Impaired renal vascular endothelial function in vitro in experimental hypercholesterolemia

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Abstract

Hypercholesterolemia (HC) induces alterations in systemic vascular reactivity, which can manifest as an attenuated endothelium-dependent relaxation, partly consequent to an impairment in nitric oxide (NO) activity. To determine whether experimental HC has a similar effect on renal vascular function, renal artery segments obtained from pigs fed a HC (n = 5) or normal (n = 5) diet were studied in vitro. Endothelium-dependent relaxation was examined using increasing concentrations of acetylcholine (Ach), calcium ionophore A23187, and Ach following pre-incubation with Nω-monomethyl-L-arginine or L-arginine (L-ARG). The NO-donor diethylamine (DEA) was used to examine smooth muscle relaxation response and cyclic GMP generation in endothelium-denuded vessels. The expression of endothelial NO synthase (eNOS) in the renal arteries was examined using Western blotting. Endothelium-dependent relaxation to Ach was significantly attenuated in the HC group compared to normal (53.3 ± 9.1 vs. 98.8 ± 3.7%, P < 0.005), but normalized after pre-incubation with L-ARG (82.3 ± 13.8%, P = 0.21). Receptor-independent endothelium-dependent relaxation to A23187 was also significantly blunted in HC (75.2 ± 10.5 vs. 115.5 ± 4.2%, P < 0.017). Smooth muscle relaxation and cyclic GMP generation in response to DEA were greater in denuded HC vessels, while relaxation of intact vessels to nitroprusside was unaltered. In the HC vessels eNOS was almost undetectable. In conclusion, experimental HC attenuates in vitro endothelium-dependent relaxation of the porcine renal artery, possibly due to low bioavailability of NO. These vascular alterations in HC could play a role in the pathogenesis of renal disease or hypertension, supporting a role for HC as a risk factor for renovascular disease. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Hypercholesterolemia (HC) is a major health risk associated with an increased risk of coronary artery disease and cardiac events [1]. Even short term experimental HC induced by cholesterol feeding has been shown to lead to dysfunction of the vascular endothelial layer, which characterizes early atherosclerosis [2]. The intact endothelium releases nitric oxide (NO), which serves to regulate both basal and reactive vascular tone, and to balance the stimulation of the blood vessel by vasoconstrictor substances [3]. However, the bioavailability of NO in HC states may be reduced, most likely due to an endothelial impairment, while systemic levels of vasoconstrictors may increase [4]. Consequently, endothelium-dependent relaxation is attenuated. The cholesterol fraction most deleterious to normal vascular reactivity is the low-density lipoprotein (LDL) fraction, most probably in its oxidized form.
Despite a large body of evidence demonstrating the injurious effects of HC on the systemic and coronary vasculature, data pertaining to its effects on the renal vasculature are rather limited. The renal vasculature shows unique responses to various vasoactive substances [5,6], and may have a differential response to risk factors compared to other vascular beds [7,8]. Hence, the effect of HC on the renal vasculature may conceivably differ from that observed in other vascular beds, where endothelial dysfunction is consistently observed. Moreover, maladjusted responses of the renal vasculature and/or a decrease in NO bioavailability, in addition to direct cellular effects of HC, may have grave repercussions on the wide spectrum of the homeostatic and endocrine functions of the kidney.

Renal synthesis of NO is governed by the NO synthase family of enzymes, of which the inducible form is expressed in epithelial cells along the nephron [9], while the endothelial (eNOS) isoenzyme is expressed constitutively in blood vessels. The renal consequences of HC-induced endothelial dysfunction or low bioavailability of NO may be multifold, and may potentially contribute to development of renal damage and hypertension [10,11]. Under normal conditions, NO plays a critical role in the local regulation of renal hemodynamics and excretory function [11]. NO contributes to the modulation of afferent arteriolar tone and mesangial relaxation, thus regulating the glomerular microcirculation, as well as renal vascular resistance [12,13]. In extreme cases, impaired NO synthesis may cause marked renal vasoconstriction, which may lead to outer medullary ischemia, tubular anoxia, and acute renal failure [14]. Ribiero et al. demonstrated that chronic (15 days) inhibition of NO synthase in rats was associated with decreased renal perfusion, hypertension, and accelerated glomerulosclerosis [15]. NO also plays a role in regulating renal sodium excretion and renin release, as well as prevention of thrombosis. Consequently, any process that compromises endothelial function and NO activity can contribute to a progression of renal disease and hypertension [16]. Nevertheless, it is yet unclear whether renal vascular function is impaired in HC, and whether NO is involved in this impairment.

