Preeclamptic sera induce nephrin shedding from podocytes through endothelin-1 release by endothelial glomerular cells

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Collino F, Bussolati B, Gerbaudo E, Marozio L, Pelissetto S, Benedetto C, Camussi G. Preeclamptic sera induce nephrin shedding from podocytes through endothelin-1 release by endothelial glomerular cells. *Am J Physiol Renal Physiol* 294: F1185–F1194, 2008. First published February 20, 2008; doi:10.1152/ajprenal.00442.2007.—In preeclampsia (PE), proteinuria has been associated with a reduced expression of nephrin by podocytes. In the present study, we investigated in vitro on human cultured podocytes the mechanism responsible for nephrin loss in PE. Sera from patients with PE did not directly downregulate the expression of nephrin. In contrast, conditioned medium obtained from glomerular endothelial cells incubated with PE sera induced loss of nephrin and synaptopodin, but not of podocin, from podocytes. Nephrin loss was related to a rapid shedding of the protein from the cell surface due to cleavage of its extracellular domain by proteases and to cytoskeleton redistribution. The absence of nephrin mRNA downregulation together with nephrin reexpression within 24 h confirm that the loss of nephrin was not related to a reduced synthesis. Studies with an endothelin-1 (ET-1) receptor antagonist that abrogated the loss of nephrin triggered by glomerular endothelial conditioned medium of PE sera indicated that ET-1 was the main effector of nephrin loss. Indeed, ET-1 was synthesized and released from glomerular endothelial cells when incubated with PE sera, and recombinant ET-1 triggered nephrin shedding from podocytes. Moreover, VEGF blockade induced ET-1 release from endothelial cells, and in turn the conditioned medium obtained triggered nephrin loss. In conclusion, the present study identifies a potential mechanism of nephrin loss in PE that may link endothelial injury with enhanced glomerular permeability.

slit diaphragm; synaptopodin; proteinuria; VEGF blockade

PREECLAMPSIA (PE) affects 1%–8% of all first pregnancies. The maternal syndrome is characterized by elevated blood pressure, proteinuria, and damage of different organ systems including the liver, kidney, brain, heart, and lungs. The systemic features can vary from mild cases with little systemic involvement to multiorgan failure. In 30% of the cases, the disease may cause placental insufficiency, leading to intrauterine growth restriction or fetal death. (46).

Defective remodeling of the spiral arteries at the time of trophoblast invasion is the most widely recognized predisposing factor for PE. As a result, perfusion of the intervillous space is impaired, leading to placental hypoxia (34). Several data support the theory that PE is the result of a generalized endothelial injury and dysfunction, due to the release of different placental factors (7, 17, 32, 38). Among those factors, an increase in PE sera of fibronectin, factor VIII antigen and thrombomodulin has been reported, all markers of vascular dysfunction (35, 42) Recently, it has been observed that serum levels of soluble vascular endothelial growth factor (VEGF) receptor-1 (sFlt1), a circulating antagonist of VEGF, were elevated in women with PE (45).

sFlt1 is a splice variant of VEGF receptor-1, which lacks the cytoplasmic and transmembrane domain, but retains the ligand-binding domain (18, 22). However, it is at present unknown whether sFlt1 and/or other circulating factors in PE induce proteinuria by a direct effect or rather by an indirect effect involving the local release of factors affecting podocytes.

It has been recently shown that slit diaphragm proteins of podocytes play a critical role in the maintenance of glomerular permeability. In particular, nephrin, a transmembrane protein of the Ig superfamily, expressed in the slit diaphragm between the podocyte foot processes, is involved in the maintenance of slit pore integrity and renal filtration capacity. 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 nephritic syndrome of the Finnish type (23, 24). Several studies have provided evidence that nephrin associates with itself, forming a zipper-like structure that acts as a size- and charge-selective filtration barrier. Moreover, nephrin has been demonstrated to have signaling functions, enabled by the intracellular domain with nine tyrosine residues, regulating podocyte cell polarity, cell survival, and cytoskeletal organization (15). The role of nephrin in proteinuria developing in PE has been confirmed by a recent report showing a reduction in the expression of nephrin and synaptopodin in glomeruli of PE patients (13).

The aim of the present study was to investigate whether sera from PE patients may downregulate the expression of nephrin from podocytes either directly or as a consequence of glomerular endothelial cell activation.

MATERIALS AND METHODS

Reagents. Polyclonal anti-nephrin guinea pig antibodies to the extracellular fibronectin domain (GP-N1) and to the intracellular domain (GP-N2) and monoclonal anti-synaptopodin mouse antibody were all purchased from Progen Biotechnik (Heidelberg, Germany). Goat polyclonal anti-podocin and anti-nephrin antibodies and anti-nephrin blocking peptide were purchased from Santa Cruz Biotech-

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nology (Heidelberg, Germany). FCS was from Euroclone (Wetherby, West Yorkshire, UK). DMEM, BSA fraction V (tested for not more than 1 ng endotoxin/mg), human ET-1, N-acetyl-[D-TRP[16]]-ET1, fragments 16–21 (ET-1A receptor antagonist), FITC-phalloidin, TRI reagent, Hoechst 33258 dye, α_1-anti-trypsin, anti-PI, and FITC anti-guinea pig secondary antibody were obtained from Sigma (St. Louis, MO). Alexa Fluor 488 anti-goat or anti-guinea pig and Texas red anti-mouse IgG were supplied by Molecular Probes (Leiden, The Netherlands). GM6001 and SB-3CT were purchased from Chemicon International (Temecula, CA). The angiotensin II receptor (AT1A) antagonist losartan, or DuP753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-(1H-tetrazole-5-yl) biphenyl-4-hy] methyl) imidazole, was kindly provided by Dr. Gabriella Gruden (University of Torino) and was supplied by DuPont Merck (West Point, PA). Human anti-VEGF polyclonal antibody was obtained from R&D Systems (Minneapolis, MN).

Patients. PE was diagnosed in the presence of two consecutive blood pressure measurements of >140 mmHg systolic or >90 mmHg diastolic and proteinuria (≥300 mg in one 24-h urine collection) after *week 20* of pregnancy in a previously normotensive and nonproteinuric woman (1, 31, 36). Healthy, normotensive pregnant women were included as controls (Table 1). Approval of the study was obtained from the Center for Molecular Biotechnology Institutional Review Board, University of Torino. Patients with chronic hypertension, preexisting proteinuria, or renal disease were excluded from the study. Blood samples were collected from pregnant patients at the time of the diagnosis of PE, after informed consent was obtained. Maternal age, parity, gestational age at delivery, birth weight, placental weight, blood pressure values, urinary protein excretion, serum creatinine, and serum uric acid levels were recorded for each patient included in the study.

Cell lines. Primary microvascular endothelial cell lines obtained from renal glomeruli were prepared. Briefly, human glomeruli were purified from specimens of normal renal tissue obtained for polar carcinomas by passages on sequential meshes, digested by trypsin (0.1%, 30 min. at 37°C), and plated onto gelatin in EBM complete medium. After 1 wk, cells were detached and endothelial cells were purified by using an anti-CD31 antibody coupled to magnetic beads by magnetic cell sorting using the MACS system (Miltenyi Biotec, Auburn, CA). Cells were characterized by morphology and expression of a panel of endothelial antigens, as described elsewhere (4).

