Nephrin Expression Is Reduced in Human Diabetic Nephropathy

Evidence for a Distinct Role for Glycated Albumin and Angiotensin II

Sophie Doublier,[1] Gennaro Salvidio,[2] Enrico Lupia,[1] Vesa Ruotsalainen,[3] Daniela Verzola,[2] Giacomo Deferrari,[2] and Giovanni Camussi[1]

We studied the distribution of nephrin in renal biopsies from 17 patients with diabetes and nephrotic syndrome (7 type 1 and 10 type 2 diabetes), 6 patients with diabetes and microalbuminuria (1 type 1 and 5 type 2 diabetes), and 10 normal subjects. Nephrin expression was semiquantitatively evaluated by measuring immunofluorescence intensity by digital image analysis. We found an extensive reduction of nephrin staining in both type 1 (67 ± 9%; $P < 0.001$) and type 2 (65 ± 10%; $P < 0.001$) diabetic patients with diabetes and nephrotic syndrome when compared with control subjects. The pattern of staining shifted from punctate/linear distribution to granular. In patients with microalbuminuria, the staining pattern of nephrin also showed granular distribution and reduction intensity of 69% in the patient with type 1 diabetes and of 62 ± 4% ($P < 0.001$) in the patients with type 2 diabetes. In vitro studies on human cultured podocytes demonstrated that glycated albumin and angiotensin II reduced nephrin expression. Glycated albumin inhibited nephrin synthesis through the engagement of receptor for advanced glycation end products, whereas angiotensin II acted on cytoskeleton redistribution, inducing the shedding of nephrin. This study indicates that the alteration in nephrin expression is an early event in proteinuric patients with diabetes and suggests that glycated albumin and angiotensin II contribute to nephrin downregulation. ***Diabetes*** **52:1023–1030, 2003**

From the [1]Department of Internal Medicine, University of Turin, Center for Experimental Research and Medical Sciences (CERMS), Turin, Italy; the [2]Department of Internal Medicine, University of Genoa, Genoa, Italy; and the [3]Department of Biochemistry, University of Oulu, Oulu, Finland.

Address correspondence and reprint requests to Dr. G. Camussi, Cattedra di Nefrologia, Dipartimento di Medicina Interna, Ospedale Maggiore S. Giovanni Battista, Corso Dogliotti 14, 10126, Torino, Italy. E-mail: giovanni.camussi@unito.it.

Received for publication 16 September 2002 and accepted in revised form 2 January 2003.

Ang II, angiotensin II; DN, diabetic nephropathy; GA, glycated albumin; GAPDH, glyceraldehyde phosphate dehydrogenase; GEC, glomerular epithelial cell; GN, glomerulonephritis; IF, immunofluorescence; mAb, monoclonal antibody; RAGE, receptor for advanced glycation end products; RAS, renin-angiotensin system.

Proteinuria is the clinical hallmark of diabetic nephropathy (DN) (1). Several studies have addressed the mechanisms involved in the loss of glomerular permselectivity in patients with diabetes (2,3). Many of them focused on the role of glomerular basement membrane components with particular regard to glomerular anionic sites (4,5). However, the central role of the podocyte slit diaphragm in maintaining the size-selective barrier has been outlined by recent studies on different slit diaphragm–associated proteins, such as nephrin, CD2-associated protein, podocin, and α-actinin-4 (6). Mutations in the nephrin gene have been found in patients with congenital nephrotic syndrome of the Finnish type, as well as in other patients with nephrotic syndrome (7,8). Moreover, a correlation between changes in nephrin expression and proteinuria has been shown in several experimental models of glomerulonephritis (GN) (9–11). We and others have recently shown a reduced expression of nephrin in patients with primary acquired nephrotic syndrome, independent from the initial pathogenic mechanism (12–14). More recently, several experimental reports showed the presence of altered nephrin expression in different models of DN, such as rats with streptozocin-induced diabetes and nonobese diabetic mice (15,16). It is at present unknown whether nephrin has a role in enhancing glomerular permeability in human DN. Therefore, the first aim of the present study was to evaluate the expression and the distribution of nephrin in renal biopsies from patients with diabetes and microalbuminuria or nephrotic syndrome. As both metabolic and hemodynamic factors have been implicated in the pathogenesis of DN, we also sought to study the effect of glycated albumin (GA) and angiotensin II (Ang II) on the expression of nephrin in human cultured podocytes.

RESEARCH DESIGN AND METHODS

Reagents. An antinephrin IgG_1 monoclonal antibody (mAb) specific for the extracellular fibronectin type III–like motif of the recombinant human nephrin produced in A293 cells and an IgG_1 mAb raised against the recombinant protein representing the complete intracellular nephrin domain produced in *Escherichia coli* were used (13). An irrelevant IgG_1 isotypic control antibody was purchased from Cedarlane (Hornby, ON, Canada). Human albumin (nonglycated and glycated), cytochalasin B, sodium azide, TRI reagent, and all of the secondary antibodies were obtained from Sigma Chemical (St. Louis,

TABLE 1
Clinical features of patients with diabetes

	Type 2 diabetes and nephrotic syndrome	Type 1 diabetes and nephrotic syndrome	Type 2 diabetes and microalbuminuria	Type 1 diabetes and microalbuminuria
n	10	7	5	1
Age (years)	60.1 ± 9.4	37.9 ± 3.9	57.6 ± 10.8	25
Sex (M/F)	7/3	4/3	3/2	0/1
Diabetes duration (years)	11.9 ± 6.9	19 ± 2.6	17 ± 11	18
HbA_{1c} (%)	6.9 ± 2.4	9.7 ± 2.6	8.3 ± 0.8	7.8
Creatinine (mg/dl)	1.7 ± 1.0	3.1 ± 1.4	1.1 ± 0.3	1.0
Creatinine clearance (ml/min)	59 ± 27	39 ± 16	102 ± 21	130
UA (mg/24 h)	ND	ND	74.2 ± 37.4	35
UP (g/24 h)	5.25 ± 3.9	7.2 ± 4.3	ND	ND
Retinopathy (%)	70	100	40	100
Hypertension (%)	100	100	100	0

Data are mean ± SD. ND, not done; UA, albuminuria; UP, proteinuria.

