Cardiovascular, Pulmonary and Renal Pathology

In Response to Protein Load Podocytes Reorganize Cytoskeleton and Modulate Endothelin-1 Gene

Implication for Permselective Dysfunction of Chronic Nephropathies

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Effacement of podocyte foot processes occurs in many proteinuric nephropathies and is accompanied by rearrangement of the actin cytoskeleton. Here, we studied whether protein overload affects intracellular pathways, leading to cytoskeletal architecture changes and ultimately to podocyte dysfunction. Mouse podocytes bound and endocytosed both albumin and IgG via receptor-specific mechanisms. Protein overload caused redistribution of F-actin fibers instrumental to up-regulation of the prepro-endothelin (ET)-1 gene and production of the corresponding peptide. Increased DNA-binding activity for nuclear factor (NF)- κ B and Ap-1 nuclear proteins was measured in nuclear extracts of podocytes exposed to excess proteins. Both Y27632, which inhibits Rho kinase-dependent stress fiber formation, and jasplakinolide, an F-actin stabilizer, decreased NF- κ B and Ap-1 activity and reduced ET-1 expression. This suggested a role for the cytoskeleton, through activated Rho, in the regulation of the ET-1 peptide. Focal adhesion kinase (FAK), an integrin-associated nonreceptor tyrosine kinase, was phosphorylated by albumin treatment via Rho kinase-triggered actin reorganization. FAK activation led to NF- κ B- and Ap-1-dependent ET-1 expression. These data suggest that reorganization of the actin cytoskeletal network in response to protein load is implicated in modulation of the ET-1 gene via Rho kinase-dependent FAK activation of NF- κ B and Ap-1 in

differentiated podocytes. Increased ET-1 generation might alter glomerular permselectivity and amplify the noxious effect of protein overload on dysfunctional podocytes. (Am J Pathol 2005, 166:1309–1320)

Glomerulosclerosis, key lesion of progressive renal disease, consists of extracellular matrix accumulation and progressive obliteration of glomerular capillaries with loss of glomerular filtration capacity. Permissive factors include high intraglomerular capillary pressure, hypertrophy, and the filtration of excess amounts of plasma proteins across the capillary barrier.^{1–6} A crucial component of the glomerular filter is the podocyte, a highly specialized epithelial cell endowed with foot processes. Podocytes possess a contractile structure, composed of actin and associated proteins and connected to the glomerular basement membrane at focal contacts via $\alpha_3\beta_1$ integrin, that stabilizes glomerular architecture by counteracting the distension of the glomerular basement membrane.^{7,8} The contractile apparatus of the foot processes responds to vasoactive hormones to control glomerular capillary surface area and in turn ultrafiltration coefficient.

Recent experimental and clinical evidence seems to imply an important role of podocytes in the pathophysiology of glomerular damage and progressive renal dysfunction.^{9–14} In this context, repeated injections of albumin in rats are followed by glomerular epithelial cell swelling, cytoplasmic protein droplets in podocytes, and extensive foot process effacement. Such events culminate in podocyte detachment from the basement mem-

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brane.¹⁵ Evidence of a causal link between podocyte protein deposition and progressive damage rests on the demonstration that in rats with renal mass reduction protein accumulation in podocytes preceded dedifferentiation and injury, documented as loss of synaptopodin and increase in desmin expression.¹⁶ Podocyte abnormalities were accompanied by transforming growth factor- β mRNA up-regulation. Concomitantly, *in vitro* experiments indicated that albumin overload in cultured podocytes caused loss of the synaptopodin differentiation marker, and enhanced transforming growth factor- β 1 mRNA and protein.¹⁶ Whether protein overload also affects the generation of other mediators of renal damage in podocytes is ill defined.

Endothelin-(ET) 1, a highly potent vasoconstrictor peptide,¹⁷ has been implicated in the pathogenesis of glomerulosclerosis¹⁸ by virtue of its action on cell proliferation, chemotaxis, and extracellular matrix accumulation.¹⁹ Among renal cells, glomerular epithelial cells constitutively express preproET-1 mRNA and synthesize the mature peptide^{20,21} whose generation is markedly up-regulated by transforming growth factor- β , C5b-9, and thrombin.²⁰ Stringent control of ET-1 gene expression is achieved through a highly regulated promoter containing consensus sequences for the binding sites of the nuclear factor-1, the activating protein-1 (Ap-1), dimers Jun-Fos, GATA-2, and nuclear factor (NF)- κ B.^{22–24} Activating protein-1 transcriptional activation is regulated by Rho-related small GTPases²⁵ involved in the remodeling of actin cytoskeleton.^{26,27} Finding that overexpression of dominant-negative mutants of RhoA and RhoB led to a significant reduction in pre-pro-ET-1 promoter activity indicates that Rho proteins modulate basal expression of ET-1 gene in endothelial cells.²⁸ No evidence is available as for the regulation of ET-1 gene transcription and related intracellular mechanisms in podocytes.

Here we test the hypothesis that protein overload alters the F-actin-based contractile podocyte apparatus resulting in modulation of ET-1 gene expression and production of the vasoactive peptide. We also provide evidence for relevant intracellular signaling evoked by cytoskeletal changes ultimately leading to ET-1 gene expression.

Materials and Methods

Cell Culture and Incubation

Immortalized mouse podocytes were grown according to the method described by Mundel and colleagues.²⁹ Briefly, cells were cultured under growth-permissive conditions on rat tail collagen type I-coated plastic dishes (BD Bioscience, Bedford, MA), at 33°C in RPMI 1640 medium (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Invitrogen), 10 U/ml mouse recombinant γ -interferon (Sigma Chemical Co., Saint Louis, MO), and 100 U/ml penicillin plus 0.1 mg/ml streptomycin (Sigma). To induce differentiation, podocytes were maintained in nonpermissive conditions at 37°C without γ -interferon for 14 days and used for the experiments. In this culture condition, cells stopped proliferating

and were identified as differentiated podocytes by their arborized morphology and the presence of high levels of synaptopodin, using indirect immunofluorescence microscopy. Cells were routinely maintained for 24 hours in serum-free medium before all of the experiments.