Therefore, the aim of this study was to define if HC has a deleterious effect on renal vascular function in the swine kidney, and whether NO availability played a role in any alterations observed. For this purpose, renal vascular function was studied in isolated arterial segments harvested from both normal pigs and pigs fed a high-cholesterol diet for 3 months. This experimental model has been previously shown to develop impairments in vascular reactivity in other vascular beds [17].

2. Methods

All procedures and handling of the animals in this study in this were in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ formulated by the US National Institutes of Health, and approved by the Institutional Animal Care and Use Committee. Five domestic pigs (60–70 kg) were fed for 3 months a 2% cholesterol diet, including 15% Lard (Harlan Teklad, Madison, WI), euthanized with an intravenous overdose of pentobarbital sodium (Sleepaway®, 30 mg/kg, Fort Dodge Laboratories), and their kidneys removed. For controls, kidneys were removed from five normal domestic pigs maintained on regular pig chow. Kidneys of all animals were immersed in cool, oxygenated physiological salt solution (Kreb’s) of the following millimolar composition: NaCl, 118.3; KCl, 4.7; MgSO4, 1.2; KH2PO4, 1.22; CaCl2, 2.5; NaHCO3, 25.0; and glucose, 11.1 (control solution). Main branches of the main renal artery (3–4 mm in diameter) were dissected from the kidneys under magnification and placed in control solution, with care taken to remove as much of the connective tissue as possible. Vessels were sectioned into rings 5–6 mm in length. In some of the rings, the endothelium was mechanically removed by inserting the tip of a watchmaker’s forceps and scraping gently, a technique that has been previously described [18].

2.1. In vitro relaxation

Rings with or without endothelium were suspended in 25 ml organ chambers (one ring from each animal in each chamber) filled with Kreb’s solution, maintained at 37°C, and aerated with 95% oxygen and 5% carbon dioxide (pH 7.4). Each ring was suspended by two stainless clips passed through the vessel lumen. One clip was attached to a stationary post, and the other was attached to a strain gauge (Statham Gould UC 2, Viggo Spectamed, Critical Care Division, Oxnard, CA) for the measurement of isometric force. Rings were placed at the optimal point of their length-tension relationship by progressively stretching them until the contraction to potassium chloride (20 mM) was maximal. In all experiments, the presence of functioning endothelium was confirmed by the response to acetylcholine (Ach, 1 × 10⁻⁶ M) following precontraction with potassium ions (20 mM). After optimal tension was determined and the presence or absence of endothelium confirmed, the rings were allowed to equilibrate for 30 min before performance of any further experiments. All experiments were performed in the presence of indomethacin (1 × 10⁻⁵ M) in order to block endogenous production of prostaglandins. Endothelial-dependent relaxation after precontraction with endothelin-1 (ET-1, 10⁻⁷ M) was examined using increasing concentrations of Ach (10⁻⁹–10⁻⁴ M)
indomethacin, which was dissolved in Na2CO3. Stock drugs were prepared with distilled water except in- 
ed ET-1 (Phoenix Pharmaceuticals). All powdered ARG and L-NMMA (Calbiochem, San Diego, CA); 
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experiments: Ach, calcium ionophore A23187, DEA, 
10
Kreb's solution. After 1 h o f incubation, 146
m

samples were then placed on dry ice, 2 ml of absolute alcohol added, and the samples homogenized. Af- 
ter being allowed to sit for 5 min, they were cen-
trifuged for 10 min at 4000 RPM at 10°C. Superna-
tant was placed separately for posterior protein assay. 
Samples were dried with Nitrogen evapo-rack and 500
m

samples were allowed to sit for 5 min. The samples were subsequently centrifuged for 10 min at 4000 RPM at 
10°C. Supernatant was discarded, 1.1 ml of cold DI 
water added, and the samples were vortexed again. 
Finally, 1 ml of the mixture was placed in a plastic scintillation vial and 15 ml of Opti-flur added. The 
samples were then mounted on the scintillator counter and tested.