MO). A specific neutralizing polyclonal antibody anti-human receptor for advanced glycation end products (RAGE) was obtained from Chemicon (Temecula, CA) (17).

Patients. The study included renal biopsies of 23 patients who were affected by type 1 or type 2 diabetes and microalbuminuria or nephrotic syndrome. In detail, we considered 10 patients with type 2 diabetes and nephrotic syndrome, 5 patients with type 2 diabetes and microalbuminuria, 7 patients with type 1 diabetes and nephrotic syndrome, and 1 patient with type 1 diabetes and microalbuminuria.

As control, 10 specimens were obtained from normal kidney portions of patients who underwent surgery for cancer. These patients were selected for absence of proteinuria and lack of glomerular abnormalities detected by light and immunofluorescence microscopy.

The study was approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa. All subjects were informed about the nature, purposes, procedures, and possible risks of the renal biopsy before their informed consent was obtained. The procedures were in accordance with the Helsinki declaration.

Biopsies included in the study presented classic histological features of diabetic nephropathy that were moderate to severe in patients with nephrotic syndrome and mild in patients with microalbuminuria. Biopsies that showed other pattern of injury, such as vascular or interstitial lesions without glomerular diabetic damage, were not included in the study.

Nephrotic syndrome was defined as proteinuria ≥3.5 g/24 h and serum albumin ≤3 g/dl. Microalbuminuria was defined as an urinary albumin excretion of 30–300 mg/24 h.

Table 1 depicts some of the clinical features of the patients included in the study. Patients who received treatment with ACE inhibitors or Ang II receptor antagonists underwent a 3-week period of washout before protein or albumin urinary excretion was assessed. The protein content of 24-h urinary samples was measured by pyrogallol red method. For measuring urinary albumin excretion, patients collected 24-h urine samples in three different occasions at least 1 week apart. Urine albumin was measured by a commercially available radioimmunoassay kit (Sclavo, Cinisello Balsamo, Italy). The intra- and interassay variability of the method were 4.5% and 6.1%, respectively.

The creatinine concentration in plasma was analyzed by kinetic Jaffé method with a Beckman Synchron CX3. Creatinine clearance was estimated on the basis of the Cockcroft-Gault formula as validated in diabetic nephropathy (18). Glycated hemoglobin was measured by ion-exchange liquid chromatography. Hypertension was defined, according to the recommendations of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC VI), as an average blood pressure ≥140/90 mmHg on at least three different occasions. The presence or absence of diabetic retinopathy was investigated by direct funduscopic examination by the same trained ophthalmologist in a darkened room and under pupil dilation.

Culture of human glomerular epithelial cells. Primary cultures of glomerular epithelial cells (GECs) were established as previously described (19). Phenotypic characterization was performed according to cell morphology (polyhedral cells with cobblestone-like appearance); positive staining for synaptopodin, Wilm's tumor antigen, podocalyxin, zonula occludens-1, cytokeratin, vimentin, and laminin; negative staining for smooth muscle–type myosin, FVIIIr:Ag, and CD45; and cytotoxicity in response to puromycin aminonucleoside (10–50 μg/ml) (20–23). Established lines of differentiated GECs were obtained by infection of pure primary cultures with a hybrid Adeno5/SV40 virus as previously described, characterized as above (19,24) and cultured in DMEM containing 25 mmol/l glucose.

Experimental in vitro conditions. For immunofluorescence (IF) studies, GECs were plated in eight-well Permanox slide at a density of 30,000 cells per well. The following day, cell layers were rinsed with PBS.

Cells were incubated in the presence of GA or native albumin at 1,200 μg/ml for 1, 24, or 48 h before fixation and staining with antinephrin mAb. In selected experiments, a neutralizing antibody anti-RAGE (1:200) was added to the cells 30 min before GA. The concentrations of GA used in this study have been shown to stimulate glomerular endothelial and mesangial cell expression of collagen type IV, fibronectin, and transforming growth factor-β1 (25) and represent those found in clinical specimens. In nondiabetic individuals, ~1% of serum albumin is in the glycated form, which is equivalent to concentrations of 300–400 μg/ml GA. The concentration of GA is increased 1.5- to 3-fold in diabetic subjects, according to recent glycemic status (26,27).

In other experiments, cells were incubated in the presence of Ang II at 100 nmol/l for 30 min, 1 h, or 24 h before fixation and staining with antinephrin mAb. In some experiments, before incubation with Ang II, the GECs were preincubated for 30 min with drugs that interfere with cytoskeletal function, such as cytochalasin B (5, 10, and 50 μg/ml) or with cell metabolism such as sodium azide (10^{-3} to 10^{-1} mol/l).

