Hypercholesterolemia in Rats Induces Podocyte Stress and Decreases Renal Cortical Nitric Oxide Synthesis via an Angiotensin II Type 1 Receptor–Sensitive Mechanism

DIANA M. ATTIA,* OLIVIER FERON,† ROEL GOLDSCHMEDING,‡ LOUK H. RADERMAKERS,‡ NOSRATOLA D. VAZIRI,§ PETER BOER,* JEAN-LUC BALLIGAND,† HEIN A. KOOMANS,* and JAAP A. JOLES*

*Department of Nephrology and Hypertension, University Medical Center, Utrecht, the Netherlands; †Department of Internal Medicine, Louvain University, Brussels, Belgium; ‡Department of Pathology, University Medical Center, Utrecht, the Netherlands; and §Division of Nephrology, Departments of Medicine, Physiology, and Biophysics, University of California Irvine, Irvine, California

Abstract. Podocyte stress precedes proteinuria in hypercholesterolemic rats. Molsidomine, a nitric oxide (NO) donor, prevented podocyte stress and proteinuria in long-term hypercholesterolemia, suggesting that podocyte stress was due to NO deficiency. Podocytes express the angiotensin II type 1 receptor, which influences their function. Because NO counteracts angiotensin II, it was hypothesized that in a setting of impaired renal NO availability, angiotensin II receptor inhibition could prevent podocyte stress. For determining the effect of NO deficiency on podocyte stress, one group of female rats were fed 2% cholesterol and another group the arginine analogue which influences their function. Because NO counteracts angiotensin II, it was hypothesized that in a setting of impaired renal NO availability, angiotensin II receptor inhibition could prevent podocyte stress. For determining the effect of NO deficiency on podocyte stress, one group of female rats were fed 2% cholesterol and another group the arginine analogue N-ω-nitro-L-arginine (L-NNA; 40 mg/kg food) for 2 wk. Another group of rats that were fed 2% cholesterol also received the NO donor molsidomine (120 mg/L water) for 2 wk before and during cholesterol feeding. For determining the influence of angiotensin II in the setting of decreased renal NO availability, rats that were treated with cholesterol or L-NNA received the angiotensin II type 1 antagonist losartan (200 mg/L water) for 2 wk before and during cholesterol or L-NNA administration. Desmin staining and electron microscopy were used to monitor podocyte activation. Glomerular caveolin was quantified by immunohistochemistry. Renal cortical NO synthase isoforms, and caveolin-1 protein mass were also measured. Both short-term cholesterol and L-NNA induced podocyte stress as evidenced by enhanced desmin staining and electron-dense fused foot processes. Podocyte stress was prevented by molsidomine in short-term hypercholesterolemia. Furthermore, losartan prevented podocyte stress in rats that were treated with cholesterol or with L-NNA. Finally, hypercholesterolemia decreased renal cortical NO synthase activity and increased caveolin-1 protein mass and glomerular caveolin staining, and these changes were also prevented by losartan. It is suggested that podocyte stress in these models of early injury results from angiotensin II, unopposed by the action of endogenous NO. This underscores the strategic role of angiotensin II blockers in early kidney disease.

Hypercholesterolemia has been found to decrease nitric oxide (NO) availability in the renal circulation (1) and renal artery (2). In a previous study, we showed that hypercholesterolemia can decrease renal NO synthase (NOS) activity and cause podocyte stress before proteinuria (3). We suggested that podocyte stress could be due to NO deficiency, because the NO donor molsidomine (4) prevented the long-term effects of cholesterol loading. Therefore, our first question was whether molsidomine could also prevent early hypercholesterolemia-induced podocyte stress.

Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 (AT-1) receptor blockers prevent hypercholesterolemia-induced vascular injury in various species (5–10). Similarly, angiotensin II receptor blockade can prevent long-term effects of hypercholesterolemia on proteinuria and renal injury. For instance, treatment of hypercholesterolemic rats with an ACE inhibitor (11) or hypercholesterolemic Imai rats with an AT-1 receptor antagonist (12) prevented the development of glomerulosclerosis. The protective effects of angiotensin II receptor blockade are thought to occur through inhibition of proteinuria and by diminishing interstitial inflammation, which are late effects. However, podocyte changes are early events. Podocytes contain AT-1 receptors, which upon stimulation increase intracellular calcium activity and, possibly, podocyte contractility (13). Furthermore, stimulation of cultured podocytes with angiotensin II increased cytoskeletal density (14). Angiotensin II infusion in vivo causes podocyte injury (15), and this can be prevented by AT-1 receptor blockade (16). In the kidney, NO counteracts angiotensin II (17,18), as is the case in the systemic circulation (19). Thus, when

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Correspondence to Dr. Jaap A. Joles, Department of Nephrology and Hypertension (Room F03.226), University Medical Center, Heidelberglaan 100, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. Phone: 31-30-2507329; Fax: 31-30-2543492; E-mail: J.A.Joles@med.uu.nl

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hypercholesterolemia or NOS inhibition reduces renal NO availability, angiotensin II may become injurious to podocytes. Therefore, the second question was whether AT-1 receptor blockade could prevent the early stress of NO deficiency, as a result of either cholesterol or NOS inhibition, in podocytes.

Finally, it has been shown that cholesterol increases caveolin-1 expression in endothelial cells, which binds to endothelial NOS (eNOS), thus inhibiting its activity (20). This notion is particularly interesting in view of the interaction of angiotensin II and hypercholesterolemia. Because angiotensin II is also known to increase caveolin-1 biosynthesis and turnover in caveola-like invaginations in cultured vascular smooth muscle cells (21) and to decrease NOS expression and activity in cultured endothelial cells (22), it might be that prevention of cholesterol effects by angiotensin II receptor blockade focuses on caveolin-1 and its effect on NOS activity. Therefore, we investigated whether in the kidney angiotensin II and hypercholesterolemia interact at the level of caveolin-1 and NOS activity.

For excluding effects secondary to proteinuria, rats were exposed to NOS inhibition or hypercholesterolemia for only 2 wk. For examining the effect of NO deficiency on podocyte stress, rats received either a low dose of N-ω-nitro-L-arginine (L-NNA) or 2% dietary cholesterol. For examining whether early podocyte stress in cholesterol-fed rats could be prevented by exogenous NO administration, rats were fed 2% cholesterol and received the NO donor molsidomine. The deleterious effects of NO deficiency, in the absence of angiotensin II, were studied in rats that received an AT-1 receptor antagonist (losartan) for 2 wk before and during either L-NNA or cholesterol administration.

Materials and Methods

Animals

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

Experimental Protocol 1: Hypercholesterolemia and Podocyte Stress

Rats were treated for 4 wk. Six groups of rats were studied, each group consisting of six to eight animals. The control group received regular diet and tap water. Water and food intake were ~80 ml/kg body wt per d and 70 g/kg body wt per d, respectively. The second group received molsidomine dissolved in drinking water (120 mg/L, resulting in a dose of ~10 mg/kg per d) for 4 wk. The third group received losartan dissolved in drinking water (200 mg/L, resulting in a dose of ~16 mg/kg per d). The fourth group was fed 2% cholesterol + 0.5% cholate for the last 2 wk, resulting in an intake of 1.4 g cholesterol/kg body wt per d. The fifth group received molsidomine for 4 wk and 2% cholesterol + 0.5% cholate for the last 2 wk. The sixth group received losartan for 4 wk and was fed 2% cholesterol + 0.5% cholate for the last 2 wk. Cholesterol and cholate were mixed through chow (RMH-TM; Hope Farms, Woerden, the Netherlands).

Experimental Protocol 2: NOS Inhibition and Podocyte Stress

Two additional groups were studied for 4 wk. The seventh group received L-NNA in food (40 mg/kg per d) for the last 2 wk. The eighth group received losartan for 4 wk and L-NNA for the last 2 wk.