Experimental Design

We first addressed whether albumin and IgG bind to podocytes through a receptor-mediated mechanism. Binding and uptake studies were performed as previously described³⁰ using human serum albumin (HSA, low endotoxin; Sigma) labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) or fluorescein isothiocyanate (FITC)-conjugated human IgG (IgG, Sigma). To investigate the effect of protein overload on F-actin cytoskeletal rearrangement, confluent differentiated podocytes were exposed for 30 minutes, and 1, 2, 6, 24, and 48 hours to medium alone or in the presence of 10 mg/ml of HSA or IgG (6 and 24 hours) (Sigma). Then cells were fixed and processed for immunofluorescence studies. Synaptopodin expression was evaluated by immunofluorescence experiments in podocytes challenged for 6 and 24 hours with HSA or IgG. The concentration of HSA and IgG was selected on the basis of previous experiments of ours and other investigators on podocytes¹⁶ and tubular cells.^{31–33} Because phenotypic changes induced by protein load have been found duration-dependent,³⁴ the relatively high concentration of plasma proteins in our short-term culture system would simulate the actual total amount of proteins handled by the cells in a chronic pathological condition.

The expression of ET-1 gene was evaluated in differentiated podocytes exposed to 10 mg/ml of albumin for different time intervals by Northern blot analysis and real-time polymerase chain reaction (PCR). Podocytes challenged with IgG (10 mg/ml) for 24 hours were also studied (real-time PCR). The time course of ET-1 protein synthesis was assessed by radioimmunoassay (RIA) in supernatants of podocytes exposed to HSA.

To study the possible role of cytoskeleton in the regulation of ET-1 gene in podocytes laden with proteins, cells were treated with Y27632 (10 μ mol/L; Calbiochem, La Jolla, CA), a specific inhibitor of Rho kinase pathway involved in stress fiber formation,³⁵ or jasplakinolide (200 nmol/L; Molecular Probes, Cambridge, UK), an F-actin stabilizer,³⁶ 30 minutes prior and during 3, 6, 15, and 24 hours of incubation with albumin, taken as representative protein. Then ET-1 mRNA transcript and protein levels were measured.

The activation of the transcription factors NF- κ B and Ap-1 was investigated in podocytes exposed to medium alone, HSA, or IgG (10 mg/ml, 30 minutes). The effect of the cytoskeleton inhibitors, Y27632 and jasplakinolide, on DNA-binding activity of these transcription factors was also assessed. To elucidate the role of focal adhesion kinase (FAK), an integrin-associated nonreceptor tyrosine kinase, in HSA-induced ET-1 expression, we first investigated the phosphorylation of FAK by Western blot in podocytes treated for 5 minutes, 30 minutes, and 1, 2,

3, and 6 hours with HSA (10 mg/ml). We then assessed the impact of cytoskeleton rearrangement on FAK activation by studying the effect of Y27632 or jasplakinolide in podocytes exposed for 30 minutes to HSA. Next, functional blockade of FAK with genistein (25 μ mol/L, Calbiochem)³⁷ on NF- κ B and AP-1 activation was investigated. Finally, the effect of FAK inhibition on ET-1 expression was evaluated by studying the effect of genistein, an inhibitor of tyrosine kinases, or the transfection with a recombinant adenovirus encoding FAK-related nonkinase (Ad-FRNK)³⁸ in podocytes challenged with HSA for 3 hours. To understand the functional significance of increased ET-1 production we evaluated the effect of exogenous ET-1 (100 nmol/L, Sigma),³⁹ added to podocytes for 2, 6, and 15 hours, on F-actin distribution.

Albumin Labeling

Ten mg of human albumin (Sigma) were dissolved in 1 ml of buffer carbonate, pH 8.5, added to a solution of 1 mg of FLUOS in dimethylsulfoxide (Sigma). Labeled HSA was separated from unbound material by gel chromatography using Sephadex G50 column (Pharmacia Fine Chemicals, Uppsala, Sweden) pre-equilibrated and eluted with carbonate buffer. The eluted fraction was analyzed with a spectrometer, UV/Vis at the wavelengths of 280 and 486 nm, and final concentration of albumin was determined by Coomassie blue G dye-binding method.

Binding and Uptake Studies

HSA Binding and Uptake

For binding experiments, podocytes were grown on collagen-coated plastic Petri dishes and used 14 days after seeding. The cells were maintained for 24 hours in serum-free condition and then washed with Ringer's solution, pH 6.0, at 4°C to remove proteins or amino acids. Binding inhibition studies were performed incubating podocytes with Ringer's solution, pH 6.0, containing 50 μ g/ml of FLUOS-HSA on ice for 15 minutes in the absence or presence of increasing concentrations of cold HSA (0 to 10 mg/ml). Unbound FLUOS-HSA was removed by washing with Ringer's solution, pH 7.4. Cells were lysed in 10 mmol/L MOPS solution (morpholinopropanesulfonic acid, Sigma) containing 0.1% Triton X-100 and the cell-associated fluorescence was measured by spectrofluorometer. Protein content was determined using the BCA Protein Assay Reagent kit (Pierce, Rockford, IL) with bovine serum albumin as standard. Binding was expressed as mg/g protein.

