

## VASCULAR BIOLOGY – HEMODYNAMICS – HYPERTENSION

## Proteinuria is preceded by decreased nitric oxide synthesis and prevented by a NO donor in cholesterol-fed rats

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**Proteinuria is preceded by decreased nitric oxide synthesis and prevented by a NO donor in cholesterol-fed rats.**

**Background.** Hypercholesterolemia decreases nitric oxide (NO) availability in the circulation and induces podocyte activation and renal injury in rats. It is unknown whether hypercholesterolemia decreases renal NO availability. To dissociate the injury-independent effect of hypercholesterolemia on renal NO availability from secondary effects of proteinuria, increasing concentrations of cholesterol were administered. To determine whether podocyte activation and renal injury were associated with NO deficiency, molsidomine, an exogenous NO donor, was administered to hypercholesterolemic rats.

**Methods.** Female rats were fed 0, 0.5, 1, or 2% cholesterol for 24 weeks. Rats fed 2% cholesterol were also studied for two weeks. In addition rats fed 0 or 1% cholesterol received 120 mg molsidomine/L drinking water. Renal NO availability was determined by measuring renal NO synthesis and superoxide activity. Podocyte activation was monitored by desmin staining.

**Results.** Hypercholesterolemia dose-dependently increased proteinuria. In the absence of proteinuria, hypercholesterolemia decreased renal NO synthesis ( $4.2 \pm 0.5$  in 0.5% cholesterol vs.  $6.8 \pm 0.6$  pmol/min/mg protein in controls;  $P < 0.05$ ). With the exception of neuronal nitric oxide synthase (nNOS), renal NOS protein mass remained unaffected. Renal superoxide activity was dose-dependently increased, thus further lowering renal NO availability. Podocyte injury was dose-dependently increased even in the absence of proteinuria (score,  $40 \pm 4$  in 0.5% cholesterol vs.  $9 \pm 4$  in controls;  $P < 0.05$ ). After two weeks, hypercholesterolemia caused no proteinuria, but did cause some podocyte injury. Renal NOS activity was decreased, but glomerular endothelial NOS (eNOS) staining was unchanged. Molsidomine prevented proteinuria, podocyte activation, and all further renal injury.

**Conclusions.** Hypercholesterolemia decreases renal NO synthesis, and induces podocyte activation before proteinuria appears. Renal superoxide activity is increased once rats are pro-

teinuric, further lowering renal NO availability. All of these changes can be prevented by a NO donor.

Alterations in lipid metabolism may be important in the pathogenesis of progressive renal injury [1]. Short-term high-dose cholesterol feeding causes lipoprotein accumulation, monocyte recruitment, and release of growth factors [2]. It is well known that hypercholesterolemia induces proteinuria, interstitial injury [3], and oxidative stress [4]. Such oxidative stress may impair renal function by decreasing renal nitric oxide (NO) availability [5]. Indeed, in isolated perfused kidneys of rats exposed to dietary hypercholesterolemia, decreased relaxation to acetylcholine was found, indicating a decreased renal NO availability [6]. Similar defects in vascular function were found in the renal circulation [7] and renal artery segments [8] from hypercholesterolemic pigs. Renal NO availability can be decreased either by decreased NO synthesis or by increased NO degradation. Studies in cultured endothelial cells have shown that exposure to high cholesterol levels decreased endothelial NO synthase (eNOS) expression [9]. Another study suggested that in endothelial cells exposed to serum from hypercholesterolemic patients, caveolin-1 expression was up-regulated and thus inhibited eNOS activity [10]. Thus, endothelial cells exposed to high cholesterol levels appear to have impaired eNOS activity, which leads to decreased endothelial NO availability. The purpose of the present study is to investigate whether hypercholesterolemia influences renal NOS activity. However, measurements in most previous studies in hypercholesterolemic rats were performed when the animals were also proteinuric. In the presence of renal injury it is impossible to dissociate the effects of hypercholesterolemia from those of injury. In view of the above results, the present study was designed to dissect the primary effect of hypercholesterolemia on renal NO availability from the secondary effect of injury.

Desmin expression is a marker of podocyte injury [11]. In a previous study we showed that dietary hypercholes-

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terolemia, characterized by marked increases in very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL), was associated with podocyte injury [12]. Podocytes are practically unable to replicate postnatally. Therefore, any injury that podocytes suffer might lead to areas of “bare” glomerular basement membrane, which are the starting points for irreversible renal injury [13]. Podocytes express the NO-dependent enzyme soluble guanylate cyclase, which probably controls their contractility [14]. Thus, by decreasing renal NO availability, hypercholesterolemia may initiate podocyte injury.

The present study investigated the injury-independent effect of dietary cholesterol on renal NO-availability after administration of a non-proteinuric low dose of dietary cholesterol (0.5%) that would cause dyslipidemia. The study further sought to investigate the injury-dependent effect of dietary cholesterol on renal NO availability after administration of proteinuric higher doses of cholesterol in the diet (1% and 2%) that would cause mild and severe hypercholesterolemia. Renal NO synthase (NOS) activity and renal superoxide activity were measured. Furthermore, we examined whether decreased renal NOS activity and podocyte activation occurred before the onset of proteinuria by administering rats 2% cholesterol for only two weeks. To investigate whether renal injury can be prevented by an exogenous NO donor, NO availability was increased with a NO donor (molsidomine [15]) in rats fed 1% cholesterol.

## METHODS

### Animals

Female Sprague-Dawley rats (weighing 150 to 175 g; Harlan-Olac, Blackthorn, UK) were exposed to a 12-hour light/dark cycle, an ambient temperature of 22°C, and a humidity of 60%. Sentinel animals were monitored regularly for infection by nematodes and pathogenic bacteria as well as for antibodies for a large number of rodent viral pathogens (International Council for Laboratory Animal Science, Nijmegen, The Netherlands), and consistently tested negative for infection throughout the experiment. The Utrecht University Board for studies in experimental animals approved the studies.

### Experimental protocol 1: Dose-dependent effects of cholesterol

Four groups of rats were studied ( $N = 8/\text{group}$ ). Cholesterol and cholate were mixed through the chow at increasing concentrations (RMH-TM; Hope Farms, Woerden, The Netherlands): 0% cholesterol + 0% cholate; 0.5% cholesterol + 0.125% cholate; 1% cholesterol + 0.25% cholate; 2% cholesterol + 0.5% cholate. Rats were treated for 24 weeks starting at the age of six weeks.

### Experimental protocol 2: Primary effect of hypercholesterolemia

Two groups of rats ( $N = 8/\text{group}$ ) were studied for two weeks. The first control group received normal chow and drinking water. The second group received 2% cholesterol + 0.5% cholate in chow. After two weeks, plasma cholesterol levels, proteinuria, renal NOS activity, renal superoxide activity, and podocyte injury were determined.

### Experimental protocol 3: NO availability and hypercholesterolemia-induced renal injury

Two additional groups of rats were studied ( $N = 8/\text{group}$ ) for 24 weeks. The first group received the NO donor molsidomine in drinking water (120 mg/L). The drinking bottles were covered with aluminum foil. This dose protected uremic rats from developing proteinuria and adverse effects were not reported [15]. The second group received 1% cholesterol + 0.25% cholate in chow and molsidomine in drinking water. These groups were compared to the control and 1% cholesterol + 0.25% cholate group from protocol 1.