For evaluating actin microfilament alterations in GA or Ang II–stimulated GECs, cells were incubated with GA (1,200 μg/ml) or Ang II (100 nmol/l) for 1 h and then fixed, permeabilized, and stained with FITC-phalloidin. For evaluating gene nephrin expression, cells were plated in 25 cm^2 dishes at a concentration of 300,000 cells per dish and grown for 2 days. They were then exposed to GA or native albumin (1,200 μg/ml) for 24 h.

IF studies. Kidney biopsies were rapidly frozen in liquid nitrogen, and 2-μm-thick cryostat sections were fixed in 3.5% paraformaldehyde for 15 min and washed in PBS. The sections were incubated with antinephrin mAb (10 μg/ml) for 2 h at room temperature, washed in PBS, and incubated with FITC-conjugated anti-mouse IgG. The number of glomeruli available on each biopsy for analysis of nephrin expression ranged between three and seven. Globally sclerosed glomeruli were not considered for IF analysis. Three nonsequential sections were examined for each specimen. The intensity of glomerular IF was detected in a 180-μm-diameter field (approximate size of glomeruli) using a 3.7-mm aperture in the light path. Nephrin expression was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis (Windows MicroImage, version 3.4 CASTI Imaging) on images obtained using a low-light video camera (Leica DC100) as previously described (13). The background fluorescence of tissue was subtracted by digital image analysis. The results were expressed as relative fluorescence intensity on a scale from 0 (fluorescence of background of tissue) to 255 (fluorescence of standard filter).

IF on cultured GECs was performed as previously described (28). Briefly, coverslip-attached GECs at subconfluent density were fixed in 3.5% paraformaldehyde containing 2% sucrose for 15 min at room temperature and washed in PBS. Cells were then incubated either with antinephrin mAb (10 μg/ml) followed by FITC-conjugated anti-mouse IgG or with anti-RAGE antibody (diluted 1:200) followed by FITC-conjugated anti-goat IgG. In experiments aimed to study cell cytoskeleton, cells were made permeable by incubation for 5 min at 0°C in HEPES-Triton X-100 buffer (20 mmol/l HEPES [pH 7.4], 300 mmol/l sucrose, 50 mmol/l NaCl, 3 mmol/l $MgCl_2$, and 0.5 Triton X-100) and stained for 30 min at 37°C with FITC-phalloidin (2 μg/ml). Control experiments included incubation of sections or cells with nonimmune isotypic

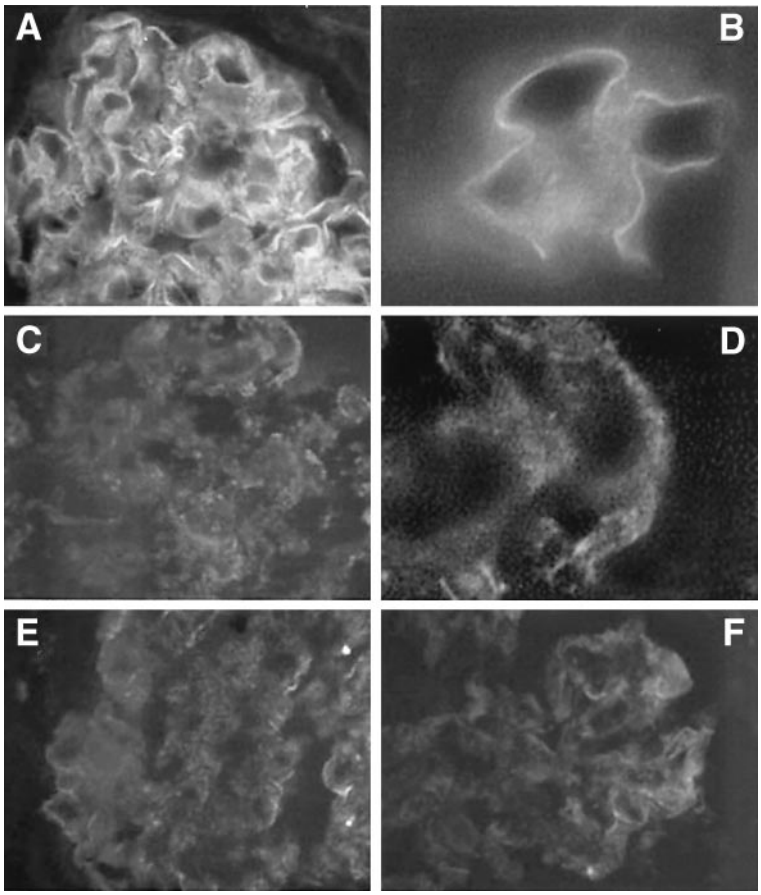

FIG. 1. IF staining for nephrin in glomeruli of control subjects (*A* and *B*), patients with type 1 diabetes and nephrotic syndrome (*C* and *D*), patients with type 2 diabetes and nephrotic syndrome (*E*), and patients with type 2 diabetes and microalbuminuria (*F*). Original magnification ×400 in *A*, *C*, *E*, and *F* and ×600 in *B* and *D*.

control antibodies or the omission of primary antibodies followed by the appropriate labeled secondary antibodies.

Nephrin expression on cultured GECs was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis on images obtained using a low-light video camera on 400× microscopic fields as described above. Seven microscopic fields were evaluated for each experimental point.