2.2. Cyclic GMP production

As a measure of NO activity, cyclic guanosine-3',5'-monophosphate (cGMP) was measured in the following 
manner: vascular rings were collected from each 
group and placed in an organ chamber filled with 
Kreb's solution. After 1 h of incubation, 146 µl of 
3-isobutyl-1-methyl-xanthine 10–4 M/l and 100 µl of 
indomethacin 10–5 M/l were added to the solution in 
the organ chamber for 30 min. Samples were then 
randomized to either standards (controls) or DEA 
(10–6 M/l) treatment for 1 min, and then shock-
frozen.

Samples were then placed on dry ice, 2 ml of abso-
lute alcohol added, and the samples homogenized. Af-
ter being allowed to sit for 5 min, they were cen-
trifuged for 10 min at 4000 RPM at 10°C. Superna-
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samples were then mounted on the scintillator counter and tested.

2.3. Western Blotting for eNOS

Arteries from four HC and five normal pigs were 
snap frozen in liquid nitrogen. Care was taken to 
ensure that the endothelial layer was untouched and 
undamaged. The vessels were subsequently homog- 
ized in lysis buffer (50 mM Tris HCl, pH 8.0, 150 
mM NaCl, 0.02% sodium azide, 0.1% SDS, PMSF 100 
µg/ml, aprotinin 1 µg/ml 1% NP-40, 0.5% sodium de-
oxycholate) using a tissue homogenizer. The lysate was 
analyzed for protein content using a Bradford assay 
(Signal Transduction Laboratory, Lexington, KY) at a dilution of 1:1000 in a non-fat milk/Tris buffer. The membrane 
was subsequently probed with a secondary anti-mouse 
antibody conjugated to horseradish peroxidase (Amer-
sham Life Sciences, IL) at a dilution of 1:5000 and 
developed with chemiluminescence (Pierce, IL). The 
membrane was then exposed to X-ray film (Kodak, 
NY) which was subsequently developed.

3. Results

3.1. Serum cholesterol levels and systemic 
hemodynamics

In normal pigs total and LDL cholesterol levels were 
74.2 ± 18.5 and 33.8 ± 18.9 mg/dl, respectively. The total serum cholesterol level in the cholesterol-fed pigs was 
454.2 ± 48.5 mg/dl (P = 0.002 compared to nor-
mal), and the LDL cholesterol fraction was 370.0 ± 
37.8 mg/dl (P = 0.009). There was no significant 
difference between the mean arterial pressures among 
the normal and HC pigs at the time of euthanasia, as 
measured using a catheter placed in the carotid artery 
(90 ± 7 and 94 ± 18 mmHg, respectively, P = 0.55).
3.2. Endothelial function

The maximal contractile response to ET-1 (10⁻⁷ M) achieved during pre-contraction were similar between normal rings and HC rings (P = 0.2). Renal arterial rings removed from HC pigs demonstrated a significantly attenuated maximal relaxation response to increasing doses of Ach compared to normal (53.3 ± 9.1% vs. 98.8 ± 3.7%, respectively, P < 0.005) (Fig. 1, top panel). Relaxation in response to calcium ionophore A23187 was also attenuated in the HC group compared to normal (75.2 ± 10.5% vs. 115.5 ± 4.2%, respectively, P < 0.017) (Fig. 1, bottom panel). On the other hand, the maximal endothelium-independent relaxation response to SNP (10⁻⁴ M) was similar between normal and HC rings (P = 0.2).

3.3. Role of NO

Pre-incubation with L-ARG prior to relaxation with Ach enhanced and normalized endothelial function in the HC group (82.3 ± 13.8% vs. 98.8 ± 3.7%, P = 0.01 compared to normal vessels without L-NMMA). Pre-incubation of normal vessels with L-NMMA resulted in a significantly attenuated response to Ach (25.6 ± 11.1% vs. 98.8 ± 3.7%, P = 0.01 compared to normal vessels without L-NMMA). Pre-incubation with L-NMMA of vessels obtained from the HC group led to a smaller and non-significant further attenuation of relaxation to Ach (31.4 ± 9.6% vs. 53.3 ± 9.1%, P = 0.21 compared to HC vessels without L-NMMA), indicating that the endogenous NO bioavailability in HC vessels was reduced. After pre-incubation with L-NMMA, the response to Ach was very similar in the HC and normal groups (P = 0.92) (Fig. 2). Subtraction of the L-NMMA pre-incubated Ach responses from the Ach responses showed that in the normal group, 71.1 ± 23.4% of the vascular relaxation was attributable to NO, while in the HC group this amounted to only 28.2 ± 25.1% (P < 0.05).

