophotometrically by using modified Greiss reaction [10] following reduction of nitrate to nitrite by the use of the reducing agent sodium tetaborate with cadmium.

Blood was obtained from tails of rats under light halothane anaesthesia after 4 and 6 weeks of dietary manipulation, for measuring fasting serum cholesterol, triglyceride, urea, creatinine, total protein and albumin, and haematocrit. Blood was immediately centrifuged and plasma was stored at −20°C until assayed. Plasma total protein was measured by biuret method, albumin by bromo-cresol green method, creatinine by a kinetic Jaffe reaction, urea, cholesterol and triglyceride was measured enzymatically by a Boehringer–Mannheim (BM) 747 analyser using BM reagents. LDL and HDL fractions were measured by isolating them by sequential ultracentrifugation.

Plasma arginine levels were measured using Biochrom 20 automated amino acid analyser (Pharmacia Biotech, St Albans, UK) by continuous flow chromatography.

Morphology of kidneys from hypercholesterolaemic rats

Histological examination of the kidneys taken from the rats on a high-cholesterol diet was performed by light microscopy to identify any structural abnormality that might have been caused by hypercholesterolaemia. When the rats were subjected to isolated perfusion of right kidneys, left kidneys were removed for this purpose. Sections were stained using haematoxylin and cosin, periodic acid–Schiff (PAS) and periodic acid–methenamine silver (PAMS) techniques.

Assessment of the activity of enzyme NO synthase (NOS)

Activity of the enzyme NOS was assessed by β-NADPH diaphorase histochemistry as described by Scherer-Singer et al. [11] and Vincent and Kimura [12]. When the rats from different groups were subjected to isolated perfusion of kidneys, contralateral non-perfused kidneys were flushed with 20 ml of 2% freshly prepared paraformaldehyde in 0.1 mol/l sodium phosphate buffer (PB), the kidneys were then removed and frozen at −70°C. Briefly, cryostat-cut slide-mounted sections of left kidneys were post-fixed in 3% paraformaldehyde in 0.1 mol/l sodium PB, pH 7.4 at 4°C for 30 min. The sections were then rinsed with 0.1 mol/l PB and blow dried. A reaction mixture was prepared consisting of (i) 50% 0.2 mg/ml nitroblue tetrazolium in 0.1 mol/l PB, (ii) 50% 2.0 mg/ml β-NADPH in 0.1 mol/l PB, and (iii) 0.3% Triton.
A negative reaction mixture was prepared for negative control omitting β-NADPH from the mixture. The sections were exposed to the reaction mixture and negative reaction mixture at 37°C for 60 min. The sections were then rinsed with 0.1 mol/l PB and stained with eosin.

**Isolated perfusion of rat kidneys**

After 6 weeks the rats were anaesthetized with thiopentone (100 mg/kg) and the right kidney removed and perfused at constant pressure (100 mmHg) in an isolated perfused kidney system as previously described [5]. Perfusion conditions were identical for kidneys taken from all four groups. The first 45 min of the perfusion was regarded as an equilibration period. Thirty-five minutes into this period, [14C] carboxy-inulin was added to the perfusate as a bolus dose (3 μCi/l) to allow the calculation of GFR. During the following 45–90-min experimental period, urine and perfusate samples were collected at 5-min intervals for measurement of GFR, urinary c-GMP, and nitrate and nitrite excretion. Renal plasma flow (RPF) and renal perfusion pressure were continuously recorded as previously described [5].

**Data analysis.** Results are all expressed as means ± SEM. ‘n’ refers to the number of independent experiments in each series. Statistical evaluation of the differences between experimental treatments were determined by performing the Mann–Whitney U test. Differences between values were considered statistically significant at \( P < 0.05 \).

**Results**

**Whole animal data**

The biochemical parameters, serum total cholesterol, triglyceride, urea, creatinine, and creatinine clearance of the rats at 6 weeks on normal and high-fat diet are summarized in Table 1. Mean baseline body weights of the rats of groups I, II, III and IV were 289 ± 12, 284 ± 11, 286 ± 11 and 291 ± 10 g respectively. Mean body weights at 4 weeks were 325 ± 14, 312 ± 16, 322 ± 13 and 332 ± 11 g of the rats of groups I, II, III and IV respectively. There was no significant difference in the body weight at baseline or at 4 weeks among four groups of rats. All rats gained weight during the 6-week period, but rats fed the high-cholesterol diet tended to gain less than rats fed standard chow, although the difference was not statistically significant. Rats fed the high-cholesterol diet had serum cholesterol levels that were 2–5 times greater than rats fed standard chow. LDL was also 3–6 times greater in rats fed high-cholesterol diet. Creatinine clearance was significantly lower in the rats on high-cholesterol diet compared to the rats on standard chow and the rats on high-cholesterol diet and L-arginine. Though serum creatinine levels were higher in the rats on high-cholesterol diet, the effect of cholesterol on serum creatinine did not achieve statistical significance. Blood haematocrit was comparable in all the groups.