Primary cultures of human podocytes were established, and lines of differentiated podocytes were obtained by infection with a hybrid Adeno5/SV40 virus. Podocytes were characterized for the positive expression of nephrin, podocin, and synaptopodin and for negative expression of von Willebrand factor, CD31, and smooth muscle cell actin, as previously described (6). Cells were cultured in DMEM containing 4,500 mg/l glucose with 10% FCS.

Experimental conditions. For immunofluorescence (IF) studies, podocytes were plated on eight-well Permanox slides at a density of 50,000 cells/well. For fluorescence-activated cell sorting (FACS) analysis, podocytes were plated in a 12-well tissue culture plate at subconfluent density. The following day, cell layers were rinsed with

PBS. Podocytes were starved for 1 h and then incubated with serum derived from PE and normal women (1:10 dilution) for different times before fixation and staining with an anti-nephrin polyclonal antibody.

In other experiments, glomerular endothelial cells were incubated in the presence of PE or normal serum or plasma (1:10) or with an anti-VEGF antibody (4.5 μg/ml) for 72 h. The obtained endothelial conditioned medium (ECM) was used (1:10 dilution) to stimulate podocytes for 1 h to assess nephrin, podocin, and synaptopodin expression. In time course experiments, podocytes were stimulated for 20 min, 1 h, or 24 h.

In selected experiments, ET-1A and AT1A receptor antagonists (N-acetyl-[D-TRP[16]]-ET1, fragment 16–21, 0.4 μM; losartan, 10^{-6} M) were added to the cells 1 h before ECM stimulation.

In addition, in some experiments podocytes were incubated with the generic protease inhibitor PMSF (1 mmol) or with two specific serine protease inhibitors, α_1-anti-trypsin (5 μg/ml) and anti-PI (5–10 μg/ml) for 20 min before ECM treatment or with two specific MMP inhibitors, GM6001 (15 nM) and SB-3CT (4 μM), during ECM stimulation.

In other experiments, podocytes were incubated in the presence of human ET-1 (10 nM) (30) for 1 h before detection of nephrin by FACS analysis. For evaluating actin microfilament alterations in PE-stimulated podocytes, cells were incubated with ECM for 1 h and then fixed, permeabilized, and stained with FITC-phalloidin.

Detection of nephrin, podocin, and synaptopodin expression by immunofluorescence and flow cytometric studies. Immunofluorescence on cultured podocytes was performed as described previously (3). Cells were fixed in 3.5% paraformaldehyde containing 2% sucrose for 15 min at 4°C and, when needed, permeabilized with HEPES-Triton X-100 buffer (Sigma). Guinea pig anti-nephrin (GP-N1, diluted 1:50), goat anti-podocin (1:50), or mouse anti-synaptopodin (undiluted) primary antibodies were applied on the cells for 1 h at 4°C. Cells were washed and subsequently incubated with fluorescein isothiocyanate or rhodamine-conjugated anti-guinea pig, goat, or mouse IgG secondary antibodies (Molecular Probes) for 1 h at room temperature. Secondary antibodies alone were used in all experiments as controls. Nephrin, synaptopodin, and podocin expression on cultured podocytes was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis (Windows Micro Image, CASTI Imaging, Venice, Italy) on images obtained using a low-light video camera (Leica DC100) on ×400 microscopic fields as described elsewhere (11). Membrane localization of slit diaphragm proteins was analyzed by confocal microscopy using a Zeiss LSM 5 Pascal model confocal microscope (Carl Zeiss, Oberkochen, Germany). Hoechst 33258 dye (Sigma) was added for nuclear staining. For flow cytometric analyses, podocytes were collected with nonenzymatic cell dissociation solution, fixed with 4% paraformaldehyde for 15 min, and stained for 1 h at 4°C with guinea pig anti-nephrin polyclonal antibody (final dilution 1:50) or corresponding isotype antibody, washed, and incubated with FITC-conjugated anti-guinea pig IgG for 1 h at 4°C.

Immunoprecipitation and Western blot analysis. Proteins of podocytes were extracted with a cold lysis buffer (50 mmol Tris·HCl, pH 8.3, containing 1% Triton X-100, 1 mmol PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 mmol sodium orthovanadate) at 4°C for 1 h and centrifuged at 15,000 g for 15 min. For the detection of nephrin, which is contained within lipid raft microdomains, the lysis buffer was supplemented with 20 mmol 3-[(3-cholamidopropyl)-dimethilammonio]-1-propanesulfonate (Sigma).

Immunoprecipitation with an anti-nephrin IgG polyclonal antibody cross-linked to Sepharose-protein A was performed as described by (8). Electrophoresis of the immunoprecipitates was performed as described previously (8).

For Western blot analysis, podocyte-free supernatants were concentrated, and 100 μg of proteins from cell lysates and cell-free supernatants were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were

Table 1. *Clinical characteristics of patients*

	PE (n = 20)	Normal (n = 20)
Maternal age, yr	30.9±4	30.6±6
Primigravida	15	11
Gestational age at delivery, wk	33.9±3*	39.7±1
Birth weight of child, g	2,166.8±823*	3,383±432
Placental weight, g	441.3±127*	587.25±97
Urinary protein, g/24 h	1.98±1.6*	0.102±0.1
Serum creatinine, mg/dl	0.77±0.3	0.72±0.1
Serum uric acid, mg/dl	6.17±1.8*	4.3±1.1

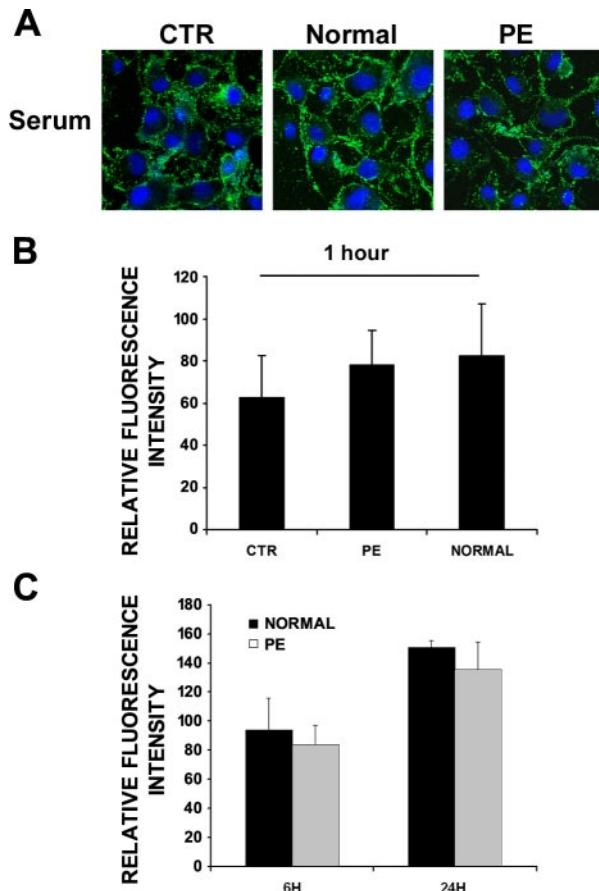Values are means ± SD of 20 patients with preeclampsia (PE) and 20 normal pregnant women. *$P < 0.005$.