Relaxation responses of endothelium-denuded arterial segments to the NO-donor DEA were significantly greater in the HC group (114.0 ± 9.9%) compared to control rings (75.9 ± 3.1%, P < 0.022) (Fig. 3). Generation of cGMP under baseline conditions in HC vessels was similar to normal (3.6 ± 2.4 and 0.91 ± 0.27 pmol/mg protein, respectively, P = 0.18), but was significantly greater in response to DEA (98.3 ± 10.2 vs. 6.3 ± 1.3 pmol/mg protein, P = 0.0004). The relative change (percent increase) in cGMP generation in response to DEA was 5-fold greater in HC than in normal pigs (P = 0.0006).
Fig. 3. Renal vascular smooth muscle relaxation in response to increasing doses of diethylamine (DEA). *P < 0.05 compared to normal arterial segments.

Fig. 4. Immunoblot of renal artery tissue homogenates for endothelial NO synthase (eNOS) protein (100 mg protein/lane) obtained from five normal (N) animals and four hypercholesterolemic (HC) pigs. The Western blot demonstrates a band in the 140 kDa range in all the renal arteries obtained from the normal group, but in none of the HC group. Molecular weight markers of 218 and 125 kDa are shown.

As Fig. 4 demonstrates, Western blotting performed in four HC renal arteries has shown markedly reduced eNOS expression compared to five normal renal arteries.

4. Discussion

The major findings of this study are that: (1) diet-induced HC in pigs resulted in an impairment of renal endothelium-dependent relaxation in response to both Ach and A23187; (2) this impairment was normalized following pre-incubation with a precursor of NO biosynthesis (L-Arg), and was accompanied by increased smooth muscle responsiveness and cGMP production to an NO-donor (DEA); (3) this impairment was also associated with markedly decreased eNOS expression in renal arteries of HC pigs. These alterations suggest involvement of the NO pathway in the observed functional impairments, and may implicate HC as a risk factor for renal vascular abnormalities.

The endothelium has emerged as a vital organ, which regulates both the tone and structure of the vasculature [19]. NO activity is a major determinant of normal vascular wall homeostasis by balancing the vasoconstrictor actions of vasoconstrictors produced by the endothelium or elsewhere [20]. Endothelial dysfunction in the coronary and systemic vasculatures has been shown to be an early event in the development of atherosclerosis, preceding any structural alterations [21]. Many mechanisms have been implicated in this early impairment of endothelial function, including uncoupling of Gt-receptor interactions, decrease in L-ARG availability due to co-factor deficiency, reduction in NOS protein due to excess oxidized LDL levels, increased NO degradation due to an increase in superoxide formation, and production of peroxynitrite that can lead to lipoprotein oxidation.

This study extends previous observations made in other vascular beds, and demonstrates that diet-induced HC in pigs imposes a deleterious insult on the renal vascular endothelium, likely due to reduced NO bioavailability, resulting in a functional impairment of the renal artery. Endothelium-dependent relaxation in response to both receptor-mediated (Ach) and non-receptor-mediated (calcium ionophore A23187) endothelium-dependent vasodilators was significantly blunted in the HC group compared with controls. This impairment was normalized when the arterial segments were incubated with L-ARG prior to exposure to Ach, supporting a role for low bioavailability of NO, and implying that at least at the early phase of diet-induced HC the functional abnormalities may be reversible. The mechanism by which L-ARG restored renal endothelial function in the setting of HC may be multifactorial. In HC states, transport of L-ARG into the cells may be decreased due to the accumulation of competitive inhibitors of L-ARG, such as oxidized LDL [22]. In addition, there is accumulation of competitive inhibitors of NOS activity, such as asymmetric dimethyl arginine (ADMA) [23]. Since this inhibition is competitive, NO production can be restored by excess supply of L-ARG [24]. Although this has been documented in other vascular beds [25], this is the first such demonstration in the renal vasculature.

The role of the NO pathway in this vascular dysfunction was further underscored by the observation of hyper-responsiveness of denuded HC renal arterial segments to DEA. Relaxation in response to the NO-donor DEA was significantly augmented in HC arterial rings compared to normal, and cGMP production was markedly augmented. This may reflect adaptation of the smooth muscle to become more sensitive to NO in NO-deficient states such as HC. This hypothesis is supported by previous studies, which demonstrated that removal of the endothelial NO synthesis pathway by denuding the endothelium resulted in an augmented response to NO-donors [26]. Moreover, blockade of the residual NO synthesis using L-NMMA had a relatively smaller effect on HC rings compared to normal rings in further attenuating the vasorelaxation response to Ach.
Thus, the observation that pre-incubation of HC renal vessels with L-NMMA did not significantly attenuate their response to Ach any further supports the hypothesis that HC in the renal circulation is associated with a decrease in endogenous NO activity.