For uptake studies, podocytes were grown on collagen-coated glass coverslips for 14 days. The monolayers were maintained for 24 hours in serum-free condition and then washed with Ringer's solution at pH 6.0. Podocytes were incubated in Ringer's solution, pH 6.0, containing 50 μ g/ml of FLUOS-HSA with or without 5 mg/ml of unlabeled HSA at 37°C for 3 hours. At the end of incubation the cells were washed with Ringer's solution, pH 7.4, and

fixed with 2% paraformaldehyde and 4% sucrose for 10 minutes at 37°C. The fixed monolayers were mounted in 1% *N*-propyl-gallate in 50% glycerol and 0.1 mol/L Tris-HCl, pH 8, and photographs were taken using immunofluorescence microscopy.

IgG Binding and Uptake

Podocytes were grown on collagen-coated coverslips for 14 days and maintained for 24 hours in serum-free conditions. For binding studies the cells were washed with Ringer's solution at pH 6.0 and incubated with human FITC-IgG (Sigma), 50 μ g/ml, with or without 5 mg/ml of unlabeled human IgG (Sigma) at 4°C for 1 hour. Uptake was performed by incubating the monolayers for 3 hours at 37°C with the concentration of FITC-human IgG and unlabeled IgG used for binding. At the end of the incubation the cells were washed and processed as described for HSA uptake.

Fluorescence Confocal Microscopy

Podocytes plated on collagen type I-coated glass coverslips were maintained in nonpermissive conditions for 14 days and incubated with medium alone, HSA, IgG, or ET-1 for different time intervals. At the end of incubation, cells were fixed in 2% paraformaldehyde plus 4% sucrose in phosphate-buffered saline (PBS), pH 7.4, for 10 minutes at 37°C, and then permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 4 minutes at room temperature. After three washings with PBS, nonspecific binding sites were saturated in blocking solution (2% fetal bovine serum, 2% bovine serum albumin, 0.2% bovine gelatin in PBS) for 30 minutes at room temperature. Podocytes were incubated with mouse monoclonal antibody anti-synaptopodin (undiluted; Progen Immunodiagnostica, Heidelberg, Germany) for 1 hour at room temperature, washed, and then incubated with FITC-conjugated goat anti-mouse antibody (30 μ g/ml; Jackson ImmunoResearch Laboratories, West Grove, PA). Negative control experiments with secondary antibody alone were performed. For F-actin staining, fixed and permeabilized cells were incubated with rhodamine-phalloidin, 20 U/ml, for 45 minutes (Molecular Probes Inc., Eugene, OR); negative control experiments without rhodamine-phalloidin were performed. For double labeling of F-actin and ZO-1, cells were preincubated overnight with polyclonal rabbit anti-ZO-1 antibody (10 μ g/ml; Zymed Laboratories, San Francisco, CA) followed by rhodamine phalloidin. Coverslips were washed and mounted in 1% *N*-propyl-gallate in 50% glycerol, 0.1 mol/L Tris-HCl, pH 8, and examined using inverted confocal laser microscopy (LSM 510 meta; Zeiss, Jena, Germany). Representative fields were digitalized with millions of colors and printed.

Northern Blot Analysis

Total RNA was isolated from podocytes by the guanidium isothiocyanate/cesium chloride procedure. Fifteen μ g of total RNA was then fractionated on 1.2% agarose gel and

blotted onto synthetic membranes (Zeta-probe; Bio-Rad, Richmond, CA). ET-1 mRNA was detected by using a 319-bp fragment of rat ET-1 cDNA. The probes were labeled with α - 32 P dCTP by random-primed method. Hybridization was performed overnight at 60°C in 0.25 mol/L Na_2HPO_4 , pH 7.2, and 7% sodium dodecyl sulfate. Filters were washed twice for 30 minutes with 20 mmol/L Na_2HPO_4 , pH 7.2, and 5% sodium dodecyl sulfate and twice for 10 minutes with 20 mmol/L Na_2HPO_4 , pH 7.2, and 1% sodium dodecyl sulfate at 60°C. Membranes were subsequently probed with β -actin cDNA, taken as internal standard of equal loading of the samples on the membrane. Expression of ET-1 mRNA was corrected for β -actin expression and quantified densitometrically.

Quantitative Real-Time PCR

Total RNA was extracted from podocytes by the guanidium isothiocyanate/cesium chloride procedure. Contaminating genomic DNA was removed by RNase-free DNase (Promega, Ingelheim, Germany) for 1 hour at 37°C. The purified RNA (1 μ g) was reverse-transcribed using random hexamers (50 ng) and 200 U of SuperScript II RT (Life Technologies, San Giuliano Milanese, Italy) for 1 hour at 42°C. No enzyme was added for reverse transcriptase-negative controls.

Real-time PCR was performed with the ABI Prism 5700 sequence detection system (PE Biosystems, Warrington, UK) using heat-activated TaqDNA polymerase (Amplitaq Gold, PE Biosystems). The TaqMan PCR reagents kit was used according to the manufacturer's protocol. After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. Ct, or threshold cycle, is used for relative quantification of the input target number. The comparative Ct method normalizes the number of target gene copies to a housekeeping gene such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Δ Ct). Gene expression was then evaluated by the quantification of cDNA corresponding with the target gene relative to a calibrator sample serving as a physiological reference (eg, untreated cells, $\Delta\Delta$ Ct). On the basis of exponential amplification of target gene as well as calibrator, the amount of amplified molecules at the threshold cycle is given by: $2^{-\Delta\Delta\text{Ct}}$. The following specific primers (300 nmol/L) were used: mouse ET-1: sense, 5'-AACTACGAAGGTTGGAGGCCA; anti-sense, 5'-CACGAAAAGATGCCTTGATGC; GAPDH sense, 5'-TCATCCCTGCATCCACTGGT; anti-sense, 5'-CTGGGATGACCTTGCCAC. All primers were obtained from Sigma Genosys (Cambridgeshire, UK).