At the end of experimental protocols 1, 2, and 3 the right kidney was removed and cut transversely into three slices. The two poles were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until being processed for NOS activity and protein mass determination. The middle slice was immersion-fixed in phosphate-buffered saline (PBS) formaldehyde (4%, pH 7.35) and embedded in paraffin for morphological studies. The left kidney was perfused at 120 mm Hg with ice-cold carbogenized Krebs-Hepes buffer (pH 7.4). After perfusion kidneys were decapsulated and four cortical slices were cut ( $5 \times 5 \times 2$  mm) using a tissue-slicing device. Renal cortical slices were kept on ice in buffer until determination of superoxide activity by lucigenin enhanced chemiluminescence (LEC).

### Plasma lipids, systolic blood pressure, body weight, and renal function

At weeks 0, 6, and 18, blood samples were taken from the tail vein for determination of plasma creatinine, cholesterol, and triglycerides. At the end of the experiment (week 24), the animals were anesthetized with 60 mg/kg sodium pentobarbital intraperitoneally to collect blood from the vena cava for determination of plasma creatinine, lipids, and lipoproteins. Plasma cholesterol and triglycerides were determined enzymatically (Roche Diagnostics GmbH, Mannheim, Germany). Systolic blood pressure (SBP) was measured every six weeks in the conscious rats, starting one week before the start of treatment (week 0) by the tail-cuff method (IITC, San Diego, CA, USA). Urine was collected every six weeks starting at week 0 for determination of urinary protein and creatinine excretion. The rats were weighed and placed in

metabolic cages for 24 hours, with free access to food and water. Urinary protein levels were determined by the Bradford method. Plasma and urinary creatinine levels were determined colorimetrically (Sigma Diagnostics Inc., St. Louis, MO, USA). The creatinine clearance, calculated by the standard formula, was used as an estimate of glomerular filtration rate (GFR).

#### **Lipoprotein isolation by density-gradient ultracentrifugation**

Lipoproteins were separated in terminal plasma samples by density-gradient ultracentrifugation [16] into five fractions [chylomicrons and VLDL,  $d < 1.006$  g/mL; IDL,  $d = 1.006$  to  $1.019$  g/mL; low-density lipoprotein (LDL1,  $d = 1.019$  to  $1.04$  g/mL and LDL2,  $d = 1.04$  to  $1.063$  g/mL); high-density lipoprotein (HDL),  $d = 1.063$  to  $1.21$  g/mL]. The subdivision of LDL into LDL1 and LDL2 was performed to separate the apolipoprotein B (apo B)-containing lipoproteins from the other particles present in the total LDL density range of  $1.019$  to  $1.063$  g/mL. Lipoprotein cholesterol was measured as described above.

#### **Renal NOS activity**

Nitric oxide synthase activity was measured by determining the formation of  $L$ - $^3$ H-citrulline from  $L$ - $^3$ H-arginine. Using an Ultraturrax, an aliquot of about 300 mg kidney tissue was homogenized in an ice-cooled homogenization buffer, pH 7.4, consisting of: 50 mmol/L Tris buffer, 320 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), 2 mg/L aprotinin, and 100 mg/L phenylmethylsulfonyl fluoride (PMSF). An aliquot of 50  $\mu$ L of homogenate was incubated in a final volume of 100  $\mu$ L at 37°C for 30 minutes in the presence of 1 mmol/L  $L$ -citrulline, 0.3 mmol/L tetrahydrobiopterin, 300 U/mL calmodulin, 0.5 mmol/L NADPH, and 1 mmol/L  $CaCl_2$ , 0.01 mmol/L  $L$ -arginine, and 3.7 kBq  $L$ -[2,3,4,5]-arginine (Amersham Pharmacia Biotech. Ltd, Buckinghamshire, UK) in 50 mmol/L  $KH_2PO_4$  phosphate buffer, pH 7.2. In an additional tube, the NADPH was substituted by 100 mmol/L  $L$ -arginine analog  $N$ -nitroso- $L$ -arginine methyl ester ( $L$ -NAME) to determine non-specific activity. The reaction was stopped by placing the tubes on ice and adding 20 mmol/L ice-cold Hepes buffer, pH 5.5, followed by separation of arginine and citrulline on Dowex 50X8-200 ( $Na^+$  form).  $^3$ H-citrulline was detected by scintillation counting. All measurements were performed in duplicate and the results are expressed as pmol per min per mg of protein.

#### **Renal NOS protein mass**

This assay was performed to determine the eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS) protein mass as previously described [17]. Monoclonal antibodies to the three NOS isoforms, and the respective

positive controls as well as the peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Transduction Laboratories. Monoclonal actin antibody was purchased from Sigma. Briefly, 100  $\mu$ g of kidney tissue preparations were size-fractionated on 4% to 12% Tris-Glycine gel (Novex) at 120 V for three hours. After electrophoresis, proteins were transferred onto Hybond-ECL membrane (Amersham Life Science Inc.) at 400 mA for 120 minutes using the Novex transfer system. The membrane was prehybridized in 10 mL of buffer A (10 mmol/L Tris hydrochloride, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for one hour and then hybridized for an additional one-hour period in the same buffer containing 10  $\mu$ L of the given NOS antibody (1:1000) or actin antibody (1:10,000). The membrane was then washed for 30 minutes in a shaking bath, with the wash buffer (buffer A without nonfat milk) changed every five minutes before one hour of incubation in buffer A plus goat anti-mouse IgG-horseradish peroxidase at the final titer of 1:1000. Experiments were carried out at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method using an ECL reagent (Amersham Inc.). The membrane was then subjected to autoluminography for one to five minutes. The autoluminographs were scanned with a laser densitometer (model PD1211; Molecular Dynamics) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain before hybridization. This step verified the uniformity of protein load and transfer efficiency across the test samples [18]. The NOS protein data were normalized against corresponding actin measurements.

#### **Glomerular eNOS immunolocalization**

Frozen tissue sections (5  $\mu$ m) of rat kidneys from protocol 2 were fixed in acetone and rinsed twice with PBST (PBS containing 0.05% Tween). Endogenous peroxidase reactions were blocked with 30%  $H_2O_2$  in a phosphate-citrate buffer, pH 5.8. Tissue sections were incubated for one hour at room temperature with eNOS antibody (1:5000 in 10% PBS; Transduction Laboratories), then rinsed twice with PBST and fixed with formalin for ten minutes. Fixed sections were incubated for 30 minutes at room temperature with rabbit anti-mouse powervision (1:100 in PBS with 10% normal rat serum; Dako, Hamburg, Germany). After rinsing with PBST, sections were incubated for 30 minutes at room temperature with goat anti-rabbit powervision (Klinipath, Duiven, The Netherlands), rinsed for ten minutes with PBS, then rinsed for five minutes with acetate buffer (100 mmol/L, pH 4.8) followed by color development in AEC substrate (Sigma) in acetate buffer. After counterstaining with hematoxylin the sections were covered with paragon. The area stained was quantified morphometrically with Optimas software



(Optimas Corporation, Seattle, Washington, USA) in 20 glomeruli per kidney at  $\times 400$  magnification and expressed as percentage of the total glomerular area.