Membrane localization of nephrin was analyzed by confocal microscopy. Confocal microscopy was performed on a Leica TCS SP2 model confocal microscope (Heidelberg, Germany) using a 63× magnification lens and 488-nm argon laser.

Semiquantitative RT-PCR analysis of nephrin expression. RT-PCR was performed using total RNA from GECs. Total RNA was extracted using TRI reagent according to the manufacturer's directions. The final RNA pellet was dissolved in 10 μl of diethyl pyrocarbonate water and stored at −70°C; 1 μg of total RNA was reverse-transcribed using a First Strand Synthesis Kit (Boehringer Mannheim, Indianapolis, IN). Fixed amounts of cDNA (8 and 2 μl from 1:10 dilution for nephrin and glyceraldehyde phosphate dehydrogenase [GAPDH] genes, respectively) and optimal PCR cycles (34 and 27, respectively) were directly used for individual PCR amplification. Sequence-specific oligonucleotide primers (purchased from TIB Molbiol, Genova, Italy) were designed (h-nephrin: 3′ reverse ggC AAA TCT gAC AAC AAg ACg, 5′ forward gga cat AgT CTg CAC TgT CgA T, h-GAPDH: 3′ reverse TCT AgA Cgg CAg gTC Agg TCC ACC, 5′ forward CCA CCC ATg gCA AAT TCC ATg gCA). Times and temperatures for denaturation, annealing, and extension were 1 min at 95°C, 1 min at 60°C, and 3 min at 72°C, respectively. Amplification products for h-nephrin (240 bp) and for h-GAPDH (598 bp) were visualized by ethidium bromide staining after agarose gel electrophoresis. Results were quantified by densitometry using Kodak 1D 3.5 and expressed in arbitrary densitometric units. We chose GAPDH as a housekeeping gene and used its gene product to normalize RT-PCR data.

Detection of nephrin expression by Western blot analysis. Proteins of GEC lysates were extracted with cold detergent-insoluble matrix buffer (50 mmol/l Pipes [pH 6.8], 100 mmol/l NaCl, 5 mmol/l $MgCl_2$, 300 mmol/l sucrose, 5 mmol/l EGTA, 2 mmol/l sodium orthovanadate) plus 1% Triton X-100 and a mixture of protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.15 units/ml aprotinin, 1 μg/ml pepstatin A). Cell-free supernatants were concentrated 100 times under speed vacuum. Thirty micrograms of proteins from cell lysates and cell-free supernatants were subjected to 8% SDS-PAGE under reducing conditions. Proteins were then transferred electrophoretically to nitrocellulose membranes that were blotted with antinephrin mAbs (at 2.5 μg/ml) as previously described (13). For reprobing, nitrocellulose filters were first stripped of antibody by 62 mmol/l Tris-HCl (pH 6.7), 2% SDS, and 100 mmol/l β2-mercaptoethanol.

Data analysis. Data were collected from three to five independent experiments. Data are presented as means ± SD. Differences between multiple groups were analyzed by one-way analysis of variance in combination with Tukey's multiple comparison test. Linear regression analysis was performed between relative fluorescence intensity for nephrin and extent of proteinuria. $P < 0.05$ was considered significant.

RESULTS

Nephrin expression in kidney biopsies from patients with diabetes. The expression of nephrin was evaluated by indirect IF in kidney biopsy samples from 23 patients with diabetes, including 10 patients with type 2 diabetes and nephrotic syndrome, 5 patients with type 2 diabetes and microalbuminuria, 7 patients with type 1 diabetes and nephrotic syndrome, and 1 patient with type 1 diabetes and microalbuminuria (Table 1). As control, 10 specimens of normal kidney portions obtained from patients who underwent surgery for renal tumors were used.

In control subjects, nephrin exhibited a glomerular epithelial staining pattern with a punctate/linear distribution along the peripheral capillary loops (Fig. 1*A* and *B*). In glomeruli of both patients with type 2 and type 1 diabetes and nephrotic syndrome, a more granular pattern and an extensive reduction of staining of nephrin were observed (Fig. 1*C*– *E*). In patients with type 2 and type 1 diabetes and microalbuminuria, the staining pattern of nephrin was also granular and reduced (Fig. 1*F*). As shown in Fig. 2,

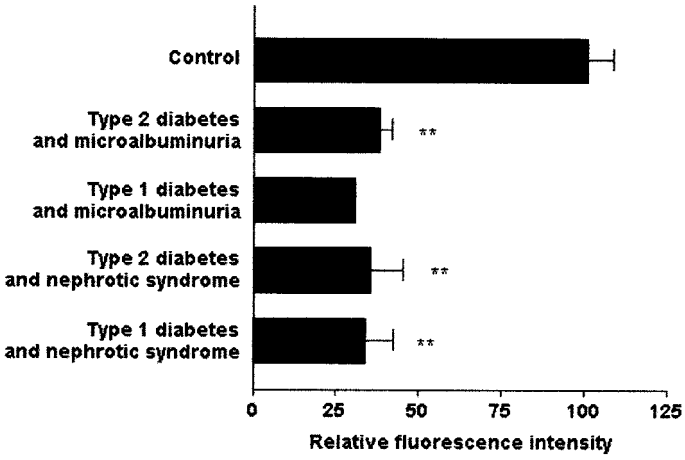

FIG. 2. Semiquantitative analysis of nephrin expression as detected by IF staining in glomeruli of control subjects, patients with type 1 diabetes and nephrotic syndrome, patients with type 2 diabetes and nephrotic syndrome, the patient with type 1 diabetes and microalbuminuria, and patients with type 2 diabetes and microalbuminuria. **$P < 0.001$ vs. control subjects.

relative fluorescence intensity was significantly reduced compared with control subjects in all groups of patients with diabetes, irrespectively of the extent of proteinuria. No difference in the extent of nephrin reduction as a function of diabetes duration and no significant correlation between relative fluorescence intensity and extent of proteinuria in patients with diabetes was found (data not shown). The average variability in nephrin staining pattern from one glomerulus to the next within individual patients with diabetes and control subjects was 16.8 ± 10.2% and 8.5 ± 2.0%, respectively. Control sections incubated with the nonimmune isotypic control antibody or with the appropriate labeled secondary antibody without the primary antibody were always negative (data not shown).