Radioimmunoassay

ET-1 production was assayed in podocyte supernatants by radioimmunoassay (RIA). Either standard compounds or unknown samples (100 μ l) were mixed with 100 μ l of antiserum (Peninsula Laboratories Inc., Belmont, CA) diluted in phosphate buffer, pH 7.2 (RIA buffer), at a final dilution of 1:72,000. The reaction mixture was incubated

for 24 hours at 4°C, then 15,000 cpm of (125 I) ET-1 in 100 μ l was added and the incubation prolonged for 24 hours at 4°C. Separation of free from antibody-bound (125 I) ET-1 was achieved by addition of a second antibody (500 μ l of immunoprecipitating mixture consisting of a sheep anti-rabbit IgG and polyethylene glycol) for 30 minutes at room temperature. Finally, 500 μ l of RIA buffer was added to stop the reaction, and the immunoprecipitates were centrifuged at $5000 \times g$ for 30 minutes. Supernatants were discarded and pellet radioactivity detected by gamma counter (Beckman, Irvine, CA). Results were expressed as pg/ 10^6 cells. The minimum detectable concentration was 0.4 pg/tube. Nonspecific binding did not exceed 2% of total radioactivity. The cross-reactivity of the antibody with other endothelins is as follows: endothelin-2, 46.9%; endothelin-3, 17%; and big endothelin-1, 9.4%. Intra-assay and interassay variability averaged 10% and 12%, respectively, throughout a range between 0.4 and 100 pg/tube.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from podocytes with the NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce/Celbio, Pero, Italy) according to the manufacturer's instructions. To minimize proteolysis, all buffers contained protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). The protein concentration was determined by the Bradford assay using the Bio-Rad protein assay reagent.

EMSAs were performed as previously described⁴⁰ with the kb DNA sequence of the immunoglobulin gene (5'-CCGGTCAGAGGGGACTTTCCGAGACT) and consensus binding site for Ap-1 (5'-CGCTTGATGACTCAGCCG-GAA). Nuclear extracts (3 μ g) were incubated with 50 kcpm of 32 P-labeled oligonucleotide in a binding reaction mixture [10 mmol/L Tris-HCl, pH 7.5, 80 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L dithiothreitol, 5% glycerol, 1.5 μ g of poly (dl-dC)] for 30 minutes on ice. In competition studies, a 100-fold molar excess of unlabeled oligonucleotide was added to the binding reaction mixture before the addition of the labeled NF- κ B or Ap-1 probes. For densitometric analysis the volume density for each band was determined in arbitrary units. The sum of the volume density of bands for a single sample was used as an indirect measure of NF- κ B or Ap-1 activation and expressed as a fold increase of the mean densitometry of respective control (represented as 1).

Western Blot Analysis

Podocytes were lysed in the lysis buffer: 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 2 mmol/L ethylenediamine tetraacetic acid, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerophosphate, plus phosphatase inhibitors 1 mmol/L Na_3VO_4 , 50 mmol/L NaF, and protease inhibitors 1 mmol/L phenylmethyl sulfonyl fluoride and 1 μ g/ml leupeptin. Protein

Figure 1. Binding and uptake of HSA and human IgG on differentiated podocytes. **Top:** Binding studies. **a:** Confluent differentiated podocytes were incubated with 50 μ g/ml of FLUOS-HSA with or without increasing concentrations of cold HSA. Complete inhibition of FLUOS-HSA binding by unlabeled HSA showed that binding was specific and not due to an interaction of FLUOS-HSA with plasma membrane ($n = 5$ experiments). Data are expressed as mean $\pm$ SE. *, $P < 0.05$ versus 0 mg/ml HSA. Representative images of binding of 50 μ g/ml of FITC-IgG on podocytes in the absence (**b**) or presence of 5 mg/ml of cold IgG (**c**). **Bottom:** Uptake studies. Cellular staining of 50 μ g/ml of FLUOS-HSA incubated without (**d**) or with (**e**) 5 mg/ml of unlabeled HSA. Endocytosis of 50 μ g/ml of FITC-IgG in podocytes in the absence (**f**) or presence (**g**) of 5 mg/ml of unlabeled IgG. Original magnifications, $\times 600$.

concentration was determined by protein assay based on bicinchoninic acid color formation (Pierce, Milan, Italy). Proteins (30 μ g) were separated on 7.5% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature

Figure 2. Reorganization of F-actin cytoskeleton in podocytes exposed to protein overload. Confluent differentiated podocytes exhibited a pattern of F-actin filaments distributed as stress fiber-like bundles along the axis of the cells after 6 hours (**a**) incubation with medium alone. Redistribution of F-actin fibers to the periphery of the cells was observed in podocytes exposed to HSA already at 30 minutes (**b**) that was maintained at 1 (**c**), 2 (**d**), 6 (**e**), and 24 hours (**f**). A similar effect was observed after podocyte exposure to IgG for 6 and 24 hours (**g**, **h**). Original magnifications, $\times 600$.

with PBS containing 5% bovine serum albumin (for p-FAK detection) or 5% nonfat dry milk (for FAK detection) and 0.1% Tween 20, and then incubated overnight at 4°C with the following primary antibodies (Biosource Europe, Nivelles, Belgium): rabbit polyclonal anti-FAK (pY397) (1:1000); rabbit polyclonal anti-FAK (1:1000). After incubation with the secondary antibodies, horseradish peroxidase-conjugated goat anti-IgG rabbit (Sigma) diluted 1:10,000, for 1 hour at room temperature in PBS with 1% bovine serum albumin or 1% nonfat dry milk and 0.1% Tween 20, proteins bands were detected by Supersignal chemiluminescent substrate (Pierce).

