Stretch reduces nephrin expression via an angiotensin II-AT₁-dependent mechanism in human podocytes: effect of rosiglitazone

Ilaria Miceli, Davina Burt, Elena Tarabra, Giovanni Camussi, Paolo Cavallo Perin, and Gabriella Gruden
Diabetic Nephropathy Laboratory, Department of Internal Medicine, University of Turin, Turin, Italy
Submitted 18 July 2008; accepted in final form 8 November 2009

Stretch reduces nephrin expression via an angiotensin II-AT₁-dependent mechanism in human podocytes: effect of rosiglitazone. Am J Physiol Renal Physiol 298: F381–F390, 2010. First published November 11, 2009; doi:10.1152/ajprenal.90423.2008.—Increased glomerular permeability to proteins is a characteristic feature of diabetic nephropathy (DN). The slit diaphragm is the major restriction site to protein filtration, and the loss of nephrin, a key component of the slit diaphragm, has been demonstrated in both human and experimental DN. Both systemic and glomerular hypertension are believed to be important in the pathogenesis of DN. Human immortalized podocytes were subjected to repeated stretch-relaxation cycles by mechanical deformation with the use of a stress unit (10% elongation, 60 cycles/min) in the presence or absence of candesartan (1 μM), PD-123319 (1 μM), and rosiglitazone (0.1 μM). Nephrin mRNA and protein expression were assessed using quantitative real-time PCR, immunoblotting, and immunofluorescence, and the protein expression of AT₁ receptor and angiotensin II secretion were evaluated. Exposure to stretch induced a significant ~50% decrease in both nephrin mRNA and protein expression. This effect was mediated by an angiotensin II-AT₁ mechanism. Indeed, podocyte stretching induced both angiotensin II secretion and AT₁ receptor overexpression, podocyte exposure to angiotensin II reduced nephrin protein expression, and both the AT-1 receptor antagonist candesartan and a specific anti-angiotensin II antibody completely abolished stretch-induced nephrin downregulation. Similar to candesartan, the peroxisome proliferator-activated receptor (PPAR)-γ agonist, rosiglitazone, also inhibited stretch-induced nephrin downregulation, suggesting interference with stretch-induced activation of the angiotensin II-AT₁ receptor system. Accordingly, rosiglitazone did not alter stretch-induced angiotensin II secretion, but it prevented AT₁ upregulation in response to stretch. These results suggest a role for hemodynamic stress in loss of nephrin expression and allude to a role of PPAR-γ agonists in the prevention of this loss.

diabetic nephropathy; proteinuria; AT₁ receptor

DIABETIC NEPHROPATHY (DN) is characterized by an abnormal increase in glomerular permeability to proteins (19). Hyperglycemia is a key determinant in the pathogenesis of the diabetic proteinuria (49). In addition, the antiproteinuric effect shown in both human and animal studies by treatment with angiotensin-converting enzyme (ACE) inhibitors (21, 26, 36, 46, 48) and angiotensin II receptor blockers (ARBs) (18) suggests a key role of the hemodynamic insult and/or the local renin-angiotensin system (1, 8).

Nephrin is a key component of the slit diaphragm (SD) (23, 40), a junction connecting foot processes of neighboring podocytes, which represents the major restriction site to protein filtration (38). Mutations of the gene encoding for nephrin are responsible for the congenital nephrotic syndrome of the Finnish type (4, 28, 31, 39), and loss of nephrin has been reported in various acquired proteinuric conditions (24). Nephrin down-regulation is also a feature of human DN and occurs in an early stage of the complication, suggesting a possible involvement in the development of albuminuria (5, 14). In vitro studies have shown that podocyte exposure to glycated albumin diminishes nephrin expression, providing a possible cellular mechanism for nephrin downregulation in diabetes (14). Podocytes are also a primary target for the mechanical insult induced by increased glomerular capillary hypertension (17, 29) and have an independent renin-angiotensin system (15, 32), but the effect of stretch, mimicking glomerular capillary hypertension, on nephrin expression is still unknown.

Recently, intervention studies in both diabetic and nondiabetic experimental nephrosis have shown that antihypertensive drugs, such as ACE inhibitors and ARBs (9, 35), and insulin-sensitizing agents of the peroxisome proliferator-activated receptor (PPAR)-γ ligand family (6, 10, 25) can both ameliorate proteinuria and prevent nephrin loss.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Flex I and Flex II plates were obtained from Flexcell International (McKeensport, PA), fetal calf serum (FCS) was acquired from Euroclone (Milan, Italy), and Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Invitrogen (Carlsbad, CA). RNeasy Mini spin columns and TAQ polymerase were purchased from Qiagen (Crawley, UK), and the reverse transcription system was obtained from Promega (Madison, WI). The guinea pig anti-human nephrin antibody was obtained from Progen (Heidelberg, Germany), and the anti-AT₁ receptor and anti-angiotensin II antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Cell Death Detection ELISA kit, oligonucleotide primers, synthetic double-stranded DNA for GAPDH, and nephrin were purchased from Roche Applied Science (Basel, Switzerland). The primers and probes used in real-time polymerase chain reaction (PCR) were obtained from Applied Biosystems. The DC-Colorimetric protein assay was from Bio-Rad (Milan, Italy), and the angiotensin II enzyme immunoassay kit was obtained from Spibio (Massay Cedex, France).