### Renal superoxide activity

Lucigenin enhanced chemiluminescence was measured using a LUMAT LB 9507 luminometer (Berthold, Wildbad, Germany) as previously described [19]. Renal cortical slices were pre-incubated for five minutes in 300  $\mu\text{L}$  buffer (300 mmol/L HEPES, 135.3 mmol/L NaCl, 4.7 mmol/L KCl, 1.8 mmol/L  $\text{CaCl}_2$ , 1.2 mmol/L  $\text{MgSO}_4$ , 1.2 mmol/L  $\text{KH}_2\text{PO}_4$ , and 11.1 mmol/L glucose; pH 7.4) in a polystyrene tube in the luminometer at room temperature. Then background LEC was measured for two minutes. Aliquots of 300  $\mu\text{L}$  of lucigenin (bis-N-methyl acridinium at a final concentration of 100  $\mu\text{mol/L}$ ) were injected and LEC was measured for 18 minutes. Subsequently, renal cortical slices were dried overnight and weighed. LEC was calculated as the average counts measured during the last five minutes minus average background counts and expressed as counts per second per 10 mg dry weight. To determine whether LEC was specific for superoxide anion,  $\text{MnCl}_2$  ( $2 \times 10^{-3}$  mol/L), a pH neutral, membrane-permeable compound with superoxide dismutase (SOD)-like activity, was added after measurement of basal  $\text{O}_2^-$  activity. Addition of  $\text{MnCl}_2$  completely abolished the LEC signal in control as well as in cholesterol-fed rats, indicating that the signal obtained with LEC is specific for superoxide. To determine which oxidative enzyme might be responsible for superoxide production, allopurinol (an inhibitor of xanthine oxidase activity, 1 mmol/L) or diphenyleioidonium (DPI; an inhibitor of flavin oxidases, 50  $\mu\text{mol/L}$ ) was added to the test tube after basal LEC measurement in renal cortical slices. The percentage decrease of signal was determined.

### Morphological studies

Light microscopy was done on 3  $\mu\text{m}$  paraffin sections of the formaldehyde-fixed kidney stained by hematoxylin-eosin. The sections were numbered. The investigators (D.M.A. Attia and M.A. Attia) were blinded to their identity. Glomerular injury (aneurysms and glomerular fibrosis) was assessed in 50 glomeruli with  $400\times$  magnification semi-quantitatively using a 0 to 4 scale: 0 = absent, 1 = slight, 2 = mild, 3 = moderate and 4 = marked. Tubulointerstitial damage (tubular dilation, casts, flattened tubular epithelium, tubular epithelial cell degeneration/necrosis), tubular cytosolic protein droplets, and peritubular fibrosis were semiquantitatively graded in twenty fields in the same way as glomerular injury. A total glomerular and tubulointerstitial injury score was determined by summing  $(1 \times \text{score } 1) + (2 \times \text{score } 2) + (3 \times \text{score } 3) + (4 \times \text{score } 4)$ .

### Immunohistochemistry

Immunohistochemistry was carried out on 3  $\mu\text{m}$  paraffin sections of formaldehyde-fixed kidney. Tissue sections were deparaffinized and dehydrated. Glomerular desmin staining was used to determine podocyte activation [11]. Paraffin sections of kidney were stained with mu072-uc (Biogenex, San Raman, CA, USA). The outer cell layer of the glomerular tuft was evaluated. The staining was scored semiquantitatively by evaluating the percentage of the glomerular edge showing positive staining: 0 = 0 to 5% stained; 1 = 5 to 25%; 2 = 25 to 50%; 3 = 50 to 75%; 4 =  $>75\%$ . 50 glomeruli were scored. A total score was determined similarly to the total injury score. Incubation with the ED-1 mouse monoclonal antibody (kindly provided by Ed Dub, Dept. of Cell Biology, Free University, Amsterdam, the Netherlands) demonstrated monocytes/macrophages. After application of ED-1 (dilution 1:2500 in PBS containing 5% BSA, 0.4% sodium azide) to the slides at  $22^\circ\text{C}$  for one hour, bound antibody was detected by the DAKO EnVision<sup>®</sup> + System (prediluted peroxidase-dextran-conjugated goat anti-mouse antibody and DAB color reaction). The number of ED-1-antigen-positive monocyte/macrophage was determined in 50 randomly distributed glomeruli and in 20 microscopic fields with  $\times 400$  magnification for determination of monocyte/macrophage infiltration into tubulointerstitium. An average score per glomerulus or per field was calculated.

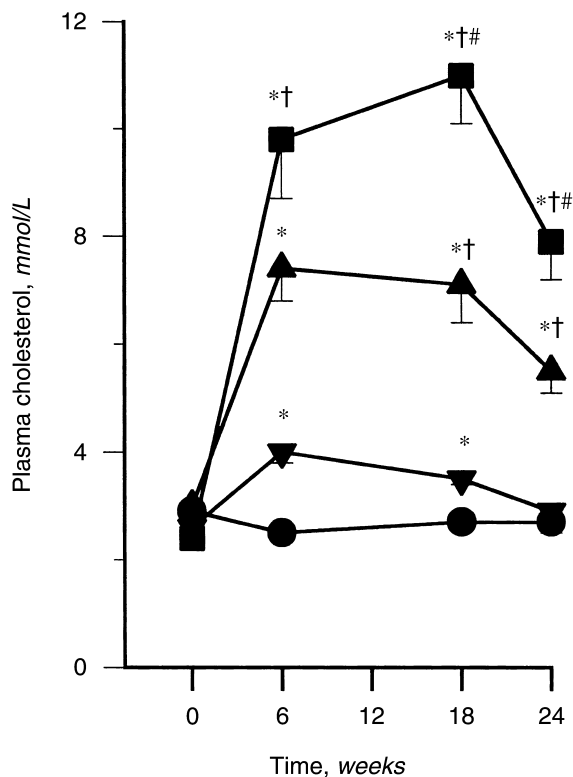
### Statistical analyses

Results are expressed as mean  $\pm$  SEM. Quantitative data were tested by one-way analysis of variance (ANOVA) in protocol 1 and by two-way ANOVA in protocol 2. The Kruskal-Wallis ANOVA tested semiquantitative data. If the variance ratio (F) reached statistical significance ( $P < 0.05$ ), quantitative data were further analyzed with the Student-Newman-Keuls test for multiple comparisons, and semiquantitative data with Dunn's test for multiple comparisons. Data in protocol 3 were compared by the *t* test.