Figure 3. Double immunolabeling for F-actin and ZO-1 in podocytes challenged with HSA. Immunofluorescence images of F-actin and ZO-1 in podocytes exposed to medium alone (**a**) or HSA for 6 hours (**b**) indicate that protein overload rearranged F-actin fibers at the periphery of the cells as outlined by ZO-1 staining (**b**). Original magnifications, $\times 1000$.

Adenoviral Constructs and Transfection Experiments

A replication-defective adenovirus encoding FAK-related nonkinase (Ad-FRNK) was constructed using a 1.2-kb wild-type chick FRNK cDNA (the gift of Dr. J.T. Parsons, Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, VA).³⁸ For adenoviral infection, podocytes were incubated with Ad-FRNK or Ad-null at a multiplicity of infection of 50 in RPMI 1640 without serum for 3 hours at 37°C. The adenoviruses were washed off and cells maintained in serum-free medium for 24 hours. Then, podocytes were exposed to HSA (10 mg/ml) for 3 hours and processed for ET-1 mRNA expression. Transfection did not affect cell viability.

Statistical Analysis

Results are expressed as mean $\pm$ SE. Statistical analysis was performed using Student's *t*-test, one-way analysis of variance, followed by Tukey test for multiple comparisons, and Kruskal Wallis test, as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Binding and Uptake of Albumin and IgG in Differentiated Mouse Podocytes

We investigated binding and uptake of HSA and IgG in differentiated mouse podocytes. FLUOS-HSA was displaced by cold HSA in a concentration-dependent manner at 4°C, indicating the presence of saturable binding sites for albumin on the podocyte surface (Figure 1a). Similarly, podocytes exposed to FITC-IgG showed (Figure 1b) a specific binding of the protein, with a granular pattern on the apical surface of podocytes that was completely displaced by cold IgG (Figure 1c). HSA and IgG endocytosis into podocytes was detected after 3 hours of exposure at 37°C to FLUOS-HSA or FITC-IgG (Figure 1, d and f, respectively). Uptake was markedly inhibited by an excess of both unlabeled proteins (Figure 1, e and g), suggesting a receptor-mediated endocytosis of HSA and IgG in podocytes.

Effect of Protein Overload on F-Actin Cytoskeleton and Synaptopodin Distribution in Differentiated Podocytes

Differentiated podocytes maintained for 14 days in non-permissive culture conditions exhibited, when confluent, a pattern of F-actin filaments distributed as stress fiber-like bundles along the axis of the cells or into the process of arborized cells, as visualized by confocal microscopy (Figure 2a). Albumin induced a marked redistribution of F-actin fibers toward the periphery of the cells already after 30 minutes (Figure 2b), which was maintained thereafter (Figure 2; c to f) until 48 hours (data not shown). A similar effect was observed after podocyte challenge with IgG (Figure 2, g and h). To demonstrate the peripheral distribution of F-actin fibers induced by excess proteins, double immunostaining of actin and ZO-1, a cell membrane marker that defines podocyte outline, was performed. Figure 3 shows that HSA induces actin rearrangement at the cell periphery in the vicinity of ZO-1 at

Figure 4. Expression of synaptopodin in podocytes exposed to HSA or IgG. **a:** High level of synaptopodin in a punctate pattern was observed along actin filament in unstimulated cells. After 6 hours of exposure of podocytes with HSA (**b**) or IgG (**c**) at the concentration of 10 mg/ml, synaptopodin was markedly reduced in respect to control cells. Original magnifications, $\times 600$.

Figure 5. ET-1 mRNA expression in podocytes exposed to protein overload. **Top:** Northern blot experiments were performed using total RNA isolated from podocytes exposed to medium alone (control) or albumin (10 mg/ml) for 6, 24, and 48 hours. The results are representative of four independent experiments. **Bottom:** Densitometric analysis of autoradiograph signals for ET-1. The optical density of the autoradiographic signals was quantified and calculated as the ratio of ET-1 to β -actin mRNA. Results (mean $\pm$ SE) are expressed as fold increase greater than control (considered as 1) in densitometric arbitrary units. °, $P < 0.01$ versus control.

the expense of transcytoplasmic filaments (Figure 3b). Cytoskeletal changes were accompanied by loss of staining for synaptopodin, an F-actin-associated protein, considered a specific marker of podocyte differentiation.⁴¹ As shown in Figure 4, unstimulated cells revealed high levels of synaptopodin in a punctate pattern along the actin filaments and at focal contact. Exposure of podocytes to HSA or IgG for 6 or 24 hours resulted in a similar marked reduction of synaptopodin staining.

Protein Overload Induces ET-1 Gene Expression and Protein via Cytoskeleton Rearrangement

Because reorganization of cytoskeletal network is functionally linked to modification of transcriptional events that regulate inflammatory and vasoactive genes, including ET-1,^{42–44} we studied the ability of a high concentration of plasma proteins to modulate the expression of ET-1 gene and protein in differentiated podocytes, and the potential role of cytoskeleton in such induction. By Northern blot, a 1.3-kb mRNA transcript specific for ET-1 was observed in unstimulated control podocytes (Figure 5). Albumin promoted after 6 hours of incubation a 1.5-fold increase in ET-1 transcript level as compared to control cells, which was further enhanced at 24 and 48

Figure 6. Effect of the cytoskeleton inhibitors Y27632 and jasplakinolide on albumin-induced ET-1 gene expression. Cells were treated with medium alone or with HSA (10 mg/ml) for 3, 6, 15, and 24 hours in the presence or absence of Y27632 (10 μ mol/L), an inhibitor of Rho kinase pathway, or jasplakinolide (200 nmol/L), an F-actin stabilizer. ET-1 mRNA was assessed by real-time PCR. The results shown are mean $\pm$ SE of five independent experiments. °, $P < 0.01$ versus control; *, $P < 0.05$; **, $P < 0.01$ versus HSA.

