Blocking Angiotensin II Synthesis/Activity Preserves Glomerular Nephrin in Rats with Severe Nephrosis

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Abstract. Angiotensin-converting enzyme inhibitors restore size-selective dysfunction of the glomerular barrier in experimental animals and humans with proteinuric nephropathies, although the structural and molecular determinants of such an effect are not completely understood. This study used an accelerated model of experimental nephrosis to assess nephrin gene and protein expression in the kidney and the possible modulating effect of drugs that block angiotensin II (AII) synthesis/activity. Passive Heymann nephritis (PHN) and control animals were studied at day 7, month 4, and month 8. Additional PHN rats were treated with lisinopril or AII receptor blocker L-158,809 and studied at 8 mo. Lisinopril and L-158,809 controlled BP, prevented proteinuria, and protected PHN animals from renal injury. An intense signal of nephrin mRNA was detected in glomeruli of control animals mainly restricted to podocytes. In PHN rats, nephrin staining progressively and remarkably decreased with time. Lisinopril and L-158,809 fully prevented the decrease in nephrin transcripts to levels comparable to those of control rats. Consistent with nephrin mRNA expression, immunostaining of the protein showed a progressive decrease in kidneys from PHN rats that was completely abolished by lisinopril and L-158,809. In summary, progressive renal injury was associated with downregulation of nephrin gene that was totally prevented by angiotensin-converting enzyme inhibitor and AII receptor blocker, suggesting that renoprotection afforded by drugs that interfere with AII synthesis/activity was related to an effect on nephrin assembly.

Terminal renal failure is the final common fate of chronic nephropathies, regardless of the type of the original insult. After the initial injury affects a critical number of nephrons, adaptive hemodynamic changes in the remaining ones—including hyperperfusion and hyperfiltration—ensure enough filtration power to the kidney but ultimately are detrimental. Such changes are mediated largely by local formation of angiotensin II (AII) and are prevented by the use of angiotensin-converting enzyme (ACE) inhibitors, which also limit forced opening of large unselective pores in the glomerular barrier restoring size selectivity (1). Such an effect can be relevant to the renoprotective effect of this class of drugs because now available is compelling evidence that exuberant protein traffic through the capillary has a pathogenetic role in disease progression (2). Structural and molecular determinants of the effect of AII on glomerular size-selective dysfunction and on the property of ACE inhibitors to restore it have not been clarified so far. In a previous study designed to investigate structural and functional changes of the glomerular filtration barrier in passive Heymann nephritis (PHN) as well as the mechanisms by which ACE inhibition results in structural and functional protection of the kidney, we found a decrease in the frequency of epithelial filtration slits (3). Theoretical analysis of glomerular hydraulic permeability predicted a reduced permeability of both the glomerular basement membrane (GBM) and the epithelial layer, responsible for a reduced filtration capacity (3). The effect of the ACE inhibitors to preserve water and macromolecule permeability was due mainly to their property of preserving epithelial filtration slit frequency and diaphragm function, the essential component of the filtration barrier (4). Little was known on the nature and molecular properties of slit diaphragm until the recent discovery of nephrin, a putative transmembrane protein of the Ig superfamily, that acts as an adhesion receptor and signaling protein. Here we used a model of experimental nephrosis reminiscent of human membranous nephropathy to evaluate (*1*) whether massive proteinuria in this model was associated with changes of the slit diaphragm protein nephrin and (*2*) whether the effect of ACE inhibitor on reducing urinary protein and restoring membrane size selectivity was related to an effect of the drug on nephrin assembly.

Materials and Methods
Experimental Design
Male Sprague-Dawley, CD-COBS rats (Charles River Italia s.p.a., Calco, Italy) with initial body weights of 250 to 300 g were used in

this study. Animal care and treatment were conducted in accordance with the institutional guidelines that are in compliance with national (D.L. n.116, G.U., suppl. 40, 18 Feb. 1992, circular No. 8, G.U., 14 July 1994) and international laws and policies (EEC Council Directive 86/609, OJL358-1, December 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All animals were housed in a room in which the temperature was kept constant on a 12-h dark/light cycle and allowed free access to standard diet containing 20% protein by weight and tap water. PHN was induced in nonanesthetized rats by a single intravenous injection of 0.4 ml/100 g body wt of rabbit anti-Fx1A antibody. The model is characterized by extensive deposits of electron-dense material stuffing the GBM space (5). Unilateral nephrectomy at day 7, when animals are proteinuric, was performed to accelerate the onset of renal damage without exacerbating hypertension (6). PHN rats were divided into five groups and killed after disease induction as follows: group 1 ($n = 5$) at 7 d, group 2 ($n = 5$) at 4 mo, group 3 ($n = 5$) at 8 mo, group 4 ($n = 5$) at 8 mo after chronic daily treatment with the ACE inhibitor lisinopril (40 mg/L; Merck Research Laboratories, West Point, PA) in the drinking water from day 7, group 5 ($n = 5$) at 8 mo after chronic daily administration of an AII receptor antagonist L-158,809 ((3,6)-dimethyl-2-ethyl-3-[(2'-(^{1}H-tetrazol-5yl) (1,1')-byphenil-4-yl]-methyl-^{3}H-imidazol[4,5-b] pyridine) (50 mg/L; Merck Research Laboratories) in the drinking water from day 7. Additional groups of normal rats (group 6, 7, 8, and 9, $n = 5$) killed at day 7 and 2, 4, and 8 mo were considered as controls.