## RESULTS

### Protocol 1

*Plasma lipids, systolic blood pressure, body weight, and renal function.* When rats were fed a high cholesterol diet, plasma cholesterol levels were dose-dependently increased (Fig. 1). There were no differences in plasma triglycerides, SBP, or creatinine clearance between the different groups (data not shown). Rats fed a 2% cholesterol diet gained significantly more weight during the 24 weeks of treatment than the other groups ( $148 \pm 9$  g in rats fed 2% cholesterol vs.  $\sim 115 \pm 3$  g in the other groups). However, no tendency to a dose-dependent ef-

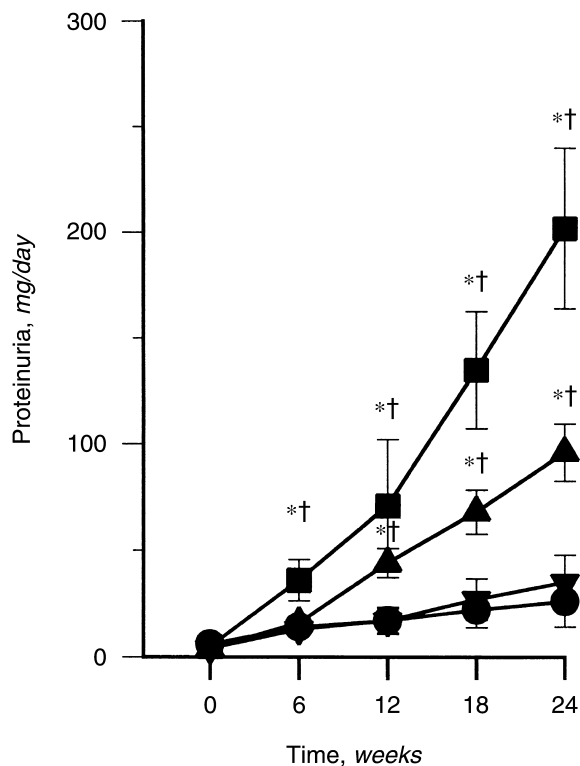


**Fig. 1.** Plasma cholesterol in control rats (●) and rats fed 0.5% cholesterol (▼), 1% cholesterol (▲), 2% cholesterol (■) for 24 weeks. \* $P < 0.05$  versus control; † $P < 0.05$  versus 0.5% cholesterol; # $P < 0.05$  versus 1% cholesterol.

fect on body weight of chow cholesterol content was observed in the groups on a lower cholesterol intake. The lowest concentration of cholesterol (0.5%) had no proteinuric effect during the study period of 24 weeks. When rats were fed 1% cholesterol, proteinuria increased in week 12 of treatment. Rats fed 2% cholesterol already showed substantial increase in proteinuria at week 6 of treatment (Fig. 2).

**Lipoprotein isolation by density-gradient ultracentrifugation.** In all cholesterol-fed animals the observed increase in terminal plasma cholesterol levels was mainly due to marked dose-dependent increases in VLDL and IDL, and to a lesser extent LDL1. However LDL2 levels were decreased, while HDL levels remained unchanged (Table 1). Note that this change in distribution also was clearly present in the rats fed 0.5% cholesterol despite the lack of increase of terminal plasma total cholesterol concentrations.

**Renal NOS activity and protein mass.** After 24 weeks of treatment renal NOS activity was significantly and similarly decreased at all levels of cholesterol intake, including rats fed only 0.5% cholesterol (Fig. 3A). In the latter, renal eNOS and iNOS protein mass were not significantly decreased, but there was a significant



**Fig. 2.** Proteinuria in control rats (●) and rats fed 0.5% cholesterol (▼), 1% cholesterol (▲), 2% cholesterol (■) for 24 weeks. \* $P < 0.05$  versus control; † $P < 0.05$  versus 0.5% cholesterol.

decrease in nNOS protein mass ( $0.92 \pm 0.05$  vs.  $0.70 \pm 0.03$  NOS/actin ratio in controls;  $P < 0.05$ ; Fig. 4).

**Renal superoxide activity.** In rats fed 0.5% cholesterol renal superoxide activity was slightly increased after 24 weeks ( $t$  test;  $P < 0.05$ ). However in rats fed 1% cholesterol renal superoxide was significantly increased and even more so in rats fed 2% cholesterol (Fig. 3B). The LEC signal was not decreased by addition of allopurinol ( $1 \pm 0.6\%$  decrease of signal), but was significantly decreased after addition of DPI ( $90.3 \pm 2.7\%$  decrease of signal), indicating that flavin oxidases rather than xanthine oxidase were responsible for superoxide activity [17].

**Glomerular and tubulointerstitial morphology.** By morphological standards, only rats fed 2% cholesterol developed significant glomerular injury (Table 2 and Fig. 5). Podocyte desmin expression and glomerular monocyte/macrophage number were dose-dependently increased in rats fed cholesterol. Rats fed 1% and 2% cholesterol developed tubulointerstitial injury (Fig. 5). The amount of protein droplets in tubular epithelial cells and tubulointerstitial monocyte/macrophage number were increased in rats fed 1% and 2% cholesterol, but not in rats fed 0.5% cholesterol (Table 2). There was no difference in peritubular fibrosis between these groups (data not shown).

**Table 1.** Cholesterol concentrations in lipoprotein fractions in rats fed increasing concentrations of cholesterol

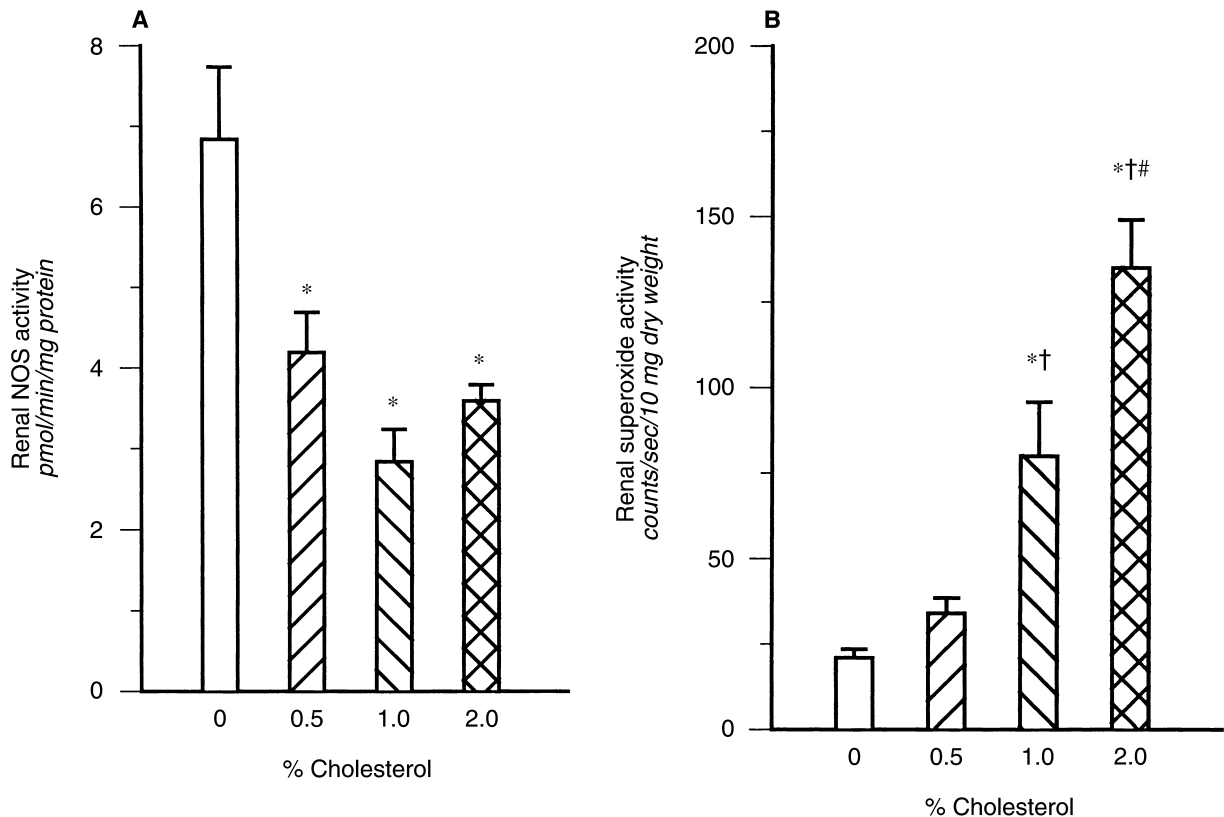
% Cholesterol	VLDL	IDL	LDL1	LDL2	HDL
	<i>μmol/L</i>				
0	0.04 ± 0.01	0.13 ± 0.04	0.18 ± 0.01	0.66 ± 0.05	1.95 ± 0.1
0.5	0.45 ± 0.08 <sup>a</sup>	0.44 ± 0.09 <sup>a</sup>	0.25 ± 0.02	0.19 ± 0.04 <sup>a</sup>	1.71 ± 0.2
1	1.53 ± 0.2 <sup>ab</sup>	1.09 ± 0.1 <sup>a</sup>	0.48 ± 0.04 <sup>ab</sup>	0.25 ± 0.06 <sup>a</sup>	1.72 ± 0.3
2	2.77 ± 0.5 <sup>ab</sup>	1.62 ± 0.6 <sup>a</sup>	0.67 ± 0.1 <sup>ab</sup>	0.42 ± 0.05 <sup>abc</sup>	1.49 ± 0.2