hours (2.5- and 2.2-fold, respectively) (Figure 5). The effect of albumin on ET-1 gene expression was consistent with data of real-time PCR experiments (Figure 6). Actually, ET-1 message levels increased within 3 hours of albumin exposure and was elevated at 6 and 15 hours with a peak at 24 hours ($P < 0.01$ versus unstimulated cells at all of the times considered). ET-1 mRNA overexpression was also observed when podocytes were challenged with IgG for 24 hours (1.5-fold increase greater than control, $P < 0.05$). As shown in Figure 6, exposure of podocytes to Y27632, a specific Rho kinase inhibitor involved in stress fiber formation, resulted in a significant inhibition of ET-1 gene expression at 3 and 6 hours. The effect was no more significant at 24 hours. Treatment with jasplakinolide, an F-actin stabilizer, normalized ET-1 mRNA expression until 15 hours. Thereafter, a less inhibitory effect was observed. The up-regulation of ET-1 mRNA was associated with a time-dependent increase of the native peptide secreted into cell supernatants (Figure 7). Unstimulated cells synthesized constitutively low levels of ET-1 protein. After HSA exposure, a significant increase in peptide secretion was found at 3 hours, which was more pronounced at 15 and 24 hours. Y27632 had an early inhibitory effect, resulting in the normalization of ET-1 secretion at 3 hours. This effect was less pro-

Figure 7. ET-1 production in podocytes exposed to albumin and effect of Y27632 and jasplakinolide. Podocytes were incubated with medium alone or with HSA (10 mg/ml) for 3, 6, 15, and 24 hours in the presence or absence of Y27632 (10 μ mol/L) and jasplakinolide (200 nmol/L). ET-1 production was measured in cell supernatants by RIA. Data are expressed as mean $\pm$ SE. °, $P < 0.01$ versus control; *, $P < 0.05$; **, $P < 0.01$ versus HSA; #, $P < 0.01$ versus HSA + Y27632.

Figure 8. Activation of NF- κ B and Ap-1 in protein-laden podocytes. NF- κ B and Ap-1 activity was evaluated by EMSA in nuclear extracts from podocytes exposed for 30 minutes to medium alone, HSA, or IgG (10 mg/ml). To demonstrate the specificity of binding of the NF- κ B and Ap-1 oligonucleotides, a 100-fold molar excess unlabeled (cold) nucleotides were used to compete with the labeled NF- κ B or Ap-1 probes for binding to nuclear proteins. Results are representative of three experiments.

nounced, although still significant until 6 hours. Podocytes exposed to jasplakinolide showed instead a long lasting inhibitory effect on ET-1 production, to the extent that a significant reduction over HSA still persisted at 24 hours (Figure 7).

Albumin and IgG Activate NF- κ B and Ap-1 in Podocytes via Cytoskeleton

The effect of HSA and IgG on NF- κ B and Ap-1 activation in podocytes is depicted in Figure 8. Nuclear extracts were assayed for NF- κ B and Ap-1 DNA binding activity using radiolabeled specific oligonucleotide probes. Unstimulated cells displayed two constitutive bands of NF- κ B: an upper complex and a faster migrating lower complex. Thirty minutes of incubation of podocytes either with albumin or IgG led to a substantial rise in NF- κ B-binding activity of the two complexes. A remarkable increase of the Ap-1-binding activity greater than control was detected after podocyte challenge with either protein. The specificity of binding reactions was confirmed in competition experiments by the ability of excess unlabeled (cold) NF- κ B or Ap-1 oligonucleotides to inhibit binding.

Next, we investigated whether the activation of NF- κ B and Ap-1 induced by protein overload could be regulated by intracellular signals evoked by cytoskeleton rearrangement. To this purpose, experiments were performed in podocytes exposed to albumin, taken as representative protein. As shown in Figure 9, densitometric analysis of five independent experiments revealed a 5.3-fold increase of NF- κ B activity greater than control. Treatment with Y27632 reduced by nearly 70% NF- κ B activation induced by albumin. A 58% inhibition was observed after jasplakinolide. Ap-1 DNA binding activity was significantly enhanced by albumin (2.2-fold greater than control) (Figure 9). In the presence of Y27632 and jasplakinolide, AP-1 activation was reduced by 30% and 44%, respectively.

Role of FAK in Protein Overload-Induced ET-1

Integrin-mediated activation of the nonreceptor tyrosine kinase FAK results in the propagation of intracellular signals that turn on transcriptional events. First, we studied

Figure 9. Effect of Y27632 and jasplakinolide on NF- κ B and Ap-1 activation induced by protein overload. **Top:** EMSA for NF- κ B and Ap-1 was performed in nuclear extracts from podocytes exposed for 30 minutes to medium alone or HSA (10 mg/ml), in the presence or absence of Y27632 (10 μ mol/L) and jasplakinolide (200 nmol/L). The results are representative of five independent experiments using different nuclear extracts. **Bottom:** Densitometric analysis of autoradiographic signals of NF- κ B and Ap-1. Results are mean $\pm$ SE. $^{\circ}$, $P < 0.01$ versus control; * , $P < 0.05$; and ** , $P < 0.01$ versus HSA.

the contribution of FAK in the regulation of ET-1 gene expression induced by HSA overload. By Western blot experiments, albumin-loaded podocytes exhibited a marked FAK phosphorylation at 5 minutes, that remained sustained until 6 hours (Figure 10a, left).