The effectiveness of L-arginine supplementation was confirmed by measurement of plasma arginine levels.
Plasma arginine level was significantly higher (approximately 5–6 times) in both the groups on 1-arginine supplementation in the drinking water compared to the other two groups. The high-cholesterol diet did not have any effect on 1-arginine concentrations.

There was no significant difference in 24-h urinary protein excretion among the four groups. Twenty-four-hour total nitrate and nitrite excretion in the hypercholesterolaemic rats (groups II and III) was 1.5–2 times greater than that of control rats (Table 2). There was no significant difference in 24-h urinary nitrate and nitrite excretion between control rats (group I) and rats on standard chow and 1-arginine (group IV). Twenty-four-hour urinary cGMP excretion was significantly lower in the rats on a high-cholesterol diet only (group II). But in the rats on a high-cholesterol diet and 1-arginine (group III), 24-h urinary cGMP excretion was not significantly different from that of the control rats (Table 2).

Light microscope examination of kidney sections taken from the rats on a high-cholesterol diet showed no obvious structural abnormality as assessed by three different staining techniques.

NOS enzyme activity as assessed by NADPH diaphorase activity showed that kidney sections taken from the rats on high-fat diet showed more intense staining, indicating increased activity than the kidney sections taken from the rats on normal diet (data not shown). NADPH diaphorase activity was similar in the kidney sections taken from rats on a high-fat diet and the rats on a high-fat diet and 1-arginine.

Renal function studies in IPRK

RPF was found to be significantly reduced in the kidneys taken from the rats on a high-fat diet as shown in Figure 1. At 45 min, RPF was 12.9 ± 1.0 ml/min/g in kidneys on the high-cholesterol diet (kidney weight 1.16 ± 0.06) in contrast to 23.7 ± 1.9 ml/min/g in kidneys on the normal diet (kidney weight 1.21 ± 0.08). 1-arginine supplementation in the drinking water almost completely reversed the effect of the high-fat diet, increasing the RPF to 20.1 ± 0.9 ml/min/g at 45 min (kidney weight 1.22 ± 0.07). RPF in the group of rats fed a normal diet and 1-arginine was not significantly different from controls (Figure 1).

Inulin clearance was 0.51 ± 0.06 ml/min/g in controls, 0.38 ± 0.04 ml/min/g in kidneys on the high-fat diet (P < 0.03 compared to control) and 0.48 ± 0.05 ml/min/g in kidneys on the high-fat diet and 1-arginine (P = n.s. compared to control)(Figure 2).

Basal cGMP excretion in urine was significantly lower in the kidneys taken from the rats on a high-fat diet compared to controls as shown in Figure 3. At 45 min of perfusion, cGMP excretion from the kidneys taken from the rats on the cholesterol-supplemented diet was 33.7 ± 2.4 fmol/min/g compared to 48.7 ± 3.1 fmol/min/g from the kidneys taken from the rats on a normal diet (P < 0.04). 1-arginine supplementation restored the basal cGMP excretion in these kidneys (45.6 ± 3.8 fmol/min/g at 45 min, P = 0.1) as

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Urinary protein excretion (mg/24 h)</th>
<th>Urinary NO2 and NO3 excretion (μmol/24 h)</th>
<th>Urinary cGMP excretion (nmol/24 h)</th>
</tr>
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<tbody>
<tr>
<td>I (control)</td>
<td>6</td>
<td>1.18 ± 0.07</td>
<td>18.33 ± 1.4</td>
<td>20.3 ± 3.4</td>
</tr>
<tr>
<td>II (chole-supp diet)</td>
<td>6</td>
<td>1.25 ± 0.08</td>
<td>31.45 ± 2.1*</td>
<td>12.3 ± 2.8**</td>
</tr>
<tr>
<td>III (chole + 1-arg)</td>
<td>6</td>
<td>1.19 ± 0.04</td>
<td>36.87 ± 3.4*</td>
<td>19.4 ± 3.1</td>
</tr>
<tr>
<td>IV (normal diet + 1-arg)</td>
<td>6</td>
<td>1.17 ± 0.05</td>
<td>24.62 ± 3.1</td>
<td>21.6 ± 3.6</td>
</tr>
</tbody>
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Results are mean ± SEM. *P < 0.05 vs group I and group IV, **P < 0.05 vs group I and group III.
Fig. 2. Effect of diet-induced hypercholesterolaemia and l-arginine supplementation on $[^{14}C]$ inulin clearance in IPRK. Results are mean ± SEM. See Figure 1 for feeding regimen. *P < 0.05 vs control and high-cholesterol diet + l-arginine.