At the end of the study, systolic BP, urinary protein excretion, and serum creatinine were measured. Rats were then anesthetized and kidneys were removed for histology, *in situ* hybridization, and immunohistochemistry for nephrin. In PHN animals at day 7 and month 8, immunohistochemistry was performed for the tight junction protein ZO-1, another component of the slit diaphragm, which seems to play a functional role in maintaining functional properties of the epithelial slit diaphragms (7).

Systolic BP was recorded by tail plethysmography in conscious rats (8). Twenty-four-h samples were collected using metabolic cages, and proteinuria was determined by modified Coomassie blue G dye-binding assay for proteins with bovine serum albumin as standard (9). Blood was collected from the tail vein of anesthetized animals. Serum was obtained after whole-blood clotting and kept frozen at $-20°C$ until assayed. Creatinine was measured by the alkaline picrate method (10).

Histology

The removed kidneys were fixed for 6 h in Dubosq-Brazil, dehydrated in alcohol, and embedded in paraffin. Kidney samples were sectioned at 3-μm intervals, and three sections per slide were mounted. Sections were stained with Masson's trichrome, hematoxylin and eosin, and periodic-acid Schiff reagent (PAS stain). Sections including superficial and juxtamedullary glomeruli were evaluated. Tubular (atrophy, casts, and dilation) and interstitial changes (fibrosis and inflammation) were graded from 0 to 4+ (0, no changes; 1+, changes affecting less than 25% of the sample; 2+, changes affecting 25 to 50% of the sample; 3+, changes affecting 50 to 75% of the sample; 4+, changes affecting 75 to 100% of the sample). At least 100 glomeruli were examined for each animal, and the extent of glomerular damage was expressed as the percentage of glomeruli presenting focal or global sclerotic lesions. All renal biopsies were analyzed by the same pathologist, who was unaware of the nature of the experimental groups.

Cloning of Mouse cDNA

Total cellular RNA was purified from 6-wk-old C57Bl/6J mouse kidneys using TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's directions. One μg of RNA was reverse-transcribed with Superscript II reverse transcriptase (Life Technologies) and oligo-dT primer. The resulting cDNA was treated with RNase H and subjected to 35 cycles of PCR with the primers created based on exon sequence derived from genomic DNA sequence. Primers used were 5'-ATG AGA CAG TCA TGG ATG GC-3' and 5'-TCT CTC CAC CTC GTC ATA CAG-3', corresponding to nucleotides 1154 to 1173 and 3526 to 3546, respectively. PCR reaction was performed as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 3 min using Expand High Fidelity enzyme cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Two μl of the reaction product was used as an insert for TA ligation into pCRII (Invitrogen, Carlsbad, CA) vector. Positive clones were analyzed by restriction analysis and verified by sequencing the entire insert.

Preparation of Digoxigenin-Labeled Mouse Nephrin Riboprobe

Mouse nephrin antisense and sense riboprobes were prepared and labeled by *in vitro* transcription using digoxigenin-labeled uridine triphosphate (Boehringer Mannheim Biochemica, Mannheim, Germany) as described previously (11).

A 2400-bp fragment of nephrin cDNA (positions 1154 to 3546) was subcloned into the *Eco*RI site of pCRII (Invitrogen BV, Groningen, The Netherlands) between SP6 and T7 promoters. After *in vitro* transcription, riboprobes were cut to approximately 300-bp fragments by alkaline hydrolysis and then used as probes (12).

Nonisotopic In Situ Hybridization

In situ hybridization was performed as described previously (11). Briefly, sections of Dubosq-Brazil–fixed, paraffin-embedded renal tissue were heat-fixed (65°C, 30 min) and deparaffinized. After permeabilization with proteinase K (40 μg/ml; Sigma-Aldrich, Milan, Italy), sections were hybridized with the RNA probes at the final concentration of 0.1 to 0.5 ng/μl in 2 × SSC, 10% dextran sulfate, 1 × Denhardt's solution, 20 mM Vanadyl Ribonucleoside Complex (Life Technologies), and 0.1 M sodium phosphate and incubated overnight in a moist chamber at 45°C. After being washed in 0.2 × SSC and blocked with a buffer-blocking solution (50 mg/ml skimmed dried milk, 150 mM NaCl in 100 mM Tris HCl [pH 7.8]) at room temperature for 30 min, the sections were incubated with antidigoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim Biochemica) at the dilution of 1:1000 for 45 min at 37°C. Colorimetric detection with nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim Biochemica) was then performed, and the sections were mounted in 60% glycerol and examined by light microscopy. The negative control included a hybridization step with the sense probe. Thirty glomeruli for each rat were observed on average by a scientist who was unaware of the nature of the experimental groups. The positivity of nephrin mRNA was scored as follows: 0, no staining; 1, weak staining; 2, staining of moderate intensity; 3, strong staining.