Fig. 1. Direct effect of preeclampsia (PE) sera on nephrin expression by human podocytes. *A*: representative micrographs of nephrin expression detected by immunofluorescence using an antibody specific for the nephrin extracellular domain in podocytes incubated for 1 h with medium alone (CTR), sera from normal pregnant women (NORMAL), or sera from women with PE. Different stimuli did not modify nephrin expression. Original magnification ×400. *B* and *C*: semiquantitative analysis of nephrin expression evaluated as relative fluorescence intensity (see MATERIALS AND METHODS) after cell stimulation for 1 (*B*) or for 6 and 24 h (*C*) showing the absence of significant variations. The incubation with sera did not modify cell viability, as detected by trypan blue dye exclusion at the different times studied. Values are means ± SD of 10 different sera for each experimental condition. CTR, control.

labeled with two different antibodies against nephrin extracellular domain (GP-N1 and N-20) and an antibody against the intracellular domain (GP-N2) at 4°C overnight. In selected experiments, the anti-nephrin polyclonal antibody N-20 was preincubated with a blocking peptide (1:5 ratio, antibody to peptide) for 3 h at room temperature before membrane labeling. Blots were probed with horseradish peroxidase-conjugated protein A (Amersham, Little Chalfont, UK) or anti-goat secondary antibody (DakoCytomation, Copenhagen, Denmark) for 1 h at room temperature and developed with chemiluminescence reagents (ECL; Amersham) (48).

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) for human ET-1 was obtained from Cayman Chemical (Ann Arbor, MI) and performed according to the manufacturer's specifications. Aliquots of supernatants from glomerular endothelial cells untreated or treated with PE and "normal" serum or with blocking anti-VEGF antibody were assayed in triplicate.

Quantitative real-time PCR. Quantitative RT-PCR was performed as described previously (28). Relative quantization by real-time PCR was performed using SYBR-green detection of PCR products in real time using the 48-well StepOne Real Time System (Applied Biosystems, Foster City, CA). Sequence-specific oligonucleotide primers

(purchased from MWG-Biotech, Ebersberg, Germany) were the following: human nephrin: forward, 5′-CAC GGT CAG CAC AAC AGA GG-3′ and reverse, 5′-GAA ACC TCG GGA ATA AGA CAC CT-3′; human GAPDH: forward, 5′-TGG AAG GAC TCA TGA CCA CAG T-3′ and reverse, 5′-CAT CAC GCC ACA GTT TCC C-3′; and human ET-1: forward, 5′-AAC CAG GTC GGA GAC CAT GA-3′ and reverse, 5′-CCG AAG GTC TGT CAC CAA TGT-3′. Power SYBR Green PCR Master Mix was purchased from Applied Biosystems. Thermal cycling conditions were as follows: activation of AmpliTaq Gold DNA Polymerase LD at 95°C for 10 min, followed by 50 cycles of amplification at 95°C for 15 s, 60°C (for nephrin, ET-1, and GAPDH) for 1 min. To detect the log phase of amplification, the fluorescence level (quantification of product) was determined at each cycle. The cycle at which the fluorescence reached threshold was recorded, averaged between triplicates, and normalized to the averaged cycle of threshold value for GAPDH. The fold-change in expression with respect to control (unstimulated cells) was calculated for all samples.
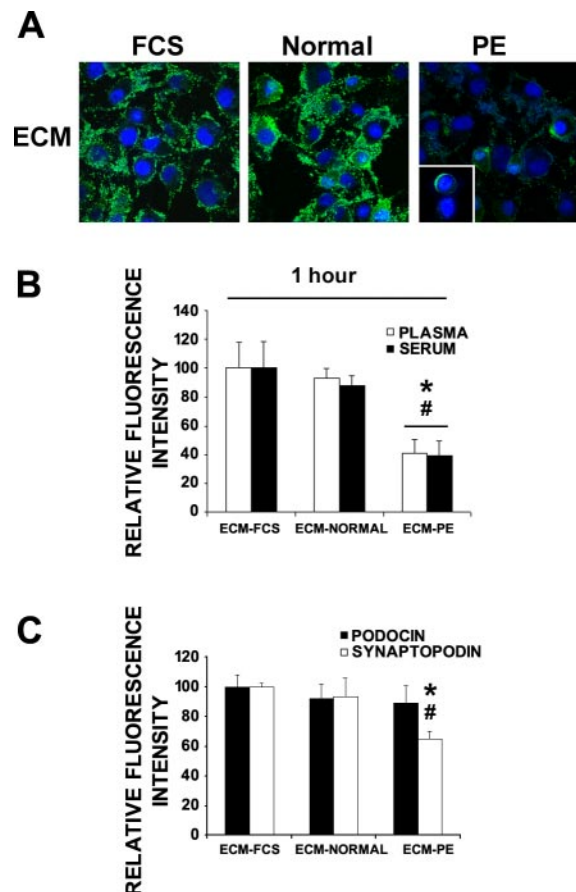

Fig. 2. Indirect effect of PE sera on nephrin expression by human podocytes. *A*: representative micrographs of nephrin expression detected by immunofluorescence in podocytes incubated with endothelial conditioned medium (ECM) derived from glomerular endothelial cells stimulated with medium alone (ECM-FCS) or with sera from normal pregnant women (ECM-NORMAL) or of women with PE (ECM-PE). ECM-PE induced a marked reduction of nephrin expression on the podocyte surface compared with ECM-FCS or ECM-NORMAL, with a protein redistribution as aspect of capping (*inset*). Original magnification ×400. *B*: semiquantitative analysis of nephrin expression evaluated as relative fluorescence intensity (see MATERIALS AND METHODS) after cell stimulation with ECM for 1 h. Values are means ± SD of 20 different sera (filled bars) or of 10 different plasmas (open bars) for each experimental condition. *C*: semiquantitative analysis of podocin and synaptopodin expression evaluated as relative fluorescence intensity after cell stimulation with ECM for 1 h. Values are means ± SD of 10 different plasmas for each experimental condition. *$P < 0.05$ vs. control. #$P < 0.05$ vs. ECM-NORMAL.

Statistical analysis. Results are representative of at least three independent experiments performed in triplicate and are expressed as means ± SD. Statistical analysis of the data was performed using Student's *t*-test. Significant differences are reported when $P < 0.05$.

RESULTS

Effects of sera from PE or normal pregnant women on nephrin expression by podocytes. It has been shown that the glomerular expression of nephrin is reduced in patients with PE (13). We evaluated whether serum factors may directly account for the reduced nephrin expression. The expression of nephrin was evaluated on human cultured podocytes stimulated with sera of PE patients and normal pregnant women. Podocytes were incu-

bated for 1, 6, or 24 h with 10% serum from PE or normal pregnant women, and nephrin expression on podocytes was evaluated by indirect IF. Unstimulated podocytes showed a surface expression of nephrin in a fine, punctuate pattern with peripheral distribution (Fig. 1A). As shown in Fig. 1, podocyte stimulation with sera from PE patients did not modify the surface expression of nephrin compared with unstimulated cells and with cells stimulated with sera from control pregnant women.