Expression of RAGE and modulation of nephrin expression in GECs. RAGE expression by immortalized GECs was shown by indirect IF (Fig. 3*A*). Unstimulated GECs expressed nephrin in a fine punctate diffuse pattern (Fig. 3*B*). Nephrin was expressed on the surface of nonpermeabilized GECs. The surface expression was confirmed by confocal microscopy (Fig. 3*B*, inset). When serial confocal optical sections of the cells were performed, it was found that the expression of nephrin was greater at the basal site of the cell membrane, in correspondence to the adhesion surface.

To determine whether nephrin expression was affected by GA, we incubated GECs with GA or native albumin for 1, 24, and 48 h. At 1 h, GA did not modify the surface expression of nephrin (Figs. 3*C* and 4). In contrast, at 24 and 48 h, GA induced a marked reduction of nephrin expression on the surface of GECs (Figs. 3*D* and 4). At any time, native albumin modified the surface expression of nephrin. Neutralizing anti-RAGE IgG but not nonimmune IgG prevented GA-induced decrease of nephrin expression (Fig. 3*E*). Moreover, GA did not induce changes in cytoskeletal organization (Fig. 3*F*).

The mRNA levels for nephrin, normalized for GAPDH expression, were decreased by 42.6 ± 7.1% in GECs that were stimulated with GA for 24 h, as compared with cells that were incubated with native albumin (Fig. 5).

At variance of GA, Ang II induced redistribution and loss

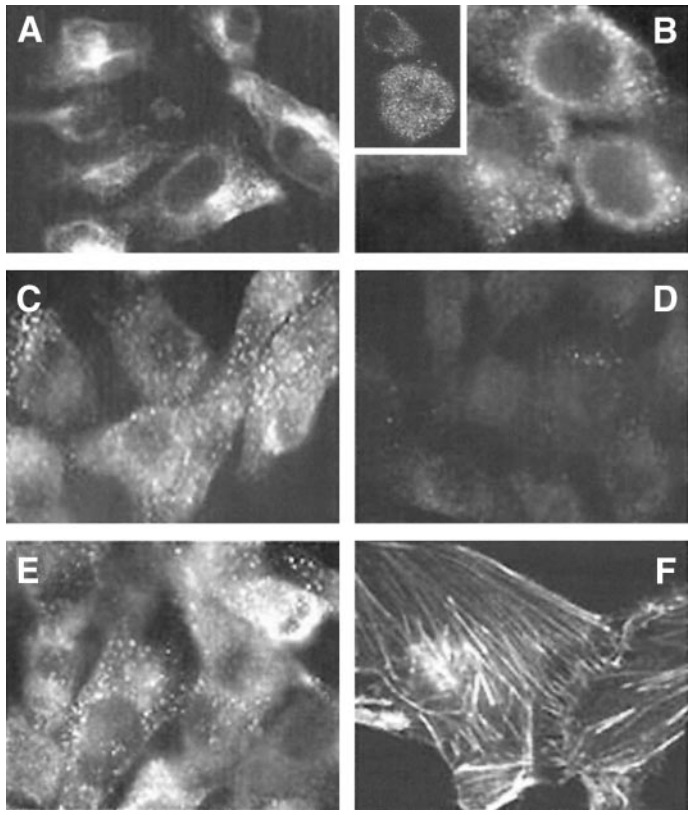

FIG. 3. IF staining for RAGE in unstimulated GECs (*A*). IF staining for nephrin in unstimulated GECs (*B*) or GECs incubated with GA (1,200 μg/ml) for 1 h (*C*) or 24 h (*D*). Anti-RAGE neutralizing antibody prevented the effect of GA on nephrin expression (*E*). FITC-phalloidin staining of actin microfilaments on permeabilized GECs was performed after incubation for 1 h with GA (1,200 μg/ml; *H*). Inset of *B* shows the surface expression of nephrin by confocal microscopy. Original magnification ×600 in *A–F* and ×630 in *B* inset.

of nephrin on GECs that was evident after 30 min and maximal after 1 h of incubation (Figs. 6*B* and 7*A*). The disappearance of nephrin from the cell surface was transient, as nephrin was reexpressed after 24 h incubation