Data are mean ± SEM. Abbreviations are: VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

<sup>a</sup>*P* < 0.05 vs. control

<sup>b</sup>*P* < 0.05 vs. 0.5% cholesterol

<sup>c</sup>*P* < 0.05 vs. 1% cholesterol



**Fig. 3.** Renal nitric oxide synthase (NOS) activity (A) and renal superoxide activity (B) in control rats and rats fed 0.5% cholesterol, 1% cholesterol, or 2% cholesterol for 24 weeks. \**P* < 0.05 versus control; †*P* < 0.05 versus 0.5% cholesterol; #*P* < 0.05 versus 1% cholesterol.

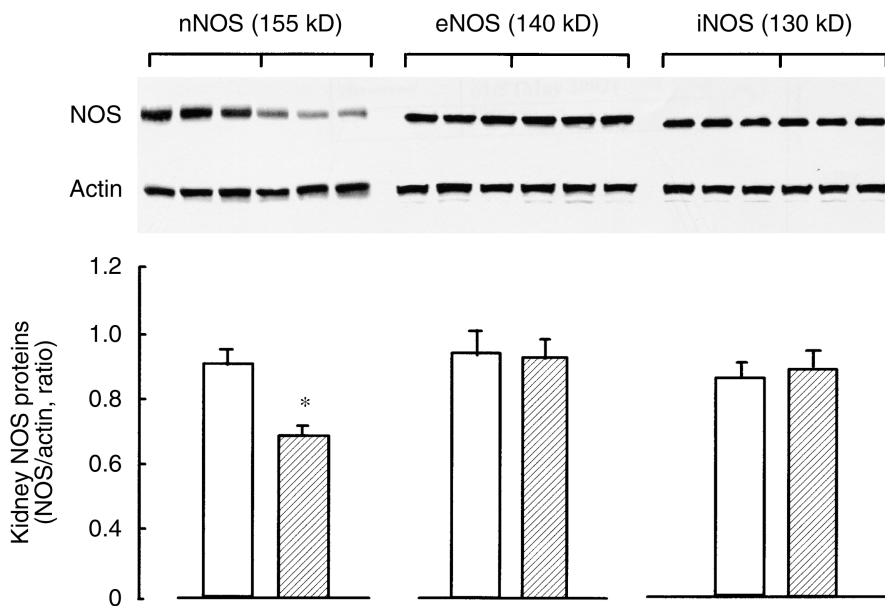
**Protocol 2**

Rats fed 2% cholesterol for two weeks had increased plasma cholesterol levels (9.4 ± 0.9 vs. 1.5 ± 0.1 mmol/L in controls; *P* < 0.05), but did not develop proteinuria (10 ± 1 vs. 7 ± 1 mg/day in controls). Renal NOS activity was significantly decreased (6.3 ± 0.3 vs. 9.1 ± 0.4 pmol/min/mg protein in controls; *P* < 0.05). In these rats renal superoxide was unchanged (12 ± 1 vs. 14 ± 2 counts/sec/10 mg dry wt in controls). Podocyte activation was significantly increased (12 ± 2 vs. 5 ± 1 in controls; *P* < 0.05). Glomerular eNOS staining in rats fed 2% cholesterol was unchanged compared to controls

(3.85 ± 0.67 vs. 4.04 ± 0.83% of glomerular area in controls; Fig. 6).

**Protocol 3**

Molsidomine had no effect on body weight, plasma lipids and lipoproteins, or on creatinine clearance (data not shown). SBP remained unchanged in molsidomine-treated rats fed normal chow, but was slightly increased in 1% cholesterol-fed rats, and this was only significant at week 18 (data not shown). Administration of molsidomine protected rats fed 1% cholesterol from development of proteinuria (Fig. 7). After 24 weeks of molsido-



**Fig. 4.** Representative NOS and actin Western blots and data depicting NOS/actin ratio for neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) nitric oxide synthases in kidneys of control rats (□) and rats fed 0.5% cholesterol (▨) for 24 weeks. \* $P < 0.05$  versus control.

**Table 2.** Glomerular and tubulointerstitial injury in control rats, and rats fed increasing concentrations of cholesterol

% Cholesterol	Glomerular injury	Podocyte activation	Glomerular monocytes #/glom	Interstitial injury	Protein droplets	Interstitial monocytes #/field
	score			score		
0	2.7 ± 1.1	9 ± 4	1.0 ± 0.1	5.3 ± 2.1	7 ± 3	4.5 ± 0.3
0.5	1.3 ± 0.7	40 ± 4 <sup>a</sup>	2.5 ± 0.2 <sup>a</sup>	4.6 ± 1.7	5 ± 2	5.0 ± 0.5
1	4.3 ± 0.5	66 ± 13 <sup>a</sup>	3.6 ± 0.2 <sup>ab</sup>	17.5 ± 3.5 <sup>ab</sup>	15 ± 2 <sup>ab</sup>	8.5 ± 0.6 <sup>ab</sup>
2	7.5 ± 2.1 <sup>ab</sup>	72 ± 11 <sup>ab</sup>	8.2 ± 0.8 <sup>abc</sup>	19.5 ± 4.6 <sup>ab</sup>	21 ± 4 <sup>ab</sup>	16.9 ± 1.7 <sup>abc</sup>

Data are mean ± SEM.