Effects of conditioned medium derived from endothelial cells incubated with sera from PE or normal pregnant women on the expression of nephrin, podocin, and synaptopodin by podocytes. Since endothelial cells are considered the primary target in PE, we tested the hypothesis that a factor(s) produced by
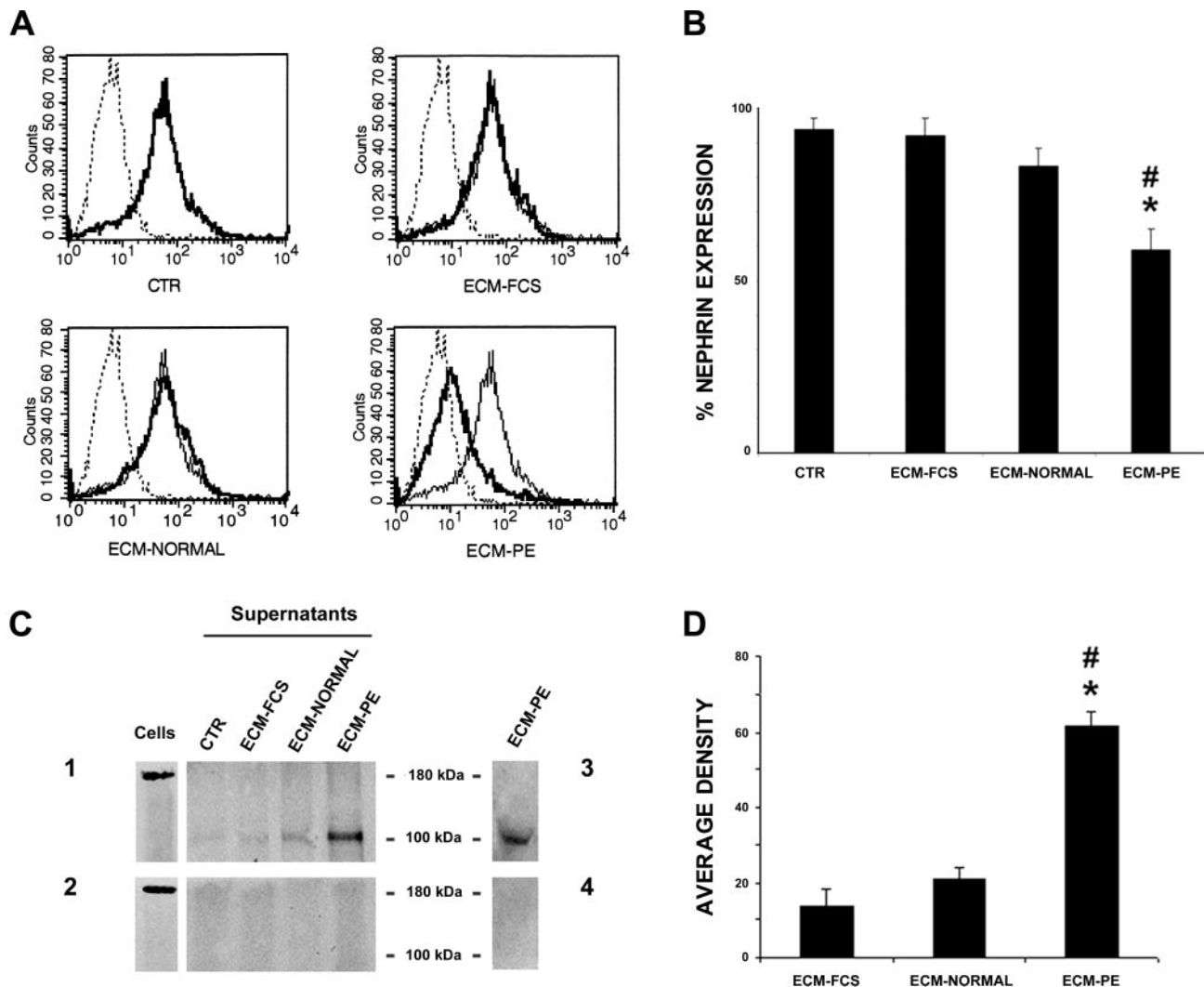

Fig. 3. Nephrin shedding from the podocyte surface after ECM-PE stimulation. *A*: representative flow cytometric analysis of nephrin expression on podocytes stimulated with ECM-FCS, ECM-PE, or ECM-NORMAL sera showing the loss of nephrin induced by ECM-PE stimulation. Thin lines represent unstimulated podocytes (positive control), dotted lines represent the corresponding isotype control antibody, and thick lines represent stimuli. *B*: semiquantitative analysis of nephrin expression evaluated by fluorescence-activated cell sorting (FACS) on podocytes treated with ECM-FCS, ECM-PE, or ECM-NORMAL. Values are means ± SD of 10 sera from PE and 10 from NORMAL and expressed as % of nephrin-expressing cells. *P < 0.05 vs. ECM-FCS. #P < 0.05 vs. ECM-NORMAL. *C*: representative Western blot analysis of nephrin detected in the cell lysates of control podocytes or in the cell-free supernatants of podocytes treated with ECM-FCS, ECM-PE, or ECM-NORMAL sera, using an antibody against the extracellular domain (GP-N1) in *blot 1* and an antibody against the intracellular domain (GP-N2) in *blot 2*. The specificity of the nephrin band was evaluated before (*blot 3*) and after (*blot 4*) preabsorption of the anti-nephrin polyclonal antibody (N-20, against the extracellular domain) with its specific peptide. Nephrin was detected as an 180-kDa band in the cell lysates of control podocytes and as a 100-kDa band, corresponding to the extracellular domain in the cell supernatants using the anti-extracellular domain antibodies. *D*: densitometric analysis of nephrin protein levels in the supernatant of podocytes treated with ECM-FCS, ECM-PE, or ECM-NORMAL sera detected by the anti-extracellular domain GP-N1 antibody. Values are means ± SD of 3 different experiments with 3 different sera. *P < 0.05 vs. ECM-FCS. #P < 0.01 vs. ECM-NORMAL.

glomerular endothelial cells stimulated with PE sera or plasmas may affect the expression of nephrin, podocin, and synaptopodin. For this purpose, glomerular endothelial cells were incubated with 10% sera or plasmas from PE or normal pregnant women and the ECM was collected after 72 h. Podocytes were then incubated for 1 h with ECM, and nephrin, podocin, and synaptopodin expression was evaluated. ECM from endothelial cells stimulated with PE sera as well as plasmas (ECM-PE) induced a significant reduction of nephrin expression on the cell surface compared with ECM from endothelial cells incubated with FCS (ECM-FCS) or with sera and plasmas from normal pregnant women (ECM-NORMAL) (Fig. 2, A and B). In addition, a significant reduction of synaptopodin but not of podocin was observed (Fig. 2C). As seen in Fig. 2A, loss of nephrin was associated with its redistribution as clusters on the podocyte surface.

Nephrin expression was also tested by flow cytofluorimetric analysis that confirmed a significant reduction of nephrin in podocytes stimulated for 1 h with ECM-PE and not with ECM-NORMAL or ECM-FCS (Fig. 3, A and B).

The release of nephrin due to podocyte stimulation with ECM-PE was confirmed by Western blot analysis of the cell supernatant. Nephrin, as a 100-kDa fragment, was detected in the podocyte-free supernant using a specific antibody against the extracellular domain (GP-N1), but not with a specific antibody against the intracellular domain (GP-N2) of nephrin (Fig. 3, C and D). These data suggested that ECM-PE stimulus induced a reduction of nephrin expression on the podocyte surface due, at least in part, to shedding of the extracellular domain of this protein.