Preparation of Anti-Human Nephrin Antibody

The antigen used for generation of the rabbit anti-human nephrin polyclonal antibody was purified in the following manner. PCR primers were designed for amplification of human nephrin nucleotides

2000 to 2769, which correspond to amino acids 668 to 923 of the extracellular domain, and subsequent cloning into the bacterial expression vector pET-22b (Novagen, Madison, WI). The resulting clone carries an in-frame, carboxy-terminal His (6) tag for purification. After purification on Ni-NTA agarose resin (Qiagen, Studio City, CA), the protein was dialyzed to phosphate-buffered saline (PBS), quantified, and used to immunize New Zealand white rabbits (Taconic, Germantown, NY). Rabbits received a subcutaneous injection of 250 μg of purified antigen and were boosted at 3-wk intervals. Three wk after the third immunization, the animals were killed, blood was harvested, and serum was then separated from the plasma for use in immunohistochemical assays. Specificity of the antiserum was checked in human nephrin transfected or untransfected HEK 293 cells. In Western blot analysis, the antibody was detected to bind a 185- to 200-kD band obtained from cell membrane preparation from transfected but not untransfected cells.

Immunohistochemical Analysis

Localization of nephrin by immunohistochemistry was performed using a rabbit anti-human nephrin antiserum raised against the extracellular domain of human nephrin (amino acid residues 668 to 923, Ig repeat domains 7 and 8). For ZO-1 immunoperoxidase, an affinity-purified rabbit anti-human ZO-1 antiserum (Zymed Laboratories Inc., San Francisco, CA) was used. Dubosq-Brazil–fixed and paraffin-embedded sections were deparaffinized, rehydrated, and incubated for 30 min with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. Tissue was permeabilized in 0.1% Triton X-100 in PBS 0.01 M (pH 7.2) for 30 min, and aspecificities were blocked by 30-min incubation with normal goat serum (Vector Laboratories, Burlingame, CA). All of the above steps were carried out at room temperature. Slides were then incubated overnight at 4°C in a moist chamber with the primary antibody (anti-nephrin antiserum preabsorbed overnight with normal rat serum and diluted 1:200 in PBS azide) followed by the secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories), avidin-biotin-peroxidase complex (ABC) solution, and finally developed with diaminobenzidine. The sections were then counterstained with Harris hematoxylin (Biooptica, Milan, Italy). Negative controls were obtained by omitting the primary antibody on a second section present on all of the slides. An average of 30 glomeruli for each rat were observed by a scientist who was unacquainted to the nature of the experimental groups. Glomerular posi-

tivity for nephrin was graded on a scale of 0 to 3 (0, no staining; 1, weak staining; 2, staining of moderate intensity; 3, strong staining).

Statistical Analyses

Data are expressed as mean $\pm$ SD. Data were analyzed using the nonparametric Kruskal-Wallis test for multiple comparisons. The statistical significance level was defined as $P < 0.05$.

Results

Systemic and Renal Parameters

Animals from the study groups behaved well and survived along the study. As shown in Table 1, untreated PHN animals developed hypertension only at 8 mo. Both the ACE inhibitor and the AII receptor blocker controlled systolic BP. Untreated PHN rats had high levels of urinary protein excretion at month 4 that augmented further at month 8. Blocking AII synthesis with lisinopril as well as the AII biologic activity with L-158,809 prevented the increase in urinary protein excretion (Table 1). Renal function in untreated PHN was already impaired at 4 mo and worsened at 8 mo. Lisinopril and L-158,809 maintained serum creatinine levels to values comparable to those of control animals (Table 1). Either the percentage of sclerotic glomeruli or the score of tubulointerstitial damage was increased to a significant extent in untreated PHN over controls and progressively increased with time from 4 to 8 mo. Both lisinopril and L-158,809 strikingly protected animals from the development of glomerular and tubulointerstitial damage (Table 1).