Fig. 3. Effects of high-cholesterol diet and l-arginine on basal cGMP excretion during isolated perfusion. l-min urine samples were collected every 5 min during isolated perfusion of the kidneys from the rats on normal diet, high-cholesterol diet, high-cholesterol diet + l-arginine, and normal diet + l-arginine. cGMP estimation was done using a radioimmunoassay. Results are mean ± SEM (n = 6 in each series). *P < 0.05 vs control and high-cholesterol diet + l-arginine.

3.4 ± 0.5 nmol/min/g from the kidneys taken from the rats fed standard chow (P < 0.05).

Discussion

The results of this study demonstrate that short-term dietary cholesterol supplementation in rats results in hypercholesterolaemia and marked vasoconstriction of renal vessels that can be reversed to a great extent by concomitant l-arginine supplementation.

A 4% cholesterol diet effectively increased the cholesterol level to about 3.5 times the level in the groups on standard chow. LDL level was increased to about five times in the rats on high-cholesterol diet compared to the rats on normal diet and there was no significant difference in the HDL and triglyceride level among the groups. l-arginine did not have any effect on the cholesterol levels. In some studies, diet-induced hypercholesterolaemia caused a severe haemolytic anaemia that may have influenced the degree of renal injury [3]. In this study, however, the haematocrit was not altered in the cholesterol-fed rats. Since the experimental diet contained 1% cholic acid as well as 4% cholesterol, it has to be considered that some of the haemodynamic changes might be due to absorbed circulating cholate rather than hypercholesterolaemia. This seems unlikely, since intravenous infusion of bile acids and bile salts in rats or dogs [13] has been shown to have no effect on creatinine clearance, inulin clearance or RBF.

Reduction of creatinine clearance in the rats on the high-cholesterol diet may be due to the reduction in the renal blood flow caused by hypercholesterolaemia...
in this group, as RPF was markedly reduced in these kidneys during subsequent perfusion. Though serum creatinine was slightly increased in this group, the rise was not statistically significant. In a study by Kaplan et al. [14], micropuncture and whole-kidney function measurements carried out at 3 weeks after 4% cholesterol diet demonstrated that GFR and renal blood flow were reduced as compared with rats on regular diet. Twenty-four-hour urinary albumin excretion was similar in all four groups in our study, so gross morphological change due to hypercholesterolaemia is unlikely. Furthermore, light microscopic appearances did not show any structural abnormality in the kidneys taken from the hypercholesterolaemic rats.

Increased vascular tone at the afferent and efferent arterioles and mesangial cell levels in rats fed a cholesterol-rich diet for a short period has been reported by other investigators [14]. These remarkable changes in renal vascular tone in this study and previous studies are presumably analogous to the increases in vascular contractility or failure of relaxation in response to various vasoactive drugs in isolated coronary blood vessels, hind-limb arteries, and aorta consequent upon hypercholesterolaemia [6,15]. In most of these previous studies as well as in the present study, the length of time of the experimental hypercholesterolaemia was too short for gross pathological lesions to develop in the blood vessels or in the kidneys. Thus, the renal vasculature of rats appears to manifest a functional response to experimental hypercholesterolaemia resembling that found in other vascular beds of other species. In humans, hypercholesterolaemia may also cause abnormalities in vascular tone in the absence of overt atherosclerosis [16]. Ferder et al. [17] measured GFR and RPF in patients with type IIa or IIb hyperlipidaemia, who had no evidence of overt renal disease or hypertension. They found significantly lower RPF and GFR as compared with control subjects matched for sex and age who had normal lipid profiles. Thus, humans with elevated LDL levels may manifest increased renal vascular tone, similar to that observed in rats with experimental hypercholesterolaemia.