Endothelial ET-1 production mediates the loss of nephrin induced by ECM-PE. In the attempt to identify a possible factor responsible for nephrin loss in the ECM-PE, we tested the effect of receptor antagonists for ET-1 and angiotensin II, vasoactive mediators known to be involved in PE. Pretreatment of podocytes with an ET-1A receptor antagonist (*N*-acetyl-[D-TRP[16]]-ET1, fragment 16–21, 0.4 μM), markedly reduced nephrin loss induced by ECM-PE stimulation (Fig. 4, A and B). In contrast, cell pretreatment with an angiotensin II receptor antagonist (losartan, 10^{-6}M) did not show a protective effect on nephrin loss from podocytes (Fig. 4A).
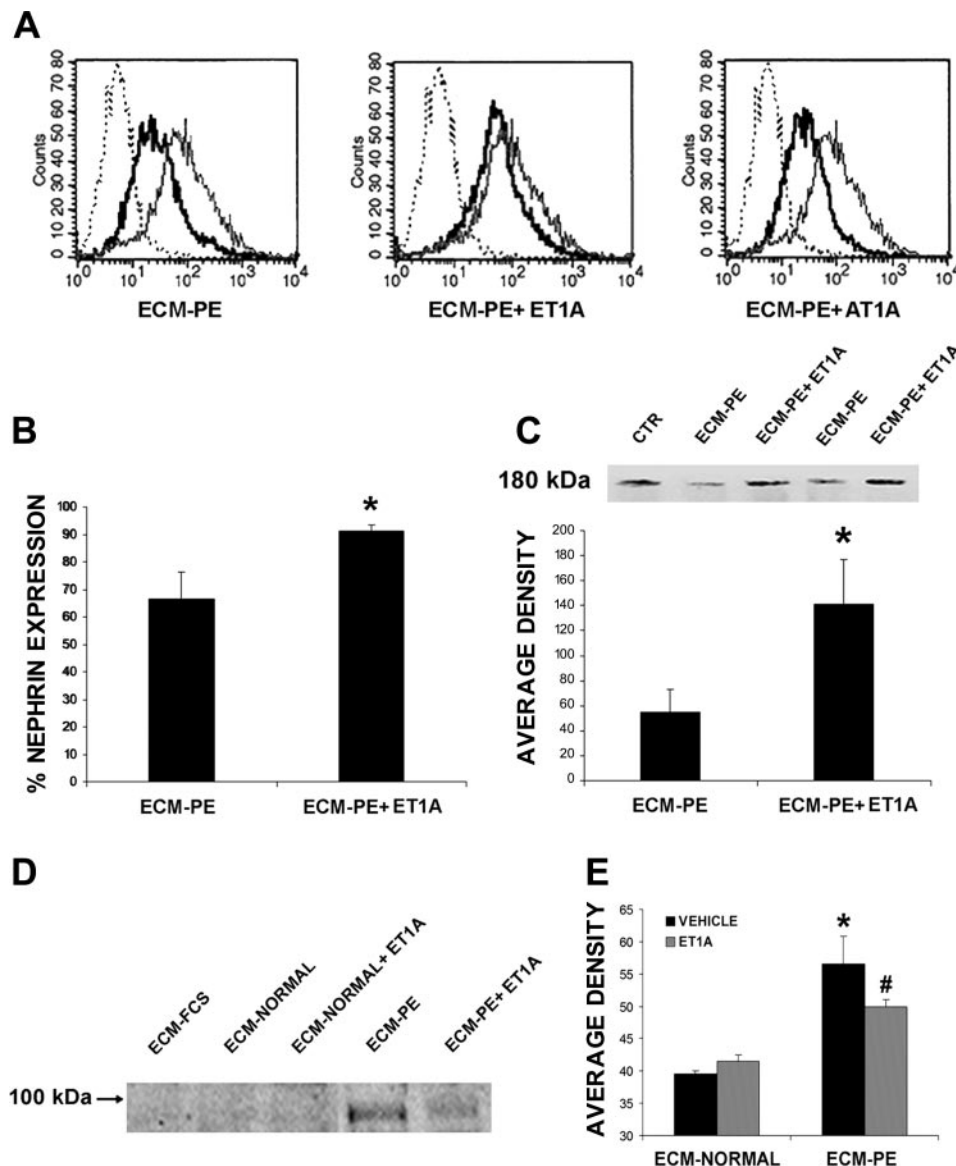

Fig. 4. Treatment with an endothelin-1A (ET-1A) receptor antagonist inhibits the effect of ECM-PE on nephrin loss. *A*: representative cytofluorimetric evaluation of nephrin expression after 1 h of stimulation with ECM-PE in the absence or presence of an ET-1 receptor antagonist (ET-1A) or an angiotensin II receptor antagonist (AT1A). Nephrin loss by ECM-PE was prevented by ET-1A. Thin lines represent unstimulated podocytes (positive control), dotted lines represent the corresponding isotype control antibody, and thick lines represent stimuli. *B*: semiquantitative analysis of nephrin expression with flow cytometric analysis of podocytes treated with ECM-PE in the absence or presence of ET-1A. Values are means ± SD of 5 experiments expressed as % of nephrin expression. *$P < 0.05$ vs. ECM-PE. *C*: immunoprecipitation with an anti-nephrin polyclonal antibody of the podocyte lysates after 1-h incubation with ECM-PE sera in the absence or presence of ET-1A. The treatment with ET-1A reverses nephrin loss induced by ECM-PE. *$P < 0.05$ vs. ECM-PE. Values are means ± SD of 3 different experiments. *D*: representative Western blot of supernatant of podocytes treated with ECM-NORMAL and ECM-PE sera in the absence or presence of ET-1A. Nephrin was detected with a GP-N1 polyclonal antibody for the extracellular domain as a fragment of 100 kDa. *E*: densitometric analysis by Western blot of nephrin protein levels in the supernatant of podocytes stimulated with ECM-NORMAL or ECM-PE sera in the absence (black bars) or presence of ET-1A (gray bars). Values are means ± SD of 3 different experiments. *$P < 0.05$ vs. ECM-NORMAL. #$P < 0.05$ vs. ECM-PE alone.

These data were confirmed by immunoprecipitation experiments with an anti-nephrin antibody showing the maintenance of cell-associated nephrin on podocyte lysates after cell incubations with ECM-PE in the presence of an ET-1A receptor antagonist with respect to ECM-PE alone (Fig. 4C). In parallel, nephrin was absent in the podocyte supernatant after ECM-PE stimulus in the presence of the ET-1A receptor antagonist, as detected by Western blot analysis (Fig. 4D), suggesting the inhibition of nephrin shedding from the surface of podocytes.

Podocyte treatment with recombinant ET-1 showed loss of nephrin that was prevented by the ET-1A receptor antagonist (Fig. 5A), supporting a possible role of ET-1 released from endothelial cells stimulated by PE sera. Indeed, glomerular endothelial cells released ET-1 after stimulation with PE. As shown in Fig. 5B, ET-1 concentration in the supernatant of cells stimulated with PE serum was significantly enhanced (106 ± 9 pg/10^6 cells) compared with cells exposed to normal serum (26 ± 16 pg/10^6 cells). This was confirmed by the enhanced expression of ET-1 mRNA by endothelial cells stimulated with PE (Fig. 5C).