In Situ Hybridization for Nephrin mRNA

The expression of nephrin mRNA was evaluated in rat kidney samples by *in situ* hybridization using alkaline-digested riboprobes and reported in Figure 1. An intense expression signal of nephrin mRNA was detected in glomeruli of control animals mainly restricted to podocytes. No specific signals were obtained with the sense control probe (Figure 1). A mild staining was observed occasionally in a few tubuli. This is not surprising in view of the fact that similar findings were also

Table 1. Systemic and renal functional parameters in control, treated, and untreated PHN rats[a]

Parameters	Control				PHN			PHN+ Lisinopril[c]	PHN+ L-158,809[c]
	7 D	2 Mo	4 Mo	8 Mo	7 D	4 Mo	8 Mo[b]		
Systolic BP (mmHg)	114 ± 10	120 ± 12	121 ± 1	134 ± 9	118 ± 10	143 ± 28	156 ± 14	103 ± 3	113 ± 11
Proteinuria (mg/d)	17 ± 3	16 ± 2	26 ± 6	36 ± 6	50 ± 21[b]	510 ± 109[b]	768 ± 147	91 ± 30	166 ± 11
Serum creatinine (mg/dl)	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	1.0 ± 0.2[b]	1.6 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
Glomeruli with sclerosis (%)	0	0	0	0.7 ± 0.8	0.4 ± 0.2	39 ± 20[b]	76 ± 7	6.0 ± 0.1	10 ± 1
Tubulointerstitial damage (score)	0	0	0	0.3 ± 0.5	0	1.8 ± 0.5[b]	3 ± 0.1	0.3 ± 0.3	0.8 ± 0.3

[a] Data are expressed as mean $\pm$ SD. PHN, passive Heymann nephritis.
[b] $P < 0.05$ *versus* control at respective time.
[c] $P < 0.05$ *versus* PHN 8 mo.
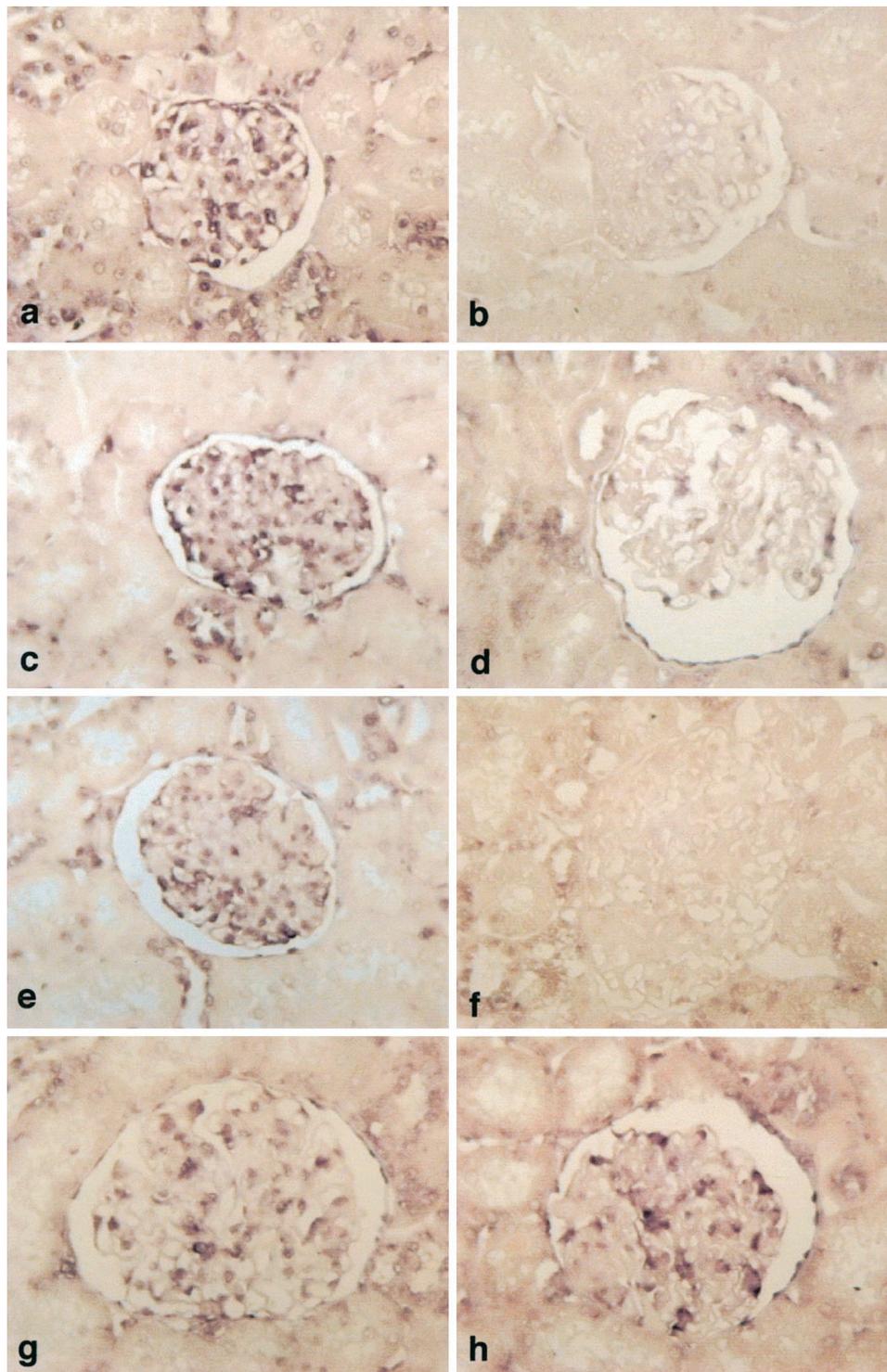
Figure 1. Representative picture of *in situ* hybridization for nephrin mRNA using alkaline-digested riboprobes. Hybridization signals (brown staining) are present in podocytes and in a few glomerular parietal epithelial cells. Staining for nephrin mRNA in control rats was comparable at 2 (a) and 4 (c) months of age and decreased at 8 mo (e). Expression of nephrin mRNA in passive Heymann nephritis (PHN) rats was remarkably decreased at 4 mo (d) and almost completely disappeared at 8 mo (f). Both lisinopril (g) and the angiotensin II (AII) receptor blocker L-158,809 (h) fairly normalized nephrin mRNA in PHN rats. No specific signals were obtained with the sense nephrin probe (b). Magnification, ×300.