Notably, an enhanced response of denuded vessels to the NO-donor DEA was not accompanied by enhanced responses of intact vessels to the NO-donor SNP. It is likely that the endothelial dysfunction observed in this study and others resulted not only from a decrease in NO bioavailability, but also from a concurrent increased release [7] or response to [27] endothelium-derived vasoconstrictors, which shifted the vasodilator/vasoconstrictor balance that controls vascular tone [28] in favor of the latter. Thus, enhanced sensitivity of the vessel to NO may be apparent only in the absence of the endothelium, which may also explain why this phenomenon is not observed in vivo.

Previous studies in HC rats have demonstrated impaired in vivo renal vasodilatory responses to acetylcholine [29], while other investigators have witnessed no change in single-nephron glomerular filtration rate and renal afferent plasma flow, but an increase in glomerular capillary pressure [30]. In cholesterol-fed rabbits, the in vivo renal vasodilatory response to acetylcholine was unaltered [31] after 8 weeks of diet, but the response of the renal artery in vitro was slightly impaired [8]. Indeed, the discrepancy among those observations may be related to the different animal models used, as well as the duration or content of the HC diet. The finding of decreased eNOS levels in the renal arteries of HC pigs also agrees with the decrease in eNOS immunoreactivity observed in the coronary arteries of HC pigs [32]. However, it contrasts with a recent study, showing decreased NO release in response to an endothelin B-receptor antagonist (BQ-3020) in the isolated, perfused kidney of HC rats, but immunostaining of eNOS comparable to that of normal controls [33]. Again, the discrepancy among these observations may be related to the different animal models used, or to the different methodology used to determine eNOS expression.

Interestingly, although chronic administration of NO inhibitors is associated with elevated blood pressure resembling the characteristics of essential hypertension [34], decreased NO-mediated responses and eNOS expression in the HC pigs was not associated with development of hypertension. The reason for this can be only speculated. The decrease in NO bioavailability in the peripheral circulation in this early phase of HC may possibly not be as complete as that obtained with L-NMMA administration. Because many regulatory mechanisms contribute to determination of basal vascular tone, it may be well-balanced under resting conditions, and the effects on vascular tone often apparent only in stimulated situations [35]. Moreover, in vivo blunted renal perfusion response to acetylcholine was recently observed in HC pigs [36], but enhanced renal tubular response and sodium excretion. The reason behind the augmented sodium excretion in response to acetylcholine is yet unclear, but by this mechanism early diet-induced HC may hypothetically blunt development of hypertension in this model, despite decreased bioavailability of NO.

Nevertheless, the alteration of renovascular endothelial function in this study may well have functional implications, and it is not unlikely that longer duration of HC in animals or in humans would lead to glomerulosclerosis [37,38] or an increase in blood pressure [16]. Furthermore, endothelial dysfunction in the renal arteries may represent a more extensive process that involves the renal microcirculation as well. Hence, partly through interference with the NO pathway [37], HC may lead to intra-renal hemodynamic and functional abnormalities, and may potentially culminate in permanent renal damage.

In summary, this study demonstrated that experimental HC in pigs resulted in renal vascular endothelial dysfunction in vitro, likely due to reduced NO bioavailability. This impairment was restored to near control values when the precursor of NO was supplied, suggesting a reversible NO-deficient state in early HC. Further evidence that supports this notion was obtained with NO-synthesis inhibition, during which normal renal arterial segments reacted to Ach similarly to those rings taken from HC pigs. In HC rings, NO-synthesis inhibition had a much smaller effect on the already attenuated Ach-induced endothelium-dependent relaxation, while the sensitivity of the vascular smooth muscle to an NO-donor was significantly augmented. This functional impairment of the renal vasculature may lead to maladaptive responses of the kidney to physiological or pharmacological challenges. Furthermore, as speculated regarding the coronary circulation, this renal dysfunction may possibly precede the development of overt atherosclerotic processes in the renal macro and microvasculature, eventuating in renal artery stenosis, glomerulosclerosis, and/or hypertension.

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