At the end of both protocols, blood was collected under anesthesia (see below), and the kidneys were removed and cut transversely into three slices. The middle slice was immersion-fixed in PBS formaldehyde (4%; pH 7.35) and embedded in paraffin for morphology and for desmin and caveolin staining. The poles were frozen in liquid nitrogen and stored at −80°C until being processed for renal cortical NOS activity, renal cortical NOS and caveolin-1 protein abundance, and glomerular lipid deposits. For transmission electron microscopy, kidneys of two cholesterol-fed and two control rats were perfused with appropriate fixative (see below) for 2 min at 160 mmHg.

Plasma Lipids, BP, Body Weight, and Renal Function

At the end of the experiment (week 4), the animals were anesthetized with 60 mg/kg sodium pentobarbital intraperitoneally to collect blood from the aorta for determination of plasma creatinine and lipids. Plasma cholesterol and triglycerides were determined enzymatically (Boehringer, Mannheim, Germany). Systolic BP (SBP) was measured in awake rats, starting 1 wk before the start of treatment (week 0) by the tail-cuff method (ITTC, San Diego, CA). Urine was collected at weeks 0 and 4 for determination of urinary protein excretion. The rats were weighed and placed in metabolic cages for 24 h, with free access to food and water. Urinary protein levels were determined by the Bradford method. Plasma creatinine levels were determined colorimetrically (Sigma Diagnostics, St. Louis, MO).

Histochemistry

Glomerular lipid deposits were determined with Sudan II/IV on frozen kidney sections. The staining was scored semiquantitatively with ×400 magnification by evaluating the percentage of the glomerulus showing positive staining, 0 = 0 to 5% stained; 1 = 5 to 25%; 2 = 25 to 50%; 3 = 50 to 75%; 4 = >75%. Twenty glomeruli were scored. A total score was determined by adding (0 × score 0) + (1 × score 1) + (2 × score 2) + (3 × score 3) + (4 × score 4).

Immunohistochemistry

Immunohistochemistry was carried out on 3-μm paraffin sections of formaldehyde-fixed kidney. Tissue sections were deparaffinized and dehydrated. Glomerular desmin staining was used to determine podocyte activation (23). Paraffin sections of formaldehyde-fixed kidney were stained with mu072-uc (Biogenex). The outer cell layer of the glomerular tuft was evaluated. The staining was scored semiquantitatively with ×400 magnification 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%. Fifty glomeruli were scored. A total score was determined as described for glomerular lipid deposits. Glomerular caveolin was evaluated morphometrically. Endogenous peroxidase reactions were blocked with citric acid (40 mmol/L) plus Na2HPO4 (120 mmol/L; pH 5.8) for 15 min. Aspecific reactions were blocked with 10% normal goat serum in PBS for 15 min. Paraffin sections were incubated overnight with the primary rabbit anti-rat polyclonal caveolin antibody (BD Transduction Laboratories, San Jose, CA; 1:150 in 10% normal goat serum/
PBS) at 4°C. The signal was enhanced by incubating for 30 min at room temperature with goat anti-rabbit Powervision (polymerized horseradish peroxidase–goat anti-rabbit; Immunologic, Duiven, The Netherlands). After rinsing for 5 min with acetate buffer (100 mmol/L; pH 4.8), color development was done with 3-amino-9-ethylcarbazole substrate (Sigma). After counterstaining with hematoxylin, sections were covered with paragon. The stained glomerular area was quantified morphometrically with Analysis software in 20 glomeruli/kidney at ×400 magnification and expressed as a percentage of total glomerular area.