reported in figures of previously published papers (13,14). In control rats, nephrin staining in glomeruli was comparable at day 7 and at 2 and 4 mo of age and decreased at 8 mo. Animals with PHN showed a reduction of glomerular mRNA for neph-rin already at day 7 (Figure 2), when glomerulosclerosis and tubulointerstitial damage were not yet present (Table 1), which progressively and remarkably decreased with time as shown by very consistent findings at month 8 (Figure 1). Lisinopril

markedly prevented the decrease in nephrin mRNA transcript in podocytes (Figure 1). To assess whether the favorable effect of ACE inhibition was actually due to a reduced activity of AII, we evaluated the action of an AII receptor blocker, L-158,809, on renal nephrin gene expression. L-158,809 was as effective as the ACE inhibitor in preventing the decrease of nephrin mRNA transcript; almost all glomeruli showed a positive staining for nephrin (Figure 1).

The intensity of the staining for nephrin mRNA was calculated by a semiquantitative score and reported in Figure 2. Intensity of the staining was comparable in control rats studied at day 7 and at 2 and 4 mo and was reduced by 33% in control rats at month 8 (Figure 2). In PHN rats, intensity of the nephrin transcript was already reduced by 17% at day 7 and further decreased by 67% at month 4 in respect to controls. At month 8, nephrin mRNA in PHN animals was virtually absent with only very few glomeruli revealing a very faint nephrin staining (Figure 2). Treatment of PHN rats with the ACE inhibitor or the AII receptor blocker induced a normalization of the staining for nephrin that reached levels comparable to that of control rats (Figure 2).

Localization of Nephrin in the Kidney from PHN Rats

Consistent with patterns of nephrin mRNA expression, immunohistochemistry of normal rat kidneys with the antibody against the extracellular nephrin portion showed a prominent glomerular localization. A linear dotted line of reactivity giving a preferentially epithelial-like staining pattern could be observed. Immunohistochemical staining of nephrin was comparable in control rats at day 7 and at 2 and 4 mo and tended to decrease at 8 mo. PHN rats studied at day 7 had a decrease

in nephrin staining that progressively lowered with time at 4 and 8 mo (Figure 3). A semiquantitative score of glomerular positivity for the light microscopy immunostaining was calculated for each animal and reported in Figure 4. At day 7 and at 2 and 4 mo, almost all glomeruli were positive for nephrin, whereas a reduction of the positivity score was observed at 8 mo (Figure 4). Untreated PHN animals exhibited a significant ($P < 0.05$) reduction in the semiquantitative score over controls already at day 7, which further exacerbated at 4 mo. Nephrin reactivity dramatically decreased in podocytes of PHN animals at 8 mo to levels that were significantly lower than those of control animals at the corresponding time ($P < 0.05$). Conversely, the administration of the ACE inhibitor markedly prevented the decrease of nephrin expression in PHN animals. The score of positivity in PHN animals that received the ACE inhibitor was remarkably higher than that of control animals of the corresponding age (month 8) and fairly comparable to the score of control animals studied at day 7 and at 2 and 4 mo (Figure 4). Consistent with the data obtained in these latter groups, PHN animals that were given the ACE inhibitor had all glomeruli positive for nephrin protein expression. The same trend was observed with the AII receptor blocker with a positivity score very comparable to that of control animals and almost all glomeruli positively stained (Figure 4), strongly indicating a role for AII in inducing changes of nephrin protein expression in the glomerulus in PHN animals.