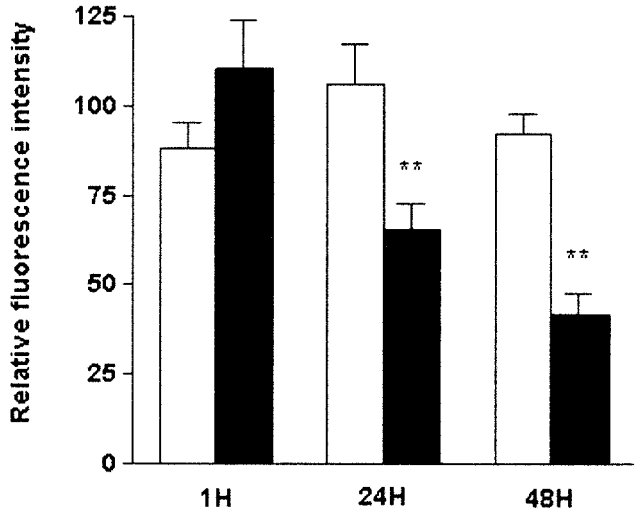

FIG. 4. Semiquantitative analysis of nephrin expression as detected by IF staining in GECs incubated with native albumin (□) or GA (■; 1,200 μg/ml) for different periods of time. **$P < 0.001$ vs. control. Results are expressed as percentage of control (GECs incubated with vehicle alone for 1 h).

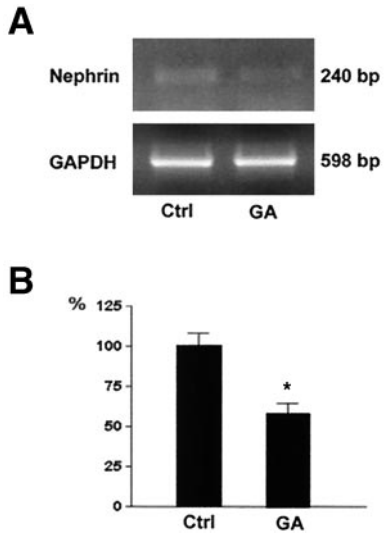

FIG. 5. Nephrin mRNA expression. *A*: Representative PCR gels of nephrin and GAPDH. *B*: Comparison of the expression of nephrin mRNA normalized to GAPDH expression in unstimulated GECs (Ctrl) and GECs stimulated with GA (1,200 μg/ml) for 24 h. Data are expressed as percentage of control. Shown are means ± SD of three independent experiments. *$P < 0.05$.

with Ang II (Figs. 6*C* and 7*A*). The effect of Ang II on nephrin was associated with changes in cytoskeleton distribution, including loss in stress fibers, cortical accumulation of F-actin, and cell retraction (Fig. 6*E*).

Cytochalasin B, a compound that affects the microfilaments of the microtubular system (29), prevented nephrin redistribution and disappearance (Figs. 6*F* and 7*B*) and the associated changes in cytoskeleton organization (Fig. 6*G*). Moreover, the process of nephrin redistribution was energy requiring, as it was inhibited by sodium azide, an inhibitor of oxidative phosphorylation and of glycolysis (Figs. 6*H* and 7*C*).

As shown in Fig. 7*D*, after treatment with Ang II, nephrin was detectable by Western blot, using antibody against the extracellular domain, into the cell-free supernatant as a fragment of ~100 kDa. In contrast, Western blot performed with antibody against the intracellular domain of nephrin was negative. These data suggested that the Ang II–induced reduction of surface expression of nephrin is, at least in part, due to cleavage and shedding of an extracellular part of this molecule.

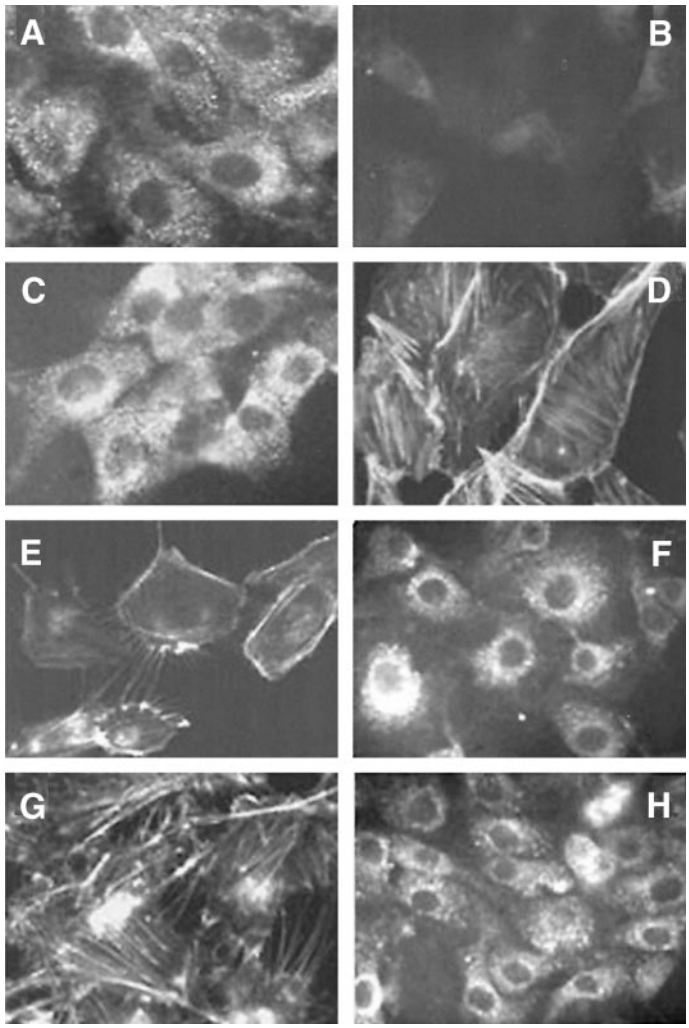

FIG. 6. IF staining for nephrin in unstimulated GECs (*A*) or GECs incubated with Ang II (100 nmol/l) for 1 h (*B*) or 24 h (*C*). FITC-phalloidin staining of actin microfilaments on permeabilized unstimulated GECs (*D*) or incubated with Ang II (100 nmol/l) for 1 h (*E*). Cytochalasin B prevented the reduction and redistribution of nephrin (*F*) as well as the cytoskeleton disorganization (*G*) induced by Ang II. Analogously, sodium azide inhibited nephrin loss and redistribution induced by Ang II (*H*). Original magnification ×600.