Transmission Electron Microscopy

For transmission electron microscopy, kidneys of two cholesterol-fed and two control rats were perfused with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) containing 5 mM CaCl₂ and 10 mM MgCl₂. Small blocks of kidney cortex were cut and postfixed in 1% OsO₄ in the same buffer for 2 h. After dehydration in a graded series of ethanol, the samples were embedded in Epon. Semithin 1-μm sections were cut with glass knives on a Reichart Ultracut ultramicrotome and stained with methylene blue and pararosanilin (24). From each kidney, three glomeruli, i.e., six per rat, were selected. Ultrathin sections were cut with a diamond knife on a Reichart Ultracut ultramicrotome and stained with uranyl acetate at 63°C for 45 min followed by lead citrate for 10 min, and evaluated in a Phillips JEOL JEM1010 electron microscope.

Renal Cortex NOS Activity

NOS activity was measured by determining the formation of L-3H-citrulline from L-3H-arginine. Using an Ultraturrax, an aliquot of ~300 μL of homogenate was subjected to electro-pherophoresis on 10% SDS-PAGE gels, then transferred onto PVDF membranes. The membranes were incubated overnight in 5% low-fat milk to block nonspecific sites and the respective positive controls as well as the peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Transduction Laboratories. Briefly, 100 μg of kidney tissue preparations were size-fractionated on 4 to 12% Tris-Glycine gel (Novex) at 120 V for 3 h. After electrophoresis, proteins were transferred onto Hybond-ECL membrane (Amersham Life Science) at 400 mA for 120 min 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 1 h and then hybridized for an additional 1-h period in the same buffer containing 10 μL of the given anti-NOS monoclonal antibody (1:1000). The membrane was then washed for 30 min in a shaking bath, with the wash buffer (buffer A without nonfat milk) changed every 5 min before 1 h 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 ECL reagent (Amersham). The membrane was then subjected to autoradiography for 1 to 5 min. The autoradiographs were scanned with a laser densitometer (model PD1211; Molecular Dynamics, Eugene, OR) 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 (26).

Renal Cortex Caveolin-1 Protein Mass

Kidney slices were ground to powder with a precooled pestle, in a mortar filled with a small amount of liquid nitrogen. After nitrogen was evaporated, samples were collected and homogenized in a glass Potter with 100 μL of buffer (composition: 20 mmol/L Tris-HCl [pH 7.4], 2.5 mmol/L EDTA, 100 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na₃VO₄, 1% NaDOC, 0.1% SDS, 1% Triton X-100, and a cocktail of protease inhibitors [Sigma]). Samples were then transferred into microtubes and centrifuged for 10 min at 10,000 × g, and the supernatants were collected for immunoblotting experiments. Protein samples (30 to 60 μg protein/lane) were subjected to electrophoresis on 10% SDS-PAGE gels, then transferred onto PVDF membranes. The membranes were incubated overnight in 5% low-fat milk in PBS-Tween buffer at 4°C. Immunoblotting was then performed as described previously (20).

Statistical Analyses

Results are expressed as mean ± SEM. Quantitative data were tested by two-way ANOVA. The Kruskal-Wallis ANOVA tested semiquantitative data. When the variance ratio (F) reached statistical significance (P < 0.05), quantitative data were analyzed further with the Student-Newman-Keuls test for multiple comparisons, and semi-
quantitative data were analyzed further with Dunn test for multiple comparisons.

Results

Protocol 1

Two weeks of cholesterol feeding significantly increased plasma cholesterol levels (Figure 1A) and glomerular lipid deposition (Figure 1B). Dietary cholesterol had no effect on SBP (Table 1). Glomerular sclerosis and interstitial fibrosis were not observed. Transmission electron microscopy of glomeruli of cholesterol-treated rats showed focal fusion of foot processes of epithelial cells of high electron density, as a result of condensed cytoskeleton (Figure 2A), which was practically absent in control rats (Figure 2B). Rats that were fed cholesterol had marked glomerular epithelial desmin staining, a marker of podocyte activation (Figure 2, C versus D), and a significant increase in glomerular edge desmin score (Figure 2E). Desmin staining was practically absent in most glomeruli of control rats (Figure 2D). Renal cortex NOS activity was significantly decreased (Figure 3A). However, renal cortical bNOS, eNOS, and iNOS protein masses were not significantly changed (Figure 3B). Cortex caveolin-1 protein mass was increased by nearly 40% in rats that were fed cholesterol (Figure 4). Glomerular caveolin, although clearly present in controls (Figure 5B), was significantly increased by cholesterol (Figure 5A), positive surface area being increased by nearly 50% (Figure 5C). Note that caveolin staining was not restricted to the visceral epithelium but also seemed to be present in endothelial and mesangial cells.