Next, to assess a possible involvement of cytoskeleton changes in FAK activation, we evaluated the effect of Y27632 or jasplakinolide in albumin-treated podocytes. As shown in Figure 10a (right), both compounds reduced FAK phosphorylation after 30 minutes of exposure with HSA, suggesting a downstream regulation of FAK by Rho kinase-triggered actin redistribution. We also observed that the tyrosine kinase inhibitor genistein markedly reduced NF- κ B- and Ap-1-binding activity (Figure 10b) in HSA-laden podocytes. As a consequence, ET-1 mRNA levels were significantly decreased by genistein in respect to HSA alone, as documented by real-time PCR assessment (Figure 10c). Transient overexpression of FAK-related nonkinase, an endogenous inhibitor of FAK activity, by adenoviral gene transfer interfered with ET-1 gene expression to the extent that ET-1 mRNA transcript levels were reduced by 50% compared to cells transfected with null gene.

Effect of Exogenous ET-1 on Podocyte Cytoskeleton

We then evaluated the functional effect of ET-1 on the distribution of F-actin. Figure 11 shows that in control cells actin stress fibers were arranged in parallel, whereas on exposure to ET-1 (100 nmol/L) for 2 and 6 hours, this pattern changed, leading to F-actin redistribution at the cell periphery, at the expense of transcytoplasmic microfilaments. The effect was transient and partially recovered at 15 hours. These data provide the first evidence that ET-1 may alter podocyte F-actin contractile

Figure 10. Role of FAK on ET-1 gene activation/expression in HSA-treated podocytes. **a: Left:** Activation of FAK in podocytes exposed to HSA (10 mg/ml) for 5 minutes, 30 minutes, and 1, 2, 3, and 6 hours. **a: Right:** Effect of Y27632 (10 μ mol/L) and jasplakinolide (jasp, 200 nmol/L) on FAK phosphorylation in podocytes treated for 30 minutes with HSA. Cell lysates were analyzed by Western blot using antibody against the phosphorylated form. The blots were stripped and reprobed with an antibody anti-nonphosphorylated FAK to confirm equal loading of the proteins on the gel. The blot is representative of three independent experiments. **b:** Effect of genistein on NF- κ B and Ap-1 activation induced by protein overload. EMSA for NF- κ B and Ap-1 was performed in nuclear extracts from podocytes exposed for 30 minutes to medium alone or HSA in the presence or absence of genistein (25 μ mol/L). The autoradiographs are representative of three independent experiments using different nuclear extracts. **c:** Effect of inhibition of FAK phosphorylation on ET-1 expression. **Left:** Cells were treated with medium alone or with HSA for 3 hours in the presence or absence of genistein. **Right:** Cells were transfected with a replication-defective adenovirus encoding FAK-related nonkinase (Ad-FRNK), an endogenous inhibitor of FAK activity or Ad-null, before HSA exposure. ET-1 mRNA was assessed by real-time PCR. *, $P < 0.01$ versus HSA + Ad-null.

apparatus relevant for the maintenance of glomerular permselectivity.

Discussion

Far from being a simple prognostic clinical parameter, proteinuria has been considered a pivotal causative factor for progressive tubular injury that precedes the relentless deterioration of renal function in progressive nephropathies.⁶ Proteins filtered in exuberant amounts as a consequence of the alteration of the size-selective function of the glomerular barrier in proteinuric conditions, have an intrinsic renal toxicity because of over-reabsorption by proximal tubular cells, which causes activation of tubular-dependent pathways of interstitial inflammation

Figure 11. Effect of ET-1 on cytoskeletal F-actin distribution in podocytes. Immunofluorescence staining of F-actin fibers in podocytes exposed for 2, 6, and 15 hours to control medium (**a**, **c**, **e**) or ET-1 (**b**, **d**, **f**). In unstimulated cells F-actin microfilaments were arranged in parallel, whereas on exposure to ET-1 (100 nmol/L) for 2 and 6 hours, this pattern changed leading to F-actin redistribution at the cell periphery. This effect partially recovered at 15 hours. Original magnifications, $\times 600$.

and fibrosis.^{6,45} Ultrafiltered plasma proteins also induce morphological changes in podocytes,⁵ which include reversible retraction and flattening of the epithelial foot processes, suggesting podocyte-GBM adhesion of critical relevance for a functional filter.^{14,46} Mechanisms and mediators underlying phenotypic changes in glomerular epithelium induced by protein overload are poorly defined.

In the present study, we found that albumin and IgG bind to cultured murine podocytes in a receptor-specific manner. Because of the recent demonstration of the presence of megalin,⁴⁷ which acts as a receptor for albumin and Ig light chain, on this very cell line, it is possible that in our setting albumin and IgG binding to the murine podocyte is mediated by megalin. The fact that megalin possesses endocytotic function in differentiated podocytes⁴⁷ would also suggest that albumin and IgG uptake, here documented, may occur through their binding to this receptor. Downstream pathway-transducing signals after protein binding to the receptors are currently poorly understood. It has been recently shown that the cytoplasmic carboxy-terminal NPXZ domain of megalin interacts with Disable protein (Dab) 2, an intracellular adaptor protein that is potentially involved in transmembrane signal transduction and regulates cellu-

Figure 12. Proposed pathway mediating protein overload-induced ET-1 expression. Ultrafiltered plasma proteins, by binding to specific receptors on podocyte surface, cause cytoskeleton rearrangement and new F-actin stress fiber formation via Rho kinase pathway. Rho kinase-triggered actin reorganization leads to FAK phosphorylation. FAK activation integrates signals from cytoskeleton to the downstream activation of NF- κ B and AP-1 which in turn translocate to the nucleus and promote pre-pro ET-1 gene transcription.

lar growth and differentiation.⁴⁸ Whether Dab 2 could transduce the signal after protein binding to megalin on podocytes is worth investigating.