To evaluate whether reduction of nephrin expression was specific, we performed the immunostaining of another component of the slit diaphragm, ZO-1, at an early and late stage of the disease of PHN. At day 7, expression of ZO-1 was fairly comparable to values observed in controls (score, 2.9 ± 0.1 *versus* 2.9 ± 0.1). At 8 mo, control animals had a slightly lower score of positivity that was on average 2.1 ± 0.6. PHN rats studied at the same time, when renal damage was very severe, revealed a lower staining for ZO-1 accounting 1.8 ± 0.2, a value numerically but not statistically different from that of controls. The appreciable difference between nephrin and ZO-1 reduction in PHN rats at month 8 (79 *versus* 15%) suggests that changes in nephrin mRNA and protein were specific and not part of a generalized phenomenon.

Discussion

The glomerular filter normally limits transcapillary traffic of albumin and other proteins in such a manner that only macromolecules of low molecular weight actually reach the urinary space and are eventually reabsorbed by proximal tubule cells (1). In glomerular disease, complete or partial loss of selective properties of the filtering structure leads to excessive filtration of albumin, and other proteins that accumulate in the lumen of proximal and distal tubuli are partly reabsorbed by and may be excreted in the urine in amounts that depend on the severity of the underlying glomerular disease (15). Crucial to the process of glomerular filtration is the integrity of the podocyte intercellular junction, slit diaphragm and its components, specifically its membrane proteins including the recently discovered nephrin, which constitutes the molecular structure of the multipore filter. Mutations of nephrin gene such as in the case of
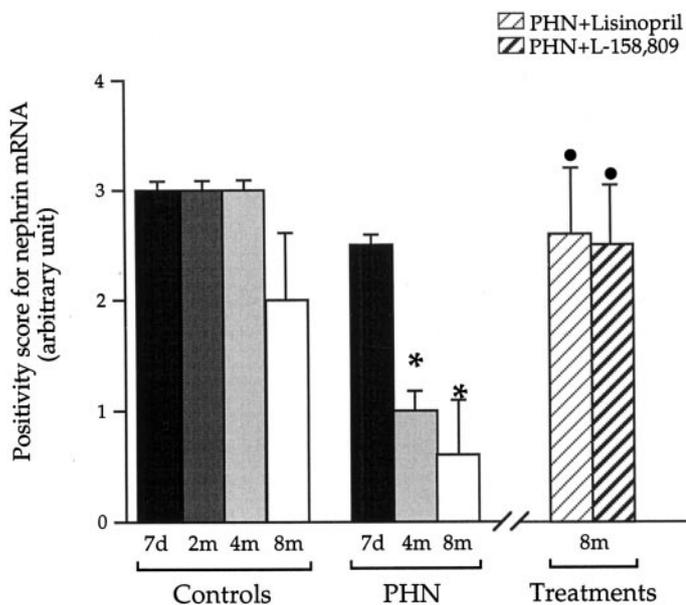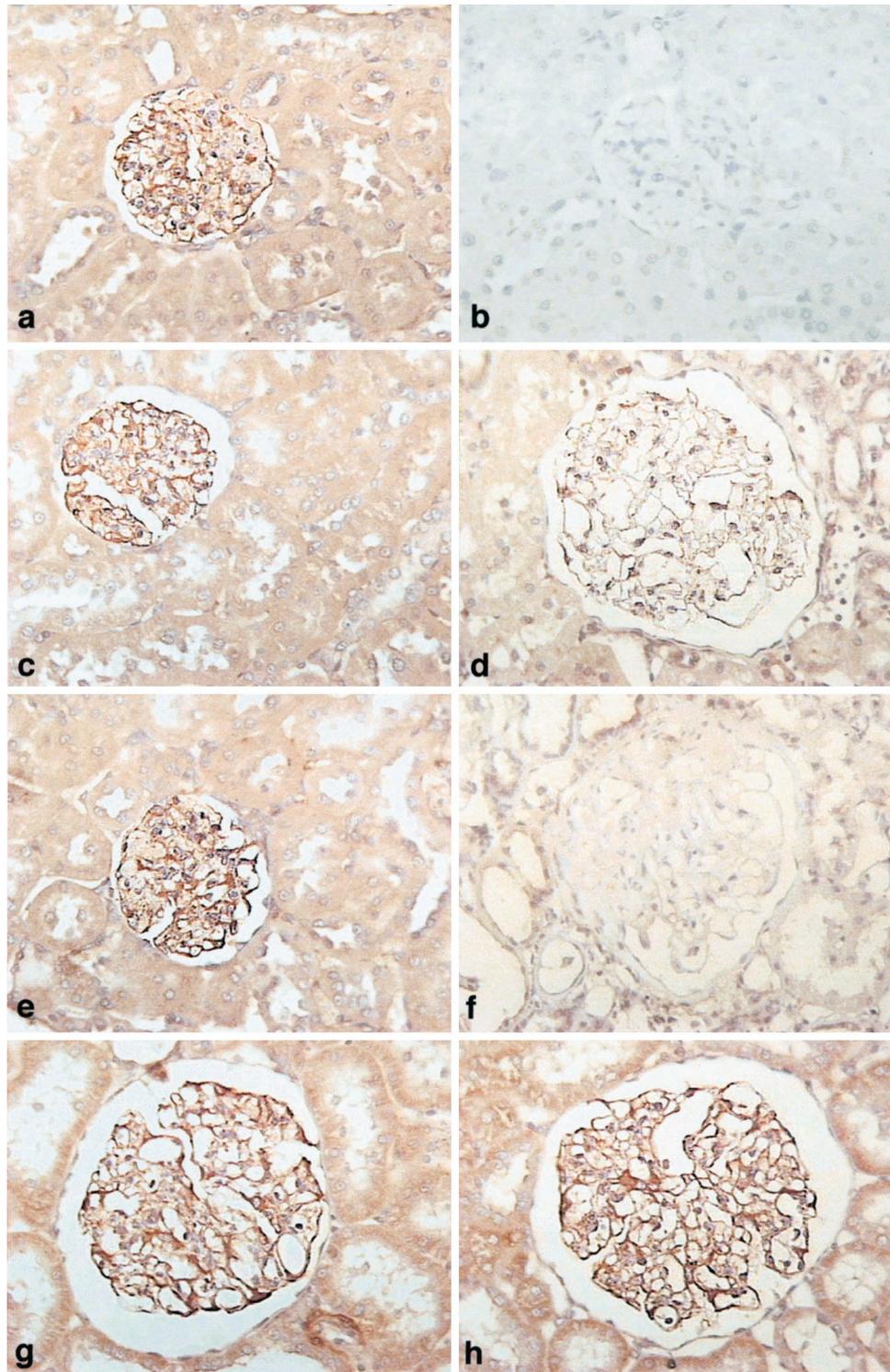