<sup>a</sup> $P < 0.05$  vs. control

<sup>b</sup> $P < 0.05$  vs. 0.5% cholesterol

<sup>c</sup> $P < 0.05$  vs. 1% cholesterol

mine administration renal NOS activity was decreased in rats fed normal chow. However, in 1% cholesterol-fed animals the low renal NOS activity was not decreased further by molsidomine treatment (Fig. 8A). The elevation of renal superoxide activity in 1% cholesterol-fed rats was prevented by molsidomine treatment. Molsidomine alone had no effect on renal superoxide activity (Fig. 8B). Combining 1% cholesterol with molsidomine completely prevented renal morphological alterations (Table 3).

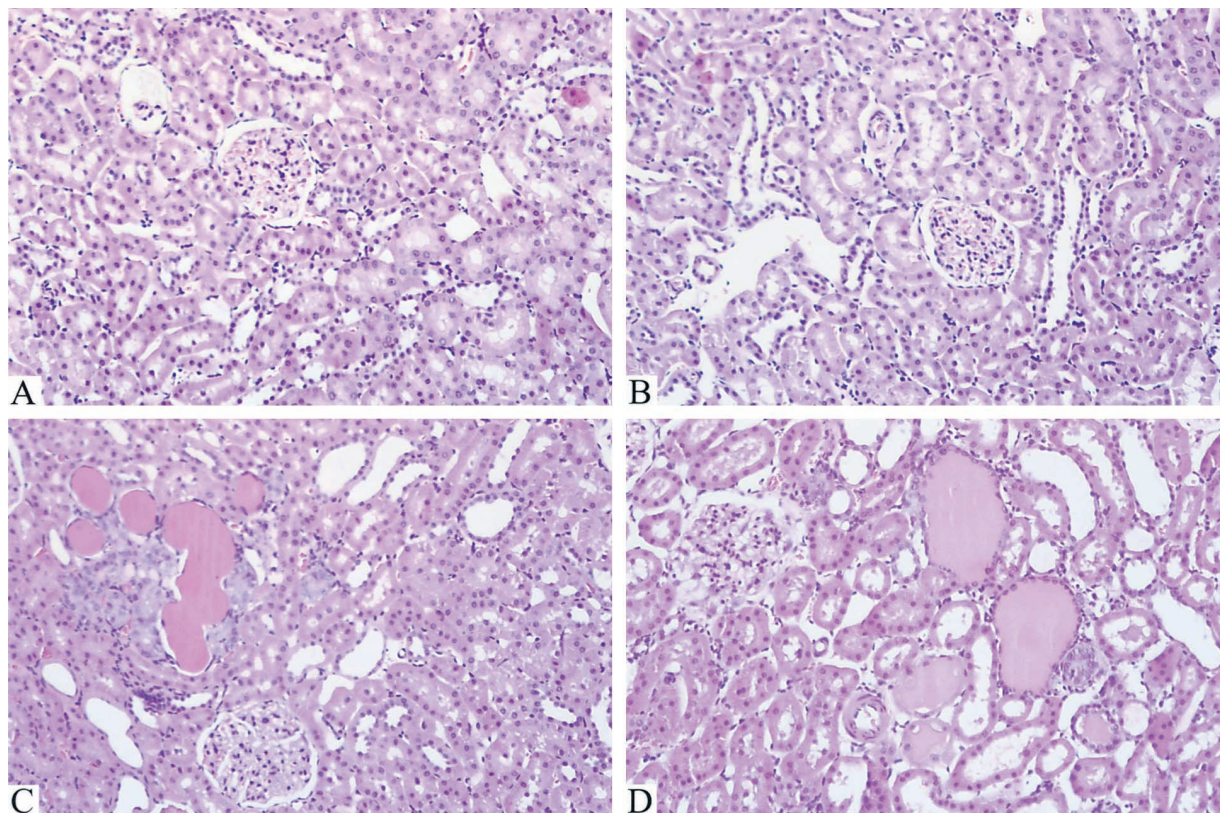
## DISCUSSION

In the present study we were able to dissociate the primary effects of hypercholesterolemia, which were decreased renal NOS activity and podocyte injury, from the secondary effects of proteinuria, which were tubulointerstitial monocyte influx, renal superoxide activity, and interstitial injury. Furthermore, hypercholesterolemia-induced podocyte injury seemed to be associated

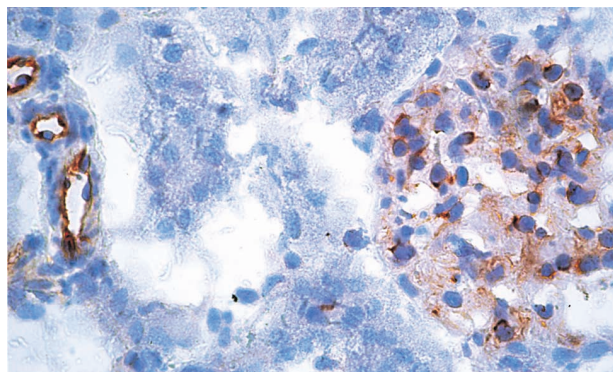
with NO deficiency since it could be prevented by exogenous NO administration.

Renal NOS activity was decreased before proteinuria was apparent. Indeed, rats fed 2% cholesterol for two weeks that were not yet proteinuric had decreased renal NOS activity. Long-term hypercholesterolemia decreased renal NOS activity even more. However, in rats fed 2% cholesterol for 24 weeks the renal NOS activity was not decreased further compared to rats fed 1% cholesterol, possibly due to a marked renal infiltration of monocytes that express high levels of iNOS [20]. Indeed, this was the group with the highest activity of superoxide in the kidney. Molsidomine also influenced renal NOS activity. It has been described that NO donors depress NOS expression and activity. For instance, NO donors induce serine phosphorylation of eNOS in endothelial cells, thereby inhibiting its activity [21]. Nitroprusside, a NO donor, decreased eNOS expression in aorta via a cGMP-mediated mechanism [22]. To our knowledge, this is the first study to demonstrate such an inhibitory effect of a NO donor on NOS in the kidney.





**Fig. 5. Representative light microscopic features of renal morphological changes in rats fed 0, 0.5, 1, and 2% cholesterol for 24 weeks.** (A) Normal glomerulus and tubulointerstitium in rats fed 0% cholesterol. (B) Rats fed 0.5% cholesterol had no glomerular or tubulointerstitial injury. (C) Rats fed 1% cholesterol showed protein casts, flattened tubular epithelium, tubular basophilia, and slight glomerular ischemia. (D) Rats fed 2% cholesterol showed more extensive tubulointerstitial damage, as well as glomerular damage including focal adhesions to Bowman's capsule.