Losartan had no influence on plasma cholesterol levels (Figure 1A) and on glomerular lipid deposits (Figure 1B) in cholesterol-fed rats and in rats that were fed normal diet (data not shown). SBP was slightly lower in all rats that received losartan (Table 1). Treatment with losartan prevented podocyte activation in cholesterol-fed rats (Figure 2E) and prevented the decrease of renal NOS activity (Figure 3A). Treatment with losartan decreased renal cortex caveolin-1 (Figure 4) and glomerular calveolin-1 (Figure 5) levels to normal. Losartan had no effect on renal cortex NOS activity (8.49 ± 0.53 versus 8.54 ± 0.38 pmol/min per mg protein in control) or glomerular caveolin-1 (Figure 5C) in rats that were fed a normal diet.

Molsidomine had no influence on plasma cholesterol levels (Figure 1A) and on glomerular lipid deposits (Figure 1B) in cholesterol-fed rats and in rats that were fed a normal diet (data not shown). Note that molsidomine also had no influence on SBP (Table 1). Treatment with molsidomine prevented podocyte activation in cholesterol-fed rats (Figure 2E) but also significantly decreased renal cortex NOS activity in rats that were fed cholesterol (Figure 3A) and in rats that were fed a normal diet (4.96 ± 0.26 versus 8.54 ± 0.38 pmol/min per mg protein in control; *P < 0.05 versus control). None of these treatments had any effect on plasma creatinine (Table 1), triglycerides (data not shown), or proteinuria (Table 1).

Protocol 2

There were no significant effects of L-NNA on plasma cholesterol (Figure 1A), glomerular lipid deposits (Figure 1B), creatinine (Table 1), and plasma triglycerides (data not shown). L-NNA for 2 wk significantly increased SBP, and losartan treatment prevented this (Table 1). L-NNA, with or without 2 wk of pretreatment with losartan, had no influence on proteinuria. L-NNA–treated rats had significantly increased podocyte activation, which was prevented by losartan treatment (Figure 2E). L-NNA–treated rats had decreased renal cortex NOS activity, which was not corrected by losartan (Figure 3A).

Discussion

The main finding of this study is that hypercholesterolemia-or NOS inhibition–induced early podocyte stress is prevented by losartan before the onset of proteinuria. Molsidomine, an NO donor, prevented podocyte stress in hypercholesterolemic rats, suggesting that podocyte stress was due to NO deficiency. Furthermore, hypercholesterolemia decreased renal cortical

Table 1. Effects of hypercholesterolemia without or with molsidomine or losartan treatment and L-NNA without or with losartan treatment on plasma creatinine, proteinuria, and SBP in female rats