Exposure of podocytes to albumin and IgG induces early phenotypic changes consisting of cytoskeleton F-actin rearrangement with marked decrease of synaptopodin staining. The ultimate effect of proteins on actin-associated synaptopodin would be taken as to indicate protein accumulation as a trigger of podocyte dedifferentiation, a phenomenon already described *in vivo* in areas of segmental sclerosis.^{49,50} Podocytes are highly differentiated cells that possess a complex contractile structure composed of F-actin microfilaments, most abundant in the foot process, exhibiting a high degree of organization.^{7,35,51,52} F-actin is connected with adaptor molecules that anchor the slit diaphragm proteins and α 3 β 1 integrins, transmembrane proteins that form focal adhesion complexes and mediate podocyte-GBM matrix interaction.^{7,46,52} Cytoskeleton F-actin rearrangement is closely associated with podocyte shape changes and dysfunction in disease.^{7,14,46} *In vitro*, actin filament disorganization affects cell adhesion to the extracellular matrix through α 3 β 1 integrin that functions as receptor-transducing signals. The consequent outside-in signals trigger specific cellular responses that lead to activation of gene expression of adhesive molecules, cytokines, and vasoactive mediators.^{42,53,54} Based on this evidence, we asked whether rearrangement of the cytoskeleton induced by protein overload was associated with up-regulation of the ET-1 gene. Our results indicate that ET-1

gene expression is efficiently enhanced by albumin and IgG load, in parallel with the generation of the protein, in a time-dependent manner, suggesting that glomerular epithelial cells in the proteinuric setting are an important source of ET-1. These data are in line with *in vivo* evidence in a murine model of protein overload that renal ET-1 transcript levels increased together with the renal synthesis of the peptide in concomitance with the development of podocyte structural damage.⁵⁵

Endothelin-1 secreted by podocytes may function in a paracrine manner on glomerular cells affecting the tone of the glomerular capillary, enhancing the vascular permeability, and stimulating mesangial cell contraction by virtue of its vasoconstrictor effects. Finding that the cytoskeleton stabilizer jasplakinolide significantly reduced expression and production of ET-1 would support the notion that protein-induced rearrangement of the actin cytoskeleton contributes to regulate ET-1. Furthermore, the addition of Y27632, an inhibitor of Rho kinases crucial for the formation of stress fibers and focal adhesions,^{26,27} also markedly decreased ET-1 expression indicating that ET-1 gene transcription is regulated by Rho-dependent pathway. In agreement with our findings are data that the Rho GTPase family modulates basal expression of preproET-1 transcript levels in vascular endothelial cells in culture.²⁸ Regulation of the preproET-1 gene is complex and has been attributed to multiple regulatory elements. It is well-known that human preproET-1 gene possesses in the promoter region consensus sequences for transcription factors including NF- κ B and AP-1.^{23,24} In the

present study, electrophoretic analysis of the nuclear extracts of podocytes loaded with albumin or IgG revealed a rapid increase in NF- κ B- and Ap-1 DNA-binding activities. Of interest, inhibition of cytoskeleton rearrangement by jasplakinolide and by Rho kinase inhibitor prevented NF- κ B and Ap-1 activation, indicating the presence of cytoskeleton-regulated signal transduction pathway of gene activation. These data are in line with previous observations of the involvement of Rho GTPase family in regulating NF- κ B and Ap-1 in genetically modified cultured fibroblasts.^{25,26,56}

We finally investigated the role of FAK, a cytoplasmic nonreceptor tyrosine kinase that localizes in integrin-extracellular matrix complexes,⁴⁶ as the effector molecule linking actin reorganization with transcriptional events. Evidence is available as to indicate that FAK activation is dependent on actin cytoskeleton.⁵⁷ Phosphorylation of FAK on Tyr397 is a docking site for activation of other tyrosine kinases such as the Src-family protein kinases⁵⁸ that transduce the signals from focal adhesions to intracellular targets resulting in gene activation. The present experiments showed that exposure of murine podocytes to albumin induced a rapid and sustained FAK phosphorylation that was substantially affected by the Rho kinase inhibitor and jasplakinolide. Inhibition of FAK activation with genistein, a tyrosine kinase inhibitor, markedly reduced NF- κ B and Ap-1 DNA-binding activity, and consequently inhibited ET-1 mRNA expression. Transfection experiments with an adenoviral construct encoding a replication-defective FAK-related nonkinase (FRNK),³⁸ further confirmed that FAK signaling contributes to ET-1 gene regulation to the extent that overexpression of FRNK resulted in a partial but significant inhibition of ET-1 mRNA expression in albumin-laden podocytes.

We finally addressed a possible effect of ET-1 on cultured podocytes that express on their surface-specific receptors for the peptide.^{59,60} Podocytes exposed to exogenous ET-1 exhibited rapid and consistent cytoskeletal changes, as revealed by the redistribution of F-actin filaments to the cell periphery. This result unravels a novel clue of the effect of ET-1 on kidney cells. So far data are available to indicate the pivotal role of ET-1 in the biology of mesangial cells, in that the peptide can elicit proliferation, hypertrophy, and contraction of the mesangium.¹⁹ In view of the fact that ET-1 acts as a local hormone whose effects are exerted in a paracrine as well as autocrine manner, it is possible that overproduction of ET-1 by podocytes may regulate the contractile status of either mesangial or glomerular epithelial cells.

In conclusion, we have demonstrated that in podocytes the abnormal uptake of plasma proteins induces Rho kinase-dependent F-actin cytoskeletal rearrangement leading to possible cell dedifferentiation. Such structural changes translate into the activation of FAK in turn responsible for NF- κ B- and Ap-1-dependent ET-1 gene up-regulation (Figure 12). Endothelin-1 overproduction may act on the podocyte contractile apparatus altering the glomerular capillary surface area thus leading to protein permeability dysfunction. These results indicate podocytes as a novel cellular target for the toxic effect of excess plasma ultrafiltered proteins.

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