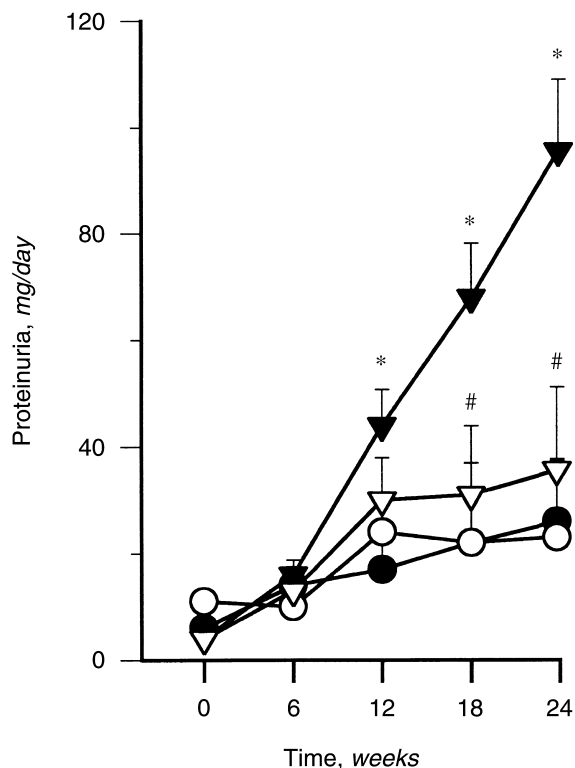


**Fig. 6. Representative light microscopic features of glomerular eNOS immunohistochemistry.** Rats fed 2% cholesterol for two weeks showed no difference in staining compared to control rats ( $3.85 \pm 0.67\%$  vs.  $4.04 \pm 0.83\%$ ).

Several mechanisms could play a role in the reduction of renal NOS activity associated with hypercholesterolemia. It has been proposed that hypercholesterolemia decreases eNOS expression in endothelial cells [9]. However, in the present study mild hypercholesterolemia, which did decrease NOS activity, did not seem to de-

crease renal eNOS or iNOS protein mass. On the other hand, nNOS protein mass was decreased. A recent study showed that both eNOS and nNOS isoforms are abundantly expressed in the renal vasculature [23]. The role of nNOS in the renal vasculature is unknown. Although it appears that hypercholesterolemia inhibited renal NOS activity, the present study does not allow us to conclude through which pathway hypercholesterolemia inhibits renal NOS activity. However, previous studies have demonstrated that hypercholesterolemia might impair tetrahydrobiopterin bioavailability, an important cofactor of NOS [24]. In patients with familial hypercholesterolemia forearm blood flow improved after tetrahydrobiopterin infusion [25]. In that case NOS becomes a superoxide instead of a NO producing enzyme. However, in the present study, both rats fed 0.5% cholesterol for 24 weeks and rats fed 2% cholesterol for two weeks had decreased renal NOS activity but no increased renal superoxide activity. Thus, it seems that in this model NOS does not become a superoxide producing enzyme. Alternatively, NOS might bind to the inhibitory protein caveolin. In endothelial cells exposed to hypercholesterolemic serum, caveolin-1 expression was up-regulated





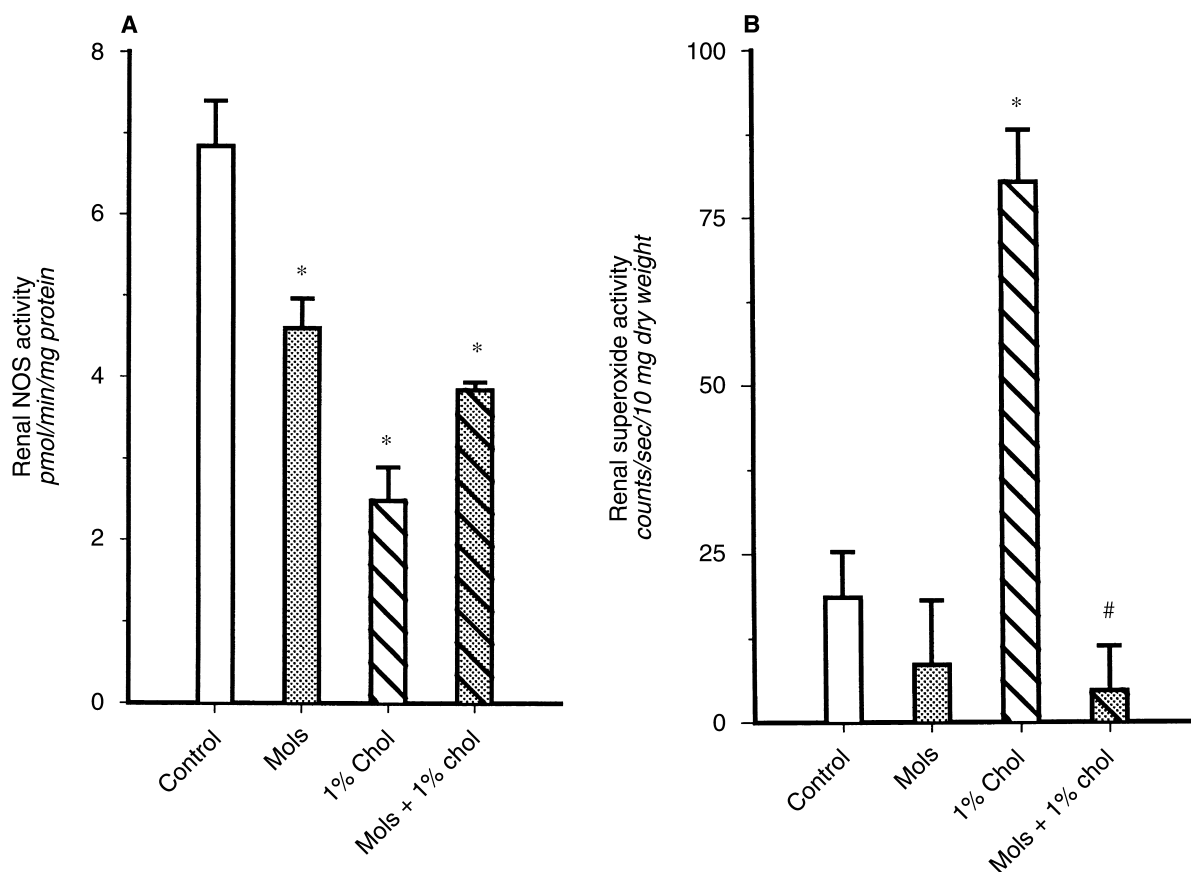
**Fig. 7.** Proteinuria in control rats (●), in rats receiving molsidomine in drinking water (○), in rats fed 1% cholesterol (▼), and in rats fed 1% cholesterol and receiving molsidomine in drinking water (▽), for 24 weeks. \* $P < 0.05$  versus control; # $P < 0.05$  versus 1% cholesterol.

[10]. Caveolin binds to NOS thereby inhibiting its NO synthesizing capacity. More calmodulin binding is required to reactivate the enzyme [10]. Another possibility is that hypercholesterolemia increases levels of an endogenous NOS inhibitor, asymmetrical dimethyl L-arginine (ADMA) [26]. Short-term dietary exposure to 4% cholesterol causing severe hypercholesterolemia increased renal NADPH diaphorase activity in rats [27]. However another short-term study using the model showed increased renal macrophage influx [28]. This may explain the increase in NADPH diaphorase activity in this model [27]. However, the somewhat milder hypercholesterolemia that we studied appeared to decrease renal NOS activity, despite practically normal NOS protein mass, thereby lowering renal NO-availability.