<table>
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<tr>
<th></th>
<th>N</th>
<th>Plasma creatinine (mmol/L)</th>
<th>Proteinuria (mg/d)</th>
<th>SBP (mmHg)</th>
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</thead>
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<td>Control</td>
<td>8</td>
<td>52 ± 2</td>
<td>5 ± 1</td>
<td>126 ± 1</td>
</tr>
<tr>
<td>Molsidomine</td>
<td>6</td>
<td>63 ± 3</td>
<td>7 ± 3</td>
<td>129 ± 2</td>
</tr>
<tr>
<td>Losartan</td>
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<td>55 ± 2</td>
<td>4 ± 1</td>
<td>120 ± 1</td>
</tr>
<tr>
<td>Cholesterol</td>
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<td>54 ± 1</td>
<td>4 ± 1</td>
<td>128 ± 1</td>
</tr>
<tr>
<td>Molsidomine + cholesterol</td>
<td>8</td>
<td>56 ± 4</td>
<td>8 ± 2</td>
<td>127 ± 2</td>
</tr>
<tr>
<td>Losartan + cholesterol</td>
<td>8</td>
<td>56 ± 2</td>
<td>7 ± 2</td>
<td>117 ± 5</td>
</tr>
<tr>
<td>L-NNA</td>
<td>6</td>
<td>57 ± 4</td>
<td>6 ± 1</td>
<td>138 ± 2</td>
</tr>
<tr>
<td>Losartan + L-NNA</td>
<td>6</td>
<td>66 ± 3</td>
<td>3 ± 0</td>
<td>110 ± 4</td>
</tr>
</tbody>
</table>

*a L-NNA, N-w-nitro-L-arginine; SBP, systolic BP. Data are mean ± SEM.

b *P < 0.05 versus control.

c *P < 0.05 versus cholesterol.

d *P < 0.05 versus L-NNA.
activity as a result of hypercholesterolemia or NOS inhibition with NO deficiency because decreased renal cortical NOS podocyte stress. This podocyte stress may have been associated with NO deficiency because decreased renal cortical NOS activity as a result of hypercholesterolemia or NOS inhibition accompanied podocyte stress. However, we must acknowledge that this association does not necessarily suggest causality and that changes in renal cortical NOS activity may not be reflected by parallel changes within the glomerulus.

Exogenous NO administration with molsidomine reduced podocyte stress. Molsidomine increases cellular levels of cGMP by stimulating soluble guanylate cyclase directly (4,28). The dose used protected hypercholesterolemic and uremic rats from developing proteinuria, and adverse effects were not reported (3,29). Podocytes do not express any of the isoforms of NOS. Whether podocytes are directly responsive to NO is debatable. Administration of NO to cultured glomerular epithelial cells increased production of cGMP, which controlled the cytoskeletal structure and limited retraction (14). However, it has recently been described that podocytes in situ do not express soluble guanylate cyclase (30). Mesangial cells express eNOS and iNOS (31) as well as soluble guanylate cyclase (30). Mesangial cell contractility is known to influence podocyte conformation (32) and hence also influences podocyte stress. Even though podocytes express LDL receptors and cultured glomerular epithelial cells bind and take up VLDL (33) and IDL (34), this is probably not relevant in the present study, because neither losartan nor molsidomine decreased plasma cholesterol levels in cholesterol-fed rats, but both prevented podocyte stress. Furthermore, glomerular lipid deposits in cholesterol-fed rats, determined by the Sudan black staining, were not altered by treatment with either losartan or molsidomine. Thus, early podocyte stress in hypercholesterolemia may be linked to NO deficiency rather than to cholesterol uptake.

In the kidney, the heart, and blood vessels, NO counteracts angiotensin II activity and is considered to be a safety factor (17–19). Hypercholesterolemia enhanced biologic activity of angiotensin II in experimental models (5–12) and in humans (35). It has been suggested that in the vasculature, angiotensin II increases superoxide production and lipid peroxidation, which decreases endothelial NO availability (19), and blockade of the renin angiotensin II system was shown to increase NOS activity in endothelial cells in aging rats (36). Treatment with AT-1 receptor blockers (10) and ACE inhibitors (37) prevented lipid peroxidation, increased endothelial NO availability, and prevented early hypercholesterolemia-induced atherosclerosis. Moreover, ACE inhibition improved endothelium-dependent relaxation in hypercholesterolemic patients (38). It has also been shown that losartan prevented renal injury during chronic NOS inhibition (18). In the current study, prevention of podocyte stress by losartan in the L-NNA rats suggests that when NO availability is low, angiotensin II may have a direct noxious effect on podocytes. Indeed, as discussed above, NO deficiency leads to podocyte stress. Podocytes are probably subject to opposing effects of angiotensin II and NO, as is the case for vascular smooth muscle cells. It has been shown that podocytes express AT-1 receptors (13), and stimulation with angiotensin II increased intracellular calcium influx and superoxide activity (39). Stimulation of cultured podocytes with angiotensin II increased cAMP levels and caused enhanced actin aggregation (14). We found previously that NOS inhibition, before the development of injury, lowers renal angiotensin II levels and