As shown for ECM-PE, ET-1 induced redistribution and shedding of nephrin from the surface of podocytes (not shown). This effect was associated with a podocyte cytoskeleton redistribution. Podocytes, when confluent, exhibited a pattern of F-actin filaments distributed as stress fiber bundles along the axis of the cells, as shown by confocal microscopy (Fig. 5D). When stimulated with ECM-PE or ET-1, we observed a loss of stress fibers and F-actin accumulation at the cell periphery compared with control and ECM-NORMAL. Pretreatment of podocytes with a ET-1A receptor antagonist
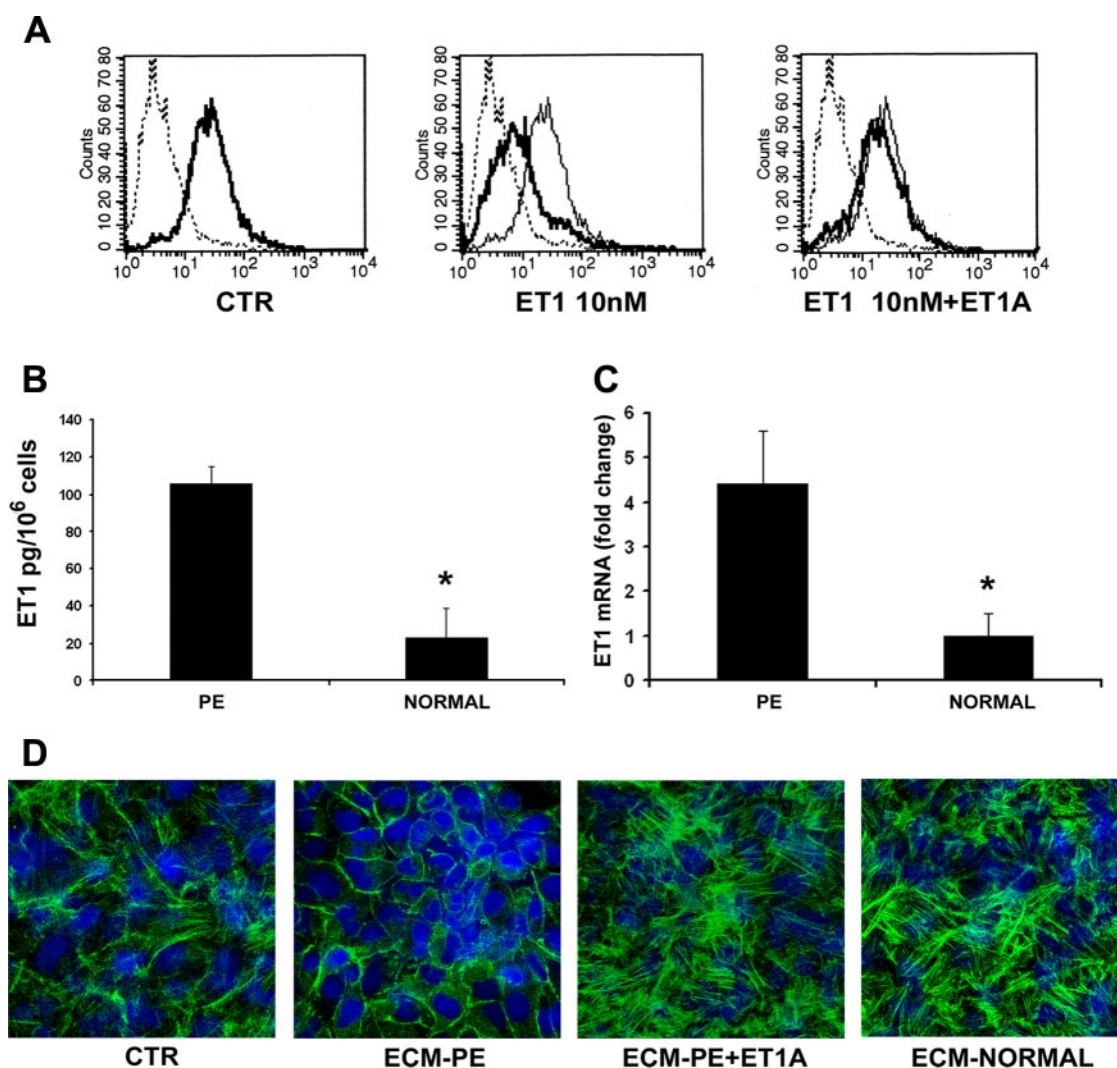

Fig. 5. Effect of ET-1 in the reduction of nephrin expression and in changes in the podocyte cytoskeleton. *A*: flow cytometric analysis of nephrin expression by podocytes incubated with a recombinant ET-1 (10 nM) in the absence or presence of an ET-1 receptor antagonist (ET-1A). Values are representative of 3 different experiments. Thin lines represent unstimulated podocytes (positive control), dotted lines represent the corresponding isotype control antibody, and thick lines represent stimuli. ET-1 induced nephrin loss that was reversed by the ET-1A. *B*: ET-1 release by endothelial glomerular cells stimulated with PE or NORMAL sera, as detected by ELISA. *$P < 0.01$ vs. NORMAL. *C*: ET-1 mRNA expression analyzed by quantitative real-time PCR in endothelial glomerular cells stimulated with PE or NORMAL sera for 72 h. The normalized expression of the ET-1 gene with respect to GAPDH was computed for all samples. *$P < 0.05$ vs. NORMAL. *D*: representative micrographs of FITC-phalloidin staining of actin microfilaments in control podocytes or in podocytes treated with ECM-PE, ECM-PE plus the ET-1A, or ECM-NORMAL sera. ECM-PE sera induced cytoskeletal reorganization at the cell periphery that was abrogated by ET-1A. Magnification ×400. The incubation with sera did not modify cell viability as detected by trypan blue dye exclusion. Three different experiments were performed with 5 different PE and NORMAL sera.

prevented changes in cytoskeleton organization and stress fiber reduction induced by ECM-PE, suggesting that ET-1 stimulates cytoskeleton redistribution (Fig. 5*D*). Moreover, real-time PCR studies showed ECM-PE or ET-1 did not affect nephrin mRNA levels after 1-h stimulation (Fig. 6*A*) nor after 24 h (not shown), indicating that nephrin loss was due to shedding rather then to an altered synthesis. In addition, after 24-h incubation nephrin expression was restored on the podocyte surface (Fig. 6, *B* and *C*).