Twenty-four-hour urinary excretion of nitrate and nitrite, the stable metabolic end-products of NO, was increased in the hypercholesterolaemic rats compared with the normal rats. During isolated perfusion of kidneys, basal excretion of nitrate and nitrite was also increased in these kidneys. A similar observation was made by Bank [7] in the rats on a 4% cholesterol diet. Minor et al. [18], using chemiluminescence, measured NO released from hypercholesterolaemic blood vessels and found it to be significantly increased. These several observations provide indirect evidence that in hypercholesterolaemia the signalling mechanisms for NO synthesis are intact and the quantity of NO being synthesized is actually increased. Increased 24-h urinary excretion of nitrate and nitrite in the rats on a high-cholesterol diet was supported by the results of the experiments for assessing NOS activity by NADPH diaphorase histochemistry. More intense NADPH diaphorase activity was observed in the kidney sections taken from the rats on high-cholesterol diet. The NADPH diaphorase reaction has been used for many years as a histochemical marker for neurons and was recently suggested to correspond to the existence of NOS [19]. So for histochemical detection of NOS, the classic nitroblue tetrazolium reaction has been used [12]. The intensity of this reaction varies with the NADPH diaphorase activity of NOS, thereby indicating NOS enzyme activity. It is believed that diaphorase staining indicates NOS activity irrespective of the particular isoforms. So with this technique it is not possible to show a direct involvement of the endothelial NOS.

Interestingly 24-h urinary excretion of cGMP—the second messenger of NO—was significantly reduced in the rats on a high-fat diet (group 1) compared to the rats on a normal diet. This is supported by the observation that during isolated perfusion of these kidneys basal urinary cGMP excretion was also decreased compared to the kidneys taken from rats fed on standard chow. These results strongly suggest that although NO production was increased in the hypercholesterolaemic rats, its activity was diminished as cGMP correlates with NO bioactivity [20]. Therefore, increased vascular tone in the rats fed only on a high-cholesterol diet may be due to accelerated inactivation of the released NO. Increased NOS activity in the hypercholesterolaemic rats may be in response to excessive inactivation of NO in these rats.

L-Arginine supplementation restored the decreased cGMP excretion in the hypercholesterolaemic rat to normal, and this suggest that L-arginine might have restored NO activity in these rats. In the isolated perfused kidney experiments, markedly reduced RPF and [14]C]nulin clearance in the kidneys taken from rats on the high-cholesterol diet was almost completely corrected in the kidneys taken from high-cholesterol diet and L-arginine rats. These observations indicate that renal blood vessels were constricted by short-term hypercholesterolaemia, which was almost completely reversed by concomitant L-arginine supplementation. It has been demonstrated that dietary supplementation of L-arginine improves or corrects endothelium-dependent vasorelaxation in hypercholesterolaemic animals and is associated with a reduction of atherogenesis [21]. L-arginine supplementation itself in the rats on standard chow did not cause any change in creatinine clearance or serum creatinine.

Correction of NO activity by L-arginine supplementation may indicate that endothelial impairment in hypercholesterolaemia is caused by a reversible reduction in intracellular arginine availability or suppression of the enzyme NOS. But as excretion of nitrate and nitrite the metabolic end-products of NO is not decreased in hypercholesterolaemia it is likely that NO signalling mechanisms and NO synthesis are intact. Since the major mechanism for inactivation of NO is oxidation to NO2 and NO3.
by reactive oxygen species [7] it seems likely that hypercholesterolaemia led to accelerated inactivation of NO by generating reactive oxygen species. Endothelial cells exposed to high levels of LDL may generate free radicals especially superoxide anions that inactivate NO [22]. L-arginine has been shown to possess free radical scavenging property by Wascher et al. [23]. L-arginine was able to diminish basal release of superoxide anion from cultured endothelial cells and aortic rings from hypercholesterolaemic rabbits [23,24]. Convincingly, endothelial-initiated degradation of extracellularly applied NO was found to be reduced when cells were preincubated with L-arginine. So L-arginine might be able to increase the bioavailability of NO, due to a reduction of release of reactive oxygen species resulting in a decreased breakdown of NO. We have previously shown that renal vasoconstriction induced by infusion of Ox-LDL in IPRK is inhibited by L-arginine and known scavengers of reactive oxygen species, superoxide dismutase and catalase [5]. These observations support the hypothesis that L-arginine acts as scavengers of reactive oxygen species. However, to prove this hypothesis in hypercholesterolaemic rats, further studies are required using specific scavengers of reactive oxygen species such as superoxide dismutase.

Conclusions

The results suggest that though the activity of NO is diminished in hypercholesterolaemic rats with impaired renal function, NO production is rather increased as evidenced by increased urinary excretion of nitrite and nitrate and increased NOS activity in the kidneys. L-arginine supplementation protects kidney from the effects of diet induced hypercholesterolaemia.

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References


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