Figure 2. Intensity score (arbitrary units) of nephrin mRNA staining in control and PHN rats studied at different time points. The effect of lisinopril and L-158,809 chronically given to PHN rats on nephrin gene expression is also shown. *, $P < 0.05$ *versus* controls at respective time; ●, $P < 0.05$ *versus* PHN 8 mo.

Figure 3. Representative photomicrographs of nephrin localization by the immunoperoxidase method. Nephrin staining (brown signal) was localized in epithelial glomerular cells with a linear-like pattern. Nephrin staining was similar in control rats that were killed at 2 (a) and 4 (c) mo and slightly decreased at 8 mo (e). In PHN rats, staining was lower in respect to control rats that were killed at 4 mo (d) and completely disappeared at 8 mo (f). Lisinopril (g) and L-158, 809 (h) fairly normalized nephrin staining in PHN rats. No specific signal was observed omitting the primary antibody (b). Magnification, ×300

congenital nephrotic syndrome of Finnish type result in the cellular alterations that lead to foot process effacement and nephrotic-range proteinuria (16).

Here we report that in an accelerated model of experimental nephrosis reminiscent of human membranous nephropathy, the glomerular expression of nephrin gene decreased over time in
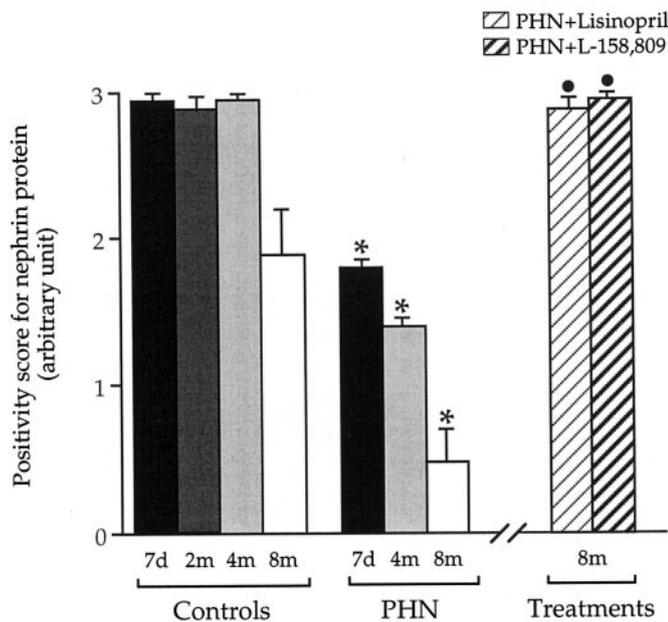
Figure 4. Intensity score (arbitrary units) of nephrin staining by immunohistochemistry in control and PHN rats evaluated at different time intervals. The effect of lisinopril and L-158,809 is also shown. *, $P < 0.05$ *versus* controls at respective time; ●, $P < 0.05$ *versus* PHN 8 mo.