**Figure 2.** (A) In a cholesterol-treated rat, focal fusion foot processes of epithelial cells of high electron density, as a result of local accumulation of condensed cytoskeleton, can be observed. (B) Part of a glomerular loop of a control rat showing electron-dense foot processes with a low density of condensed cytoskeleton. Larger processes are also present. Bar = 1 μm. (C) Desmin in a glomerulus of a female rat with dietary hypercholesterolemia. (D) Desmin staining was virtually absent in the glomerulus of a control rat. (E) Desmin staining in controls, rats that were fed 2% cholesterol for 2 wk, rats that were fed 2% cholesterol for 2 wk and were pretreated for 2 wk with molsidomine or losartan, and rats that received 40 mg L-NNA/kg diet for 2 wk without or with pretreatment with losartan for 2 wk. *P < 0.05 versus control; †P < 0.05 versus 2% cholesterol; #P < 0.05 versus L-NNA.

NOS activity and increased renal cortical and glomerular caveolin protein, which were also prevented by losartan.

Despite a similar degree of hypercholesterolemia, podocyte injury was much less pronounced at this early stage than in our previous studies, in which after 3 mo hypercholesterolemia had lead to proteinuria (3,27). Transmission electron microscopy showed only occasional fusion of some epithelial foot processes with focal accumulation of condensed cytoskeleton. At a later stage when proteinuria was present, we previously observed pseudocyst formation and extensive foot process effacement (27). Nevertheless, these mild changes in morphology were accompanied by a clear increase in desmin staining, suggesting that hypercholesterolemia as such already leads to podocyte stress. This podocyte stress may have been associated with NO deficiency because decreased renal cortical NOS activity as a result of hypercholesterolemia or NOS inhibition
that, nevertheless, losartan was an effective protective agent (18). In double-knockout eNOS- and apolipoprotein E–deficient mice, dietary hypercholesterolemia induced atherosclerosis and kidney dysfunction that were abrogated by an ACE inhibitor (40). Thus, the hypercholesterolemia-induced podocyte stress can be due to direct effects of angiotensin II, unopposed by endogenous NO. However, podocyte stress is often associated with glomerular hypertension (41), and we cannot exclude that the hypercholesterolemia induced glomerular hypertension (42). In addition, there was a slight decrease in SBP induced by losartan in the hypercholesterolemic rats (117 versus 128 mmHg), and in the L-NNA–treated rats, the antihypertensive effect of losartan was stronger (110 versus 138 mmHg). Thus, in the cholesterol plus losartan and L-NNA plus losartan groups, reduction of podocyte stress by a hemodynamic component is possible. However, it should be noted that molsidomine prevented podocyte stress without affecting BP.

Hypercholesterolemia decreased renal cortical NOS activity and increased cortical and glomerular caveolin protein, and this