Since a recent study suggested that VEGF blockade is responsible for proteinuria (43) in PE, we evaluated whether blockade of VEGF stimulated ET-1 production from endothelial cells and loss of nephrin induced by the endothelial conditioned medium. As shown in Fig. 7*A*, blocking an anti-VEGF antibody induced a significant production of ET-1 in glomerular endothelial cells. In turn, the conditioned medium
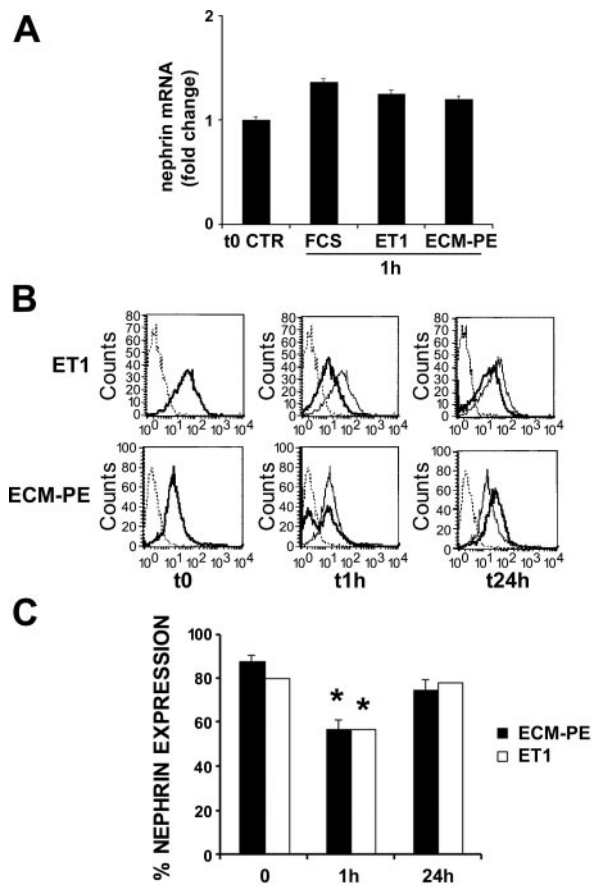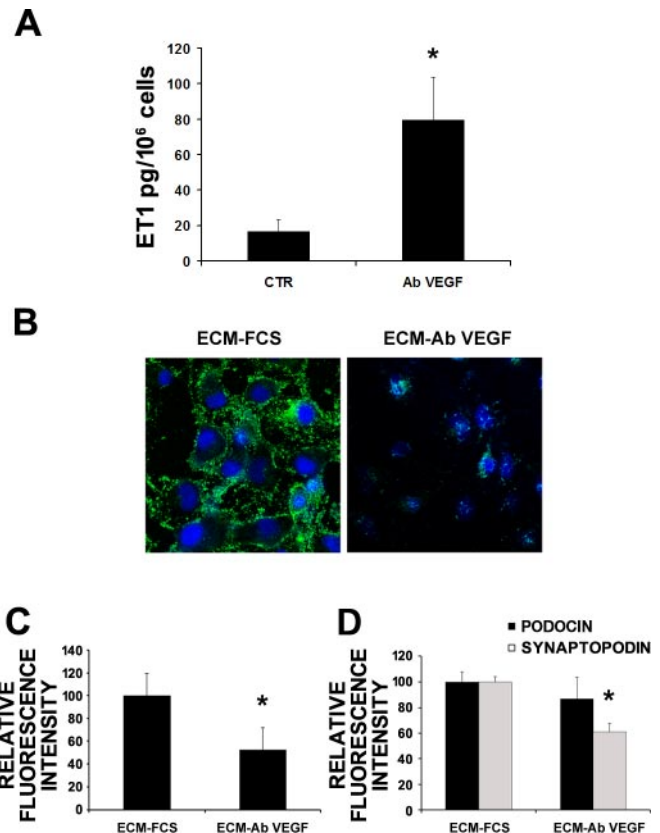

Fig. 7. Effect of VEGF blockade on ET-1 production and slit diaphragm protein expression. *A*: ET-1 release by endothelial glomerular cells stimulated with anti-VEGF antibody (Ab VEGF), as detected by ELISA. *$P < 0.01$ vs. CTR. *B*: representative micrographs of nephrin expression detected by immunofluorescence in podocytes incubated with ECM derived from glomerular endothelial cells stimulated with anti-VEGF antibody (ECM-Ab VEGF). Nephrin loss was observed in respect to ECM-FCS. *C* and *D*: semiquantitative analysis of the expression of nephrin (*C*), podocin, and synaptopodin (*D*) evaluated as relative fluorescence intensity after podocyte stimulation with ECM-FCS or ECM-Ab VEGF for 1 h. *$P < 0.05$ vs. ECM-FCS. Values are representative of 5 different experiments.

Fig. 6. Nephrin reduction by ECM-PE or ET-1 is transient and does not affect nephrin mRNA levels. *A*: nephrin mRNA expression analyzed by quantitative real-time PCR in unstimulated podocytes or podocytes stimulated with ECM-FCS, ECM-PE, or ET-1 for 1 h. The normalized expression of the nephrin gene with respect to GAPDH was computed for all samples. Values are means ± SD of 2 independent experiments performed in triplicate and expressed as fold-changes with respect to control. *B*: representative flow cytometric panels showing the time course of nephrin expression by podocytes treated with ET-1 or ECM-PE. Thin lines represent unstimulated podocytes (positive control), dotted lines represent the corresponding isotype control antibody, and thick lines represent stimuli. Nephrin expression was reacquired at 24 h. *C*: semiquantitative analysis of nephrin expression by podocytes stimulated with ECM-PE (filled bars) or ET-1 (open bars). Values are means ± SD of 4 experiments. *$P < 0.05$ vs. *time 0*.

obtained by endothelial cells after VEGF blockade induced a significant loss of nephrin and synaptopodin, but not podocin from podocytes (Fig. 7, *B–D*). No direct effect of the anti-VEGF antibody on nephrin and synaptopodin loss was observed (not shown).

Effect of protease inhibitors on expression of nephrin after ECM-PE stimulation. As we have detected nephrin shedding in the cell-free supernatant after ECM-PE stimulation, we analyzed the possible role of proteases as cleaving enzymes of the nephrin extracellular domain.

For this purpose, we tested two metalloproteases inhibitors: GM6001, a collagenase inhibitor, and SB-3CT, a gelatinase inhibitor (16, 21). Pretreatment of podocytes with GM6001 or SB-3CT did not induce a reduction of nephrin loss caused by ECM-PE stimulus, as indicated by FACS analysis (Fig. 8*A*) nor inhibition of nephrin shedding (not shown). In contrast, pretreatment with a generic serine protease inhibitor, PMSF, prevented nephrin reduction induced by ECM-PE stimulus (Fig. 8). We next tested the effect of two more specific serine protease inhibitors, α1-anti-trypsin and anti-protease inhibitor (anti-PI), on nephrin reduction induced by the ECM-PE stimulus.
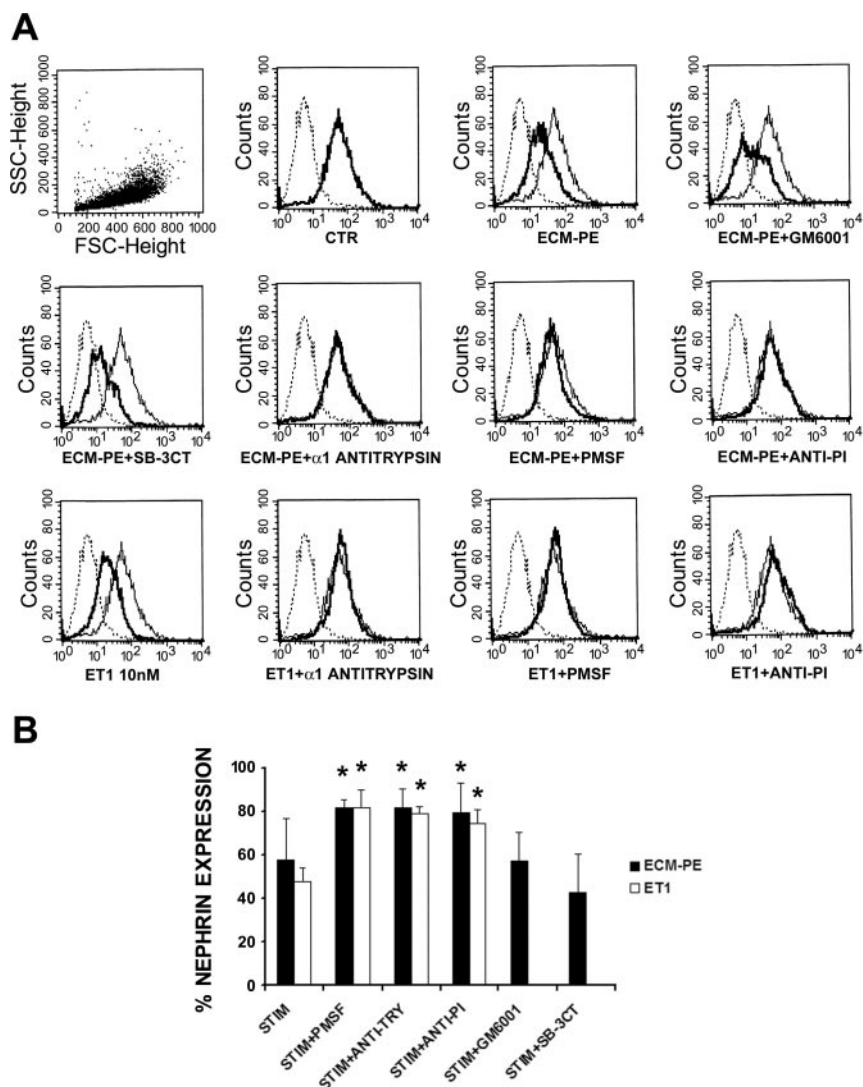
Fig. 8. Serine protease inhibitors block nephrin reduction induced by ECM-PE. *A*: representative flow cytometric panels of nephrin expression by podocytes untreated or treated with ECM-PE or ET-1 in the absence or presence of 3 different serine protease inhibitors [PMSF, α_1-anti-trypsin, and anti-protease inhibitor (PI)] or 2 specific metalloprotease inhibitors (GM6001 and SB-3CT). Thin lines represent unstimulated podocytes (positive control), dotted lines represent the corresponding isotype control antibody, and thick lines represent stimuli Treatment with serine proteases but not with metalloproteases inhibitors prevented the reduction in nephrin loss. *B*: semiquantitative analysis of nephrin expression by podocytes stimulated with ECM-PE (filled bars) or ET-1 (open bars). Values are means ± SD of 5 experiments expressed as % of nephrin expression. *$P < 0.05$ vs. ECM-PE and ET-1 alone.