parallel to the development of proteinuria and the subsequent renal damage. Concomitant with the time-dependent reduction in gene expression, nephrin formation was also impaired. This phenomenon was reasonably specific for nephrin because the expression of another protein of the slit diaphragm ZO-1 was not affected substantially. Downregulation of glomerular nephrin production as early as 7 d after the induction of the disease indicates a pathogenetic role in permeability changes. A time-dependent reduction of nephrin mRNA together with a decreased staining of the cortical kidney with an antibody to the intracellular nephrin domain was observed previously in glomeruli from rats with puromycin amino nucleoside nephrosis (17). Altered expression of nephrin mRNA was reported also in nephrotic syndrome patients with idiopathic membranous glomerulonephritis or minimal change nephropathy whose glomerular nephrin mRNA was reduced in comparison with the transcript of normal, nonproteinuric glomeruli (18).

PHN was commonly used in the past to understand the mechanisms underlying the disease pathology of the human counterpart membranous nephropathy. Changes in the ultrastructure of the glomerular capillary wall components including a decreased frequency of the filtration slits and an increased GBM thickness has been described in PHN rats (3). Theoretical analysis of morphometric parameters allowed an appreciation of a reduction of hydraulic permeability to water and albumin of the GBM as well as the epithelial layer, which have been considered as possibly being responsible for the filtration power in this model (3). Treatment with lisinopril was associated with a preserved frequency of epithelial slits in the face of abnormal GBM thickness. Lisinopril also prevented, at least in

part, GFR decline and ameliorated the hydraulic permeability of the GBM (3). The above study already suggested that the clue for the effect of ACE inhibitor of preserving membrane filtration barrier must reside on slit diaphragm structure or function. Our previous studies also documented significant changes in the distribution of the tight junction protein occludens-1 (ZO-1)—which plays a crucial role in maintaining the functional properties of the epithelial slit diaphragm—in rats genetically programmed to develop spontaneous proteinuria with age (19). This alteration was not accompanied by any apparent ultrastructural change of the foot processes and the epithelial slit diaphragms and was prevented by ACE inhibition that also effectively reduced protein excretion rate in these animals (19). Interestingly, in this model, amelioration of glomerular ultrafiltration coefficient induced by the ACE inhibitor was not a consequence of changes in filtering surface area but rather reflects an increase in membrane hydraulic permeability properties (19). In the present study, we also found that blocking the biologic effects of AII either with an ACE inhibitor or with an AII receptor antagonist fully prevented nephrin rearrangement observed in untreated animals with a severe form of PHN. The resulting well-known effect of these drugs of limiting urinary proteins in this and other animal models of progressive nephropathy therefore may depend on the capacity of AII blockers to preserve slit diaphragm functional integrity of which nephrin seems to represent a crucial aspect. Indirectly, these data imply a role for AII in modifying the size-selective properties of the glomerular capillary barrier. This is supported further by direct evidence from our laboratory and others' that continuous infusion of AII into the renal artery induces a progressive and significant increase in urinary protein excretion rate accompanied by enhanced fractional clearance of tracer molecules corresponding to the size of albumin as a function of intracellular signaling through the angiotensin II type 1 receptor (5,20).

Potential AII-independent mechanisms of preservation of nephrin expression by AII blockade also could be considered. Those include modification in the development of immune deposits and changes in epithelial cell morphology. Concerning the former, a significant reduction in the number and the extension of electron-dense deposits within the GBM was observed previously in PHN rats given lisinopril in respect to untreated animals (3). As far as podocyte morphology, some very recent data obtained in the same model by Yuan *et al.* (21) found changes in nephrin distribution before the appearance of proteinuria with subsequent disruption of nephrin staining in the more proteinuric phase. Changes in nephrin corresponded to the deposition of C5b-9, suggesting the possibility that complement-mediated podocyte injury alters the slit diaphragm as to eventually account for proteinuria.

The effect of ACE inhibitors and AII receptor antagonists on nephrin biosynthesis is an unprecedented finding. Currently, the mechanism responsible for downregulation of nephrin mRNA and protein in conditions of excessive renal AII is unknown. Whatever the mechanism by which AII blockade maintains nephrin expression, either by directly reducing AII or by preserving podocyte and/or foot process architecture, the

present study discloses a novel pathway linking ACE inhibitors and AII receptor antagonist to renoprotection. The time-dependent downregulation of the nephrin seems to be the molecular equivalent of the recruitment of large unselective pores responsible for proteinuria in this and other model of progressive nephropathies. Recent findings that the slit area can be modified by enhancing intraglomerular pressure offer a molecular explanation for the old observation that glomerular hemodynamic changes translate into opening of large glomerular pores and protein trafficking and for the protective effect of limiting AII on both parameters.

Acknowledgments

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