DISCUSSION

The pivotal role of nephrin in the regulation of glomerular filter integrity has recently emerged from genetic studies, showing that mutations in the nephrin gene (NPHS1) underline the development of the congenital nephrotic syndrome of the Finnish type (7,30). Mutations in the nephrin gene have been also described in proteinuric patients who do not exhibit the classic severe congenital nephrotic syndrome (8). Moreover, a correlation between changes in nephrin expression and proteinuria has been shown in experimental models of GN, such as that induced by injection into rats of mAb antinephrin (9), puromycin aminonucleoside-induced nephrosis (10), and mercury chloride–GN (11). We and others recently showed that nephrin is redistributed and its expression is reduced in glomeruli of patients with primary acquired nephrotic syndrome, including membranous GN, minimal-change GN, and focal segmental glomerulosclerosis (12–14). In vitro, stimuli acting on the cell cytoskeleton, known to be involved in the pathogenesis of GN, such as aggregated IgG_4, complement, tumor necrosis factor-α, and puromycin, induced redistribution of nephrin on the surface of human cultured podocytes (13). This suggests that the activation of cell cytoskeleton may modify surface expression of nephrin, thus allowing its dislocation from plasma membrane to an extracellular site. Indeed, it has been shown that nephrin is linked to the actin cytoskeleton and dissociates from actin in early experimental membranous nephropathy (31,32). The role of nephrin in the enhancement of glomerular permeability in diabetes remains to be clarified. In a recent experimental study, it was shown that diabetic spontaneously hypertensive rats develop albuminuria in concomitance with a reduction in both gene and protein expression of nephrin (16). Analogously, in rats with streptozotocin-induced diabetes and in nonobese diabetic mice, nephrin was redistributed and lost in the

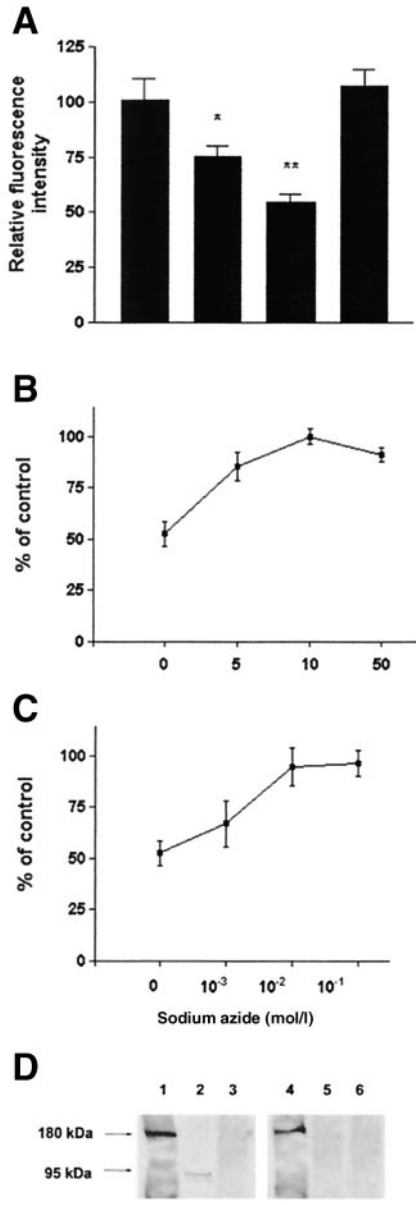

FIG. 7. ***A*****: Semiquantitative analysis of nephrin expression as detected by IF staining in GECs incubated with vehicle alone for 1 h (Ctrl) or with Ang II (100 nmol/l) for different periods of time; *****P*** **< 0.001 vs. control.** ***B*****: Dose-effect of cytochalasin B preincubation on IF staining for nephrin in GECs incubated with Ang II (100 nmol/l) for 1 h.** ***C*****: Dose-effect of sodium azide preincubation on IF staining for nephrin in GECs incubated with Ang II (100 nmol/l) for 1 h. Results are expressed as percentage of control (GECs incubated with vehicle alone for 1 h).** ***D*****: Representative Western blot analysis of nephrin expression by cell lysates and cell-free supernatants of GECs treated with Ang II.** ***Lanes 1*** **and** ***4*****, cell lysates of unstimulated GECs;** ***lanes 2*** **and** ***5*****, supernatants of GECs stimulated with Ang II (100 nmol/l) for 1 h at 37°C;** ***lanes 3*** **and** ***6*****, supernatants of GECs incubated with vehicle alone. Nephrin was detected using an antibody against the extracellular domain in** ***lanes 1-3*** **and an antibody against the intracellular domain in** ***lanes 4-6*****.**

urine (15). In the present study, we observed that the expression of nephrin in glomeruli of both patients with type 1 and type 2 diabetes and nephropathy is markedly reduced and altered in its distribution. At variance of patients with primary GN (13), no significant correlation between the reduction of nephrin expression and the extent of proteinuria was observed in patients with diabetes. Moreover, a marked reduction and redistribution of nephrin was observed also in glomeruli of patients with diabetes and microalbuminuria without significant histological glomerular lesions. This observation suggests that nephrin loss and redistribution may precede the development of glomerular lesions and be an early event in the progression of diabetic nephropathy, thus conditioning the onset of proteinuria. These data are in agreement with a previous report that showed that patients with type 1 diabetes and nephropathy present structural alterations of the glomerular filtration unit, consisting of increased width of podocyte foot processes and of filtration slits, demonstrable already in the stage of microalbuminuria (33).