Figure 3. (A) Renal cortex nitric oxide synthase (NOS) activity in controls, rats that were fed 2% cholesterol for 2 wk, rats that were fed 2% cholesterol for 2 wk and were pretreated for 2 wk with molsidomine or losartan, and rats that received 40 mg of L-NNA/kg diet for 2 wk without or with pretreatment with losartan for 2 wk. *P < 0.05 versus control; †P < 0.05 versus 2% cholesterol. (B) Representative Western blot and data depicting NOS isoform protein abundance in renal cortex of control rats and rats that were fed 2% cholesterol for 2 wk.
was prevented by losartan. The upregulation of renal cortical caveolin-1 may account for decreased renal cortical NOS activity, analogous to what we have observed in endothelial cells (20). However, it should be noted that we did not measure cortical NOS activity in a single compartment. The caveolin–NOS interaction maybe angiotensin II sensitive, because losartan treatment prevented the increase in caveolin-1 protein abundance and decrease in renal cortex NOS activity induced by hypercholesterolemia. Caveolin-1 binds to eNOS, thereby inhibiting its activity and decreasing NO availability. Thus, if caveolin-1 is upregulated, then more calmodulin is required to destabilize the inhibitory caveolin/eNOS complex. Lack of caveolin-1 expression, both in kidney cells and in mice, prevents trafficking of the AT-1 receptor from the endoplasmic reticulum to the cell surface (43). The present study has, to our knowledge, for the first time demonstrated hypercholesterolemia-induced increase of renal cortical and, more specific, glomerular, caveolin protein abundance and decrease of renal cortical NOS activity and that these changes may be angiotensin II sensitive. Losartan treatment alone had no effect on renal cortical and glomerular caveolin, suggesting a low baseline activity of angiotensin II in the kidney. In vascular smooth cells, angiotensin II was found to increase caveolin-1 biosynthesis (21), and in cultured endothelial cells, angiotensin II was found to decrease NOS activity (22). After 2 wk of hypercholesterolemia, the protein abundance of all NOS isoforms in the renal cortex was unchanged (this study), and previously we also found that morphometric quantification of glomerular eNOS was unaltered (3). Thus, it is possible that hypercholesterolemia decreased renal cortical NOS activity by increasing caveolin-1 protein abundance via an AT-1–sensitive mechanism. However, although podocyte stress was also prevented by losartan in L-NNA–treated rats, it is unlikely that losartan could restore L-NNA–induced NO deficiency. Hence, even though in hypercholesterolemia losartan improved renal cortical NOS activity and decreased caveolin-1 protein mass and molsidine and losartan were equally capable of preventing podocyte stress in cholesterol-fed rats, increasing NO availability is probably not the only protective action of AT-1 blockade.

An alternative candidate pathway involved in podocyte stress that might be targeted by AT-1 blockade is oxidative stress. In a previous study, we found that high-dose cholesterol feeding for a longer period increased renal superoxide activity in the presence of proteinuria (3). Similarly, in the porcine renal vasculature, chronic hypercholesterolemia increased oxidative stress (2). The same authors recently showed that chronic antioxidant supplementation preserved renal vascular function in hypercholesterolemia (44). However, in our model of short-term hypercholesterolemia, proteinuria and renal su-
peroxide activity were not increased (3), which makes it unlikely that these targets were involved in the protective effect of losartan observed at this early stage.

In summary, the present study demonstrated that hypercholesterolemia increased podocyte stress, which was associated with NO deficiency and angiotensin II sensitivity. All of these events were early and occurred without proteinuria. We suggest that the decreased renal NO availability induced by hypercholesterolemia may leave the hemodynamic or direct effects of angiotensin II on podocytes unopposed. Furthermore, hypercholesterolemia decreased renal cortical NOS activity and increased renal cortical and glomerular caveolin protein abundance. Caveolin-1 protein abundance seemed to be increased via an angiotensin II–sensitive mechanism. It is interesting that in the present study, angiotensin II receptor blockade was applied before the onset of proteinuria. Proteinuria has been established as a risk factor for renal injury, and clinical trials have used AT-1 receptor blockade (45) and ACE inhibition (358:113) to reduce proteinuria and stabilize the rate of progression of renal injury. The clinical relevance of this study may be that in patients without proteinuria but with other risk factors for renal injury, early treatment with an AT-1 blocker or an ACE inhibitor might serve to prevent podocyte stress and the onset of proteinuria.

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