Renal superoxide activity was only significantly increased in rats with significant proteinuria, tubulointerstitial monocyte influx, and tubulointerstitial injury. Thus, increased superoxide activity seemed to be secondary to proteinuria. Indeed, rats receiving 1% cholesterol plus molsidomine did not develop proteinuria and had no increased renal superoxide activity, whereas rats fed 1% cholesterol alone did. Furthermore, rats fed 2% cholesterol for two weeks had neither proteinuria nor increased renal superoxide activity, which is in contrast to

cultured endothelial cells where hypercholesterolemia increased superoxide production in parallel with decreased NO production [29]. However, unlike isolated endothelial cells in culture, the intact kidney contains additional powerful scavenging systems, such as hemoglobin. Superoxide activity may only become apparent when its production overwhelms these scavenger systems. In the present study, we found increased cytosolic protein droplets in tubular epithelial cells and tubulointerstitial infiltration of macrophages in the presence of proteinuria. Indeed, tubular reabsorption of leaking proteins increases tubulointerstitial macrophage influx [30]. Both macrophages [31] and renal epithelial cells [32, 33] are potential sources of superoxide production in the kidney. In the female rats used in the present study, renal superoxide production was only increased when proteinuria increased fourfold, and when tubulointerstitial injury and monocyte influx became apparent. Once renal NOS activity is already decreased, superoxide anions may cause a further decrease in renal NO-availability due to peroxynitrite formation [5]. It has been shown in severely proteinuric nephrotic rats that administration of molsidomine worsened cortical lipid peroxidation and injury [34]. This suggests that when renal superoxide activity is increased, exogenous NO can increase peroxynitrite production. Hence, NO donors such as molsidomine may only be useful to ascertain primary renal NO deficiency, that is, to supplement NO before the onset of severe renal injury.

Desmin expression is a marker of podocyte injury [11]. In a previous study we showed that dietary hypercholesterolemia, characterized by marked increases in VLDL and IDL [35, 36], was associated with podocyte injury [12]. However, in that study increased desmin expression coincided with increased proteinuria [12]. In the present study desmin expression was increased in rats that had no proteinuria, or that had not yet developed proteinuria. Podocytes express LDL receptors and show deposition of apolipoproteins B and E in glomeruli of patients with kidney diseases [37], which is in accordance with our finding that VLDL and IDL were increased after cholesterol feeding. Furthermore, cultured glomerular epithelial cells bind and take up VLDL [38] and IDL [39]. Besides lipoprotein receptors, podocytes also express the NO-dependent enzyme soluble guanylate cyclase, although they do not express any isoform of NOS [14]. It also has been demonstrated that administration of NO to cultured podocytes increased production of cGMP, which controls the cytoskeletal structure of the podocyte and thus limits retraction [40]. Although we did not find a decrease in glomerular eNOS staining, whole kidney NOS activity was decreased and molsidomine could prevent podocyte injury. Thus, hypercholesterolemia appears to decrease renal NO-availability by stimulating endogenous inhibitors. Podocytes might be tonically sub-



**Fig. 8.** Renal NOS activity (A) and renal superoxide activity (B) in control rats, in rats receiving molsidomine in drinking water, in rats fed 1% cholesterol, and in rats fed 1% cholesterol and receiving molsidomine in drinking water for 24 weeks. \* $P < 0.05$  versus control; # $P < 0.05$  versus 1% cholesterol.

**Table 3.** Glomerular and tubulointerstitial injury in control rats, rats treated with molsidomine, 1% cholesterol, or cholesterol in combination with molsidomine

Groups	Glomerular injury	Podocyte activation	Glomerular monocytes #/glom	Interstitial injury	Protein droplets	Interstitial monocytes #/field
	score			score		
Control	2.7 ± 1.1	9 ± 4	1.0 ± 0.1	5.3 ± 2.1	7 ± 3	4.5 ± 0.3
Molsidomine	0.5 ± 0.3	18 ± 4	0.5 ± 0.1	3.8 ± 2.6	3 ± 2	3.2 ± 0.3
1% Cholesterol	4.3 ± 0.5	66 ± 13 <sup>a</sup>	3.6 ± 0.2 <sup>a</sup>	17.5 ± 3.5 <sup>a</sup>	15 ± 2 <sup>a</sup>	8.5 ± 0.6 <sup>a</sup>
1% Cholesterol + molsidomine	1.1 ± 0.6 <sup>b</sup>	16 ± 5 <sup>b</sup>	0.8 ± 0.2 <sup>b</sup>	5.8 ± 1.6 <sup>b</sup>	3 ± 2 <sup>b</sup>	2.5 ± 0.6 <sup>b</sup>

Data are mean ± SEM.

<sup>a</sup> $P < 0.05$  vs. control

<sup>b</sup> $P < 0.05$  vs. 1% cholesterol

jected to oxygen radicals as they contain the superoxide-producing enzyme NAD(P)H oxidase, which is activated by ATP [41]. Because NO normally counteracts oxidative stress, when hypercholesterolemia decreases renal NO-availability, podocyte injury may occur. Podocytes are practically unable to replicate postnatally. Therefore, any injury that podocytes suffer might lead to areas of “bare” glomerular basement membrane, which are the starting points for irreversible renal injury [13]. In the present study glomerular monocyte influx did not seem

to be required for podocyte injury, since podocytes were activated in the absence of such an influx. The change in podocyte phenotype appeared to be due to decreased NO-availability, since rats receiving 1% cholesterol plus molsidomine had high plasma cholesterol levels but no podocyte activation.

As mentioned above, podocyte injury leads to proteinuria and further renal damage via a cascade of events. At the ultrastructural level increased desmin expression accompanied by retraction of podocytes results in bare

areas of the glomerular basement membrane and thus increased glomerular protein permeability [13, 42], and secondary protein reabsorption by proximal tubular cells [43], which lead to macrophage influx, and secondary tubulointerstitial injury [30]. Only hypercholesterolemic, proteinuric rats had increased cytosolic protein droplets in tubular epithelial cells and tubulointerstitial infiltration of macrophages. Marked hypercholesterolemia also can cause tubulointerstitial injury directly by triggering proinflammatory and profibrogenic events [2]. However, the absence of tubulointerstitial injury after two weeks of hypercholesterolemia indicates that tubulointerstitial injury was, at least partly, secondary to proteinuria, and to increased oxidative stress. Apparently, glomerular protein leakage in rats fed 0.5% cholesterol or 2% cholesterol for only two weeks was so low that tubular protein reabsorption did not lead to extensive inflammation. The presence of hypercholesterolemia for a longer period does lead to podocyte injury, proteinuria, and interstitial injury. Treatment with molsidomine prevented all these changes. Glomerular and interstitial monocyte influx was remarkably suppressed by molsidomine to levels numerically lower than those found in controls. It is well known from endothelial cell culture studies that NO reduces the expression of adhesion molecules [44] and that L-arginine reduces monocyte adhesion ex vivo [45]. However, a direct effect of a NO donor on monocyte influx to our knowledge has not been shown previously in vivo.

In summary, the present study demonstrates dose- and time-dependent effects of hypercholesterolemia, allowing us to dissect the primary effects of hypercholesterolemia, namely decreased renal NOS activity and podocyte activation, from the secondary effects of proteinuria, which are tubulointerstitial monocyte influx, renal superoxide activity, and interstitial injury. Hypercholesterolemia-induced podocyte activation seems to be associated with NO deficiency, as it can be prevented by exogenous NO administration. Podocyte activation, the first step in a pathway common to many models of renal injury, leads to proteinuria, tubulointerstitial monocyte influx, renal superoxide activity, and interstitial injury.

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