Podocyte treatment with anti-PI (5–10 µg/ml) and α1-anti-trypsin (5 µg/ml) (5) before ECM-PE addition induced a marked inhibition of nephrin loss (Fig. 8), suggesting that nephrin shedding is mediated by specific serine proteases activated in podocytes after ECM-PE stimulation.

Moreover, podocytes pretreated with PMSF, α1-anti-trypsin, and anti-PI before recombinant ET-1 stimulus showed the same reduction of nephrin loss (Fig. 8), confirming that the effect of ET-1 on nephrin shedding was mediated by serine protease release.

DISCUSSION

In the present study, we showed that sera as well as plasmas from patients with PE downregulated the expression of nephrin by inducing ET-1 release from endothelial cells. The loss of nephrin from the podocyte membrane induced by ET-1 was associated with cytoskeletal activation and due to protease activation, leading to its cleavage from the cell membrane and shedding into the cell supernatant.

Increased maternal vascular permeability and enhanced vasoconstriction are major underlying pathophysiological events in PE (17, 38). Clinically, proteinuria, and interstitial edema

are mainly considered manifestations of endothelial dysfunction. In this context, a number of studies have confirmed that sera from PE women cause endothelial cell activation (2, 37). However, no information is present on the effect of PE factors on the slit diaphragm of podocytes. We and others recently showed that nephrin expression is reduced in glomeruli of patients with primary acquired nephritic syndrome, including membranous glomerulonephritis (GN), minimal-change GN, and focal segmental glomerulosclerosis, as well as in diabetic patients with proteinuria (9–11). In some, but not in all, of these diseases, the altered expression of slit diaphragm proteins is related to primary mutations. It is debated whether the loss of nephrin and related proteins is the cause or the consequence of proteinuria; however, experimental studies of nephrin redistribution induced by antibodies (44) suggest that a cytoskeleton-dependent shedding of nephrin may be instrumental in inducing proteinuria. Although a severe foot process effacement has not been documented in PE, a recent report showed that the expression of the glomerular proteins of the slit membrane nephrin and synaptopodin was reduced in patients with PE (13). Sugimoto et al. (43) in an experimental model of PE based on VEGF blockade found occasional but significant

damage in podocytes. Recently, urinary podocyte excretion has been reported in PE with a predictive value (14). Taken together, these data indicate that an alteration of slit diaphragm proteins could also be relevant in the pathogenesis of proteinuria in PE. In the present study, we showed that PE sera did not exert a direct effect on nephrin loss from the podocyte surface. We therefore investigated the possibility that sera and plasma from patients with PE stimulate the release of endothelial factors able to cross the basal membrane and to affect nephrin expression on podocytes. Indeed, endothelial dysfunction has a primary role in the pathogenesis of PE (17, 38). Recent studies indicate that decrease in free VEGF due to the presence of soluble VEGFR-1 (sFlt-1) in the sera of PE patients may account for such endothelial dysfunction (25). Moreover, the infusion of sFlt-1 in pregnant rats induced hypertension, proteinuria, and glomerular endotheliosis (43). In the present study, we found that VEGF blockade did not directly induce nephrin loss from podocytes. Indeed, podocytes do not express the VEGF receptor (12, 41); thus it is conceivable that the in vivo observed effect of VEGF blockade on podocytes may be indirect (43). In support of an indirect mechanism of proteinuria, we found that sera from PE patients stimulated the release of an endothelial factor/s able to trigger the loss of nephrin and synaptopodin from cultured human podocytes. We previously demonstrated that angiotensin II is able to induce nephrin loss (11). However, inhibitors of the angiotensin II receptor did not prevent the effect of the endothelial factor/s elicited by PE sera. Searching for the possible mediator, we found that ET-1 was responsible for the loss of nephrin as this was abrogated by an ET-1 receptor inhibitor. ET-1, a potent endogenous vasoconstrictor peptide, has been previously shown to be released from endothelial cells in PE (29). Indeed, sera from pregnant rats with decreased uterine perfusion induced ET-1 release from endothelial cells (39). In our study, we found that PE sera induced ET-1 production by glomerular endothelial cells and that the administration of recombinant ET-1 to podocytes, which are known to express the ET-1 receptor (26, 27, 33), triggered the loss of nephrin. Moreover, we found that VEGF blockade stimulated production of ET-1 by endothelial cells that, in turn, may induce nephrin and synaptopodin loss from podocytes. The data of the present study indicate that in PE circulating factors, such as sFlt1 or cytokines, may cause an endothelial activation able to locally affect podocyte homeostasis.

The loss of nephrin was not due to an inhibition of its synthesis, but rather to shedding from the cell surface. A similar reduction of surface expression was observed for synaptopodin, but not for podocin, which presents an intracellular NH_2 and COOH terminal (19, 20). The shedding of surface molecules is known to depend on their redistribution on the plasma membrane and the cleavage of their extracellular portion. Several mediators were reported to induce nephrin shedding by activation of the cell cytoskeleton and by cleavage of the extracellular domain (3, 10, 11). In PE, this mechanism is probably shared by ET-1, which was able to induce nephrin loss and in parallel acted on cytoskeletal redistribution. This suggests that the activation of the cell cytoskeleton modifies surface expression of nephrin, thus allowing its dislocation from the 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 (40, 47). It has been previously shown that Neph2, a slit diaphragm component, is cleaved from the podocyte surface by metalloproteases (16). However, in our experimental condition, nephrin shedding was not dependent on metalloprotease, but rather on serine protease activation.

In conclusion, this study identifies a mechanism of nephrin loss that may account for the enhanced glomerular permeability in PE. A serum factor or factors present in PE patients trigger the production of ET-1 from glomerular endothelial cells and ET-1 in turn may induce shedding of nephrin from the surface of podocytes. These results suggest that interfering with ET-1 or nephrin shedding may inhibit proteinuria.

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