It has been postulated that the development and the progression of DN is largely determined by the presence of a genetic prone background (34). DN may occur as a result of the interplay between genetic and nongenetic factors, such as the metabolic and hemodynamic ones (34,35). Hyperglycemia has been shown to be an independent risk factor for DN (36), and in patients with overt nephropathy, the mean level of glycosylated hemoglobin is correlated with the loss of renal function (37). Nonenzymatic glycation of proteins represents a major mechanism by which hyperglycemia leads to diabetic renal disease. Indeed, Amadori-modified proteins and advanced glycation end products elicit pathobiologic effects in cultured renal cells and in in vivo experimental models that are identical to those of high ambient glucose (38–40). However, several studies sustain the importance of not only systemic hypertension but also local hemodynamic changes in the renal microcirculation for the development and the progression of renal lesions in diabetes (35). In particular, the renin-angiotensin system (RAS) has been shown to be a key factor in this setting (41). Indeed, pharmacological blockade of the RAS system, using ACE inhibitors or Ang II type 1 receptor antagonists, reduces the risk of end-stage renal disease and decreases the level of urinary protein excretion (42).

To investigate whether metabolic and hemodynamic factors are potentially involved in the reduced expression of nephrin in patients with diabetes, we studied the effect of GA and Ang II on nephrin expression in human cultured GECs. The cell line used in this study was initially selected and grown in high glucose, thus not allowing us to conduct experiments to test the effect of hyperglycemic culture conditions on nephrin expression. Because Amadori-modified proteins, such as GA, have been shown to produce, in vivo and in vitro, the same pathobiologic effects than high ambient glucose, we studied the effect of GA incubation on nephrin expression. The incubation of podocytes in the presence of GA, at concentrations similar to those measured in the serum of patients with diabetes (26,27), did not induce any change in nephrin expression and cytoskeleton organization after 1 h, whereas it induced a marked reduction after 24 h. After incubation with GA for 24 h, the mRNA levels for nephrin were also diminished, showing that the decrease in nephrin expression was at least partially due to reduced gene transcription.

The biological effects of glycated proteins have been shown to be mainly mediated by the interaction with specific cell surface receptors, the best characterized of which is the RAGE (40). Several studies have shown that blockade of RAGE, using soluble RAGE, specific antibod-

ies anti-RAGE, or RAGE inhibitors, prevents many complications of diabetes, including chronic inflammation, vascular hyperpermeability, and impaired wound healing (43,44). It was shown recently that RAGE is expressed in kidney on normal podocytes and upregulated in DN (45). Here we showed that human cultured podocytes express RAGE and that the blockade of RAGE by a neutralizing antibody prevented the loss of nephrin expression induced by GA, suggesting the involvement of this receptor in mediating the effect of GA on nephrin expression.

At variance of GA, Ang II induced a rapid disappearance of nephrin from the surface of GECs, which was evident at 30 min, peaked at 1 h, and completely reversed at 24 h. The kinetic of this phenomenon suggests that it is independent of an effect on mRNA expression. On the contrary, the loss of nephrin induced by Ang II involves the cytoskeleton activation, and it requires metabolic energy, as it was prevented by cytochalasin B and sodium azide. These results are similar to those obtained with other agents that affect the cytoskeleton, such as immune complexes, tumor necrosis factor-α, and puromycin, which were shown to induce redistribution and shedding of nephrin (13). Indeed, it was shown recently that nephrin interacts with cellular actin (31,32). On the basis of these analogies, one can hypothesize that the loss of nephrin expression induced by Ang II is also due to a mechanism of shedding from the cellular surface to an extracellular site. The relevant role of Ang II in modulating the expression of nephrin is confirmed by several experimental reports, showing that the inhibition of RAS system, either by ACE inhibitors or Ang II receptor antagonists, prevents the loss of nephrin expression in severe nephrosis as well as in diabetic nephropathy (46–48).

In conclusion, the present study demonstrates that nephrin is lost and redistributed in glomeruli of both patients with type 1 and type 2 diabetes and nephropathy. These alterations are evident already at the stage of microalbuminuria, suggesting that nephrin loss may be an early event in the progression of DN. The in vitro experiments suggest at least two possible mechanisms of nephrin alteration in diabetic conditions, the first related to a rapid redistribution and shedding from the cell surface depending on Ang II stimulation of cell cytoskeleton and the second mediated by GA-RAGE interaction involving the inhibition of nephrin gene transcription.

ACKNOWLEDGMENTS

This work was supported by the National Research Council (CNR), Targeted Project Biotechnology, and by MURST, FIRB projects, and Local University Grants (ex 60%). Dr. Sophie Doublier is the recipient of a Marie Curie individual fellowship from the European Community. We thank Professor Karl Tryggvason (Karolinska Institute, Stockholm, Sweden) for giving us the antibodies antinephrin.

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