Culture of Podocytes

Primary cultures of human podocytes were established and characterized as previously described (13). Established lines of differentiated podocytes were obtained by infection of pure primary cultures with a hybrid Adeno5/SV40 virus (12). Phenotypic characterization of immortalized podocytes was performed according to cell morphology (polyhedral cells with cobblestone-like appearance); positive staining for synaptotoglin, podocalyxin, zona occludens-1, cytokeratin, vimentin, and laminin; negative staining for smooth muscle-type myosin, FVIIIr:Ag, and CD45; and cytotoxicity in response to puromycin
aminonucleoside. Cells were cultured in DMEM containing t-glutamine, 6.8 mM glucose, 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humified 5% CO2 incubator at 37°C. Cell number was determined using a standard counting chamber after cell harvesting with 0.25% trypsin and 0.5% EDTA.

Podocyte Exposure to Mechanical Stretch or Angiotensin II

Stretch. Human immortalized podocytes were seeded in equal number (20,000/cm2) into six-well type I collagen-coated silicon elastomer-base culture plates (Flex I plates) and control plates (Flex II plates). Cells were subjected to repeated stretch-relaxation cycles by mechanical deformation using a stress unit. The stress unit is a macroscale device that consists of a vacuum unit and a baseplate. A vacuum was cyclically applied (60 cycles/min) to the rubber baseplates via the baseplate, which was placed in a humidified incubator with 5% CO2 at 37°C. Cells were exposed to an average 10% uniaxial elongation, which mimics that present in vivo in glomeruli exposed to supraphysiological pressure levels. Stretch and control experiments were carried out simultaneously with cells derived from a single pool. Control cells were grown in nondeformable, but otherwise identical, plates in parallel. In some experiments, podocytes were exposed to stretch in the presence and absence of candesartan (1 μM), an AT1 receptor antagonist; PD-123319 (1 μM), an AT2 receptor antagonist; rosiglitazone, a PPAR-γ ligand (BRL 49655; 0.1 μM); or a specific anti-angiotensin II antibody (1, 10, or 20 μM). Control experiments with cells exposed to vehicle or to an irrelevant antibody were performed in parallel.

Exogenous angiotensin II. Podocytes were incubated with increasing concentrations of angiotensin II (0.01, 1, or 10 nM) or vehicle for 24–48 h.

mRNA Analysis

Total RNA was extracted using RNAasy Mini spin columns and reverse transcribed (2 μg) according to standard protocols using avian myeloblastosis virus reverse transcriptase and oligo(dT). Nephrin, GADPH, and hypoxanthine phosphoribosyltransferase (HPRT) mRNA expression were measured by quantitative real-time PCR using the TaqMan system based on real-time detection of the accumulated fluorescence (Applied Biosystems, Monza, Italy). Fluorescence for each cycle was quantitatively analyzed using an ABI Prism 7300 sequence detection system. PCR amplification was done in a 25-μl volume, including two sequence-specific PCR primers and a TaqMan-VIC/ FAM dye-labeled MGB probe (Nephrin, hs00190446_m1; GADPH, hs99999905_m1; and HPRT, hs99999909_m1). Assays were run in triplicate with Universal PCR Master Mix containing AmpErase-UNG on an Applied Biosystems 7300 real-time PCR system. To quantify target mRNA abundance, differences in threshold cycles between nephrin and GAPDH were calculated and then relative mRNA abundance was calculated using the 2-ΔΔCT method.

Immunoblotting

Podocytes were fixed in 3.5% paraformaldehyde, 0.025% glutaraldehyde, and 2% sucrose for 15 min at room temperature. After being blocked in 3% bovine serum albumin in phosphate-buffered saline (PBS) for 15 min at room temperature, cells were incubated with a guinea pig anti-human nephrin antibody (GP-N1; 1:50), raised against the extracellular domain of nephrin and then with a biotinylated anti-guinea pig antibody for 60 min at room temperature. After washing, fluorescein isothiocyanate-conjugated streptavidin was added for 60 min. Images were obtained using a low-light video camera (Leica DMIL). A permeabilization step with Triton X-100 (0.1%) was included in some staining.

Immunofluorescence

Podocytes were fixed in 3.5% paraformaldehyde, 0.025% glutaraldehyde, and 2% sucrose for 15 min at room temperature. After being blocked in 3% bovine serum albumin in phosphate-buffered saline (PBS) for 15 min at room temperature, cells were incubated with a guinea pig anti-human nephrin antibody (GP-N1; 1:50) raised against the extracellular domain of nephrin and then with a biotinylated anti-guinea pig antibody for 60 min at room temperature. After washing, fluorescein isothiocyanate-conjugated streptavidin was added for 60 min. Images were obtained using a low-light video camera (Leica DMIL). A permeabilization step with Triton X-100 (0.1%) was included in some staining.

Data Presentation and Statistical Analysis

The number of independent experiments is reported. Each experiment was carried out at least in triplicate. All data are means ± SE. Data are expressed as relative change compared with control. Student’s t-test was used for comparison between two groups. When more than two groups were studied, data were analyzed by ANOVA followed by Bonferroni’s t-test. Values with P < 0.05 were considered significant.
RESULTS

Mechanical Stretch Reduces Both Nephrin mRNA and Protein Expression in Human Podocytes

To test whether mechanical stretch alters nephrin expression, we subjected human podocytes to mechanical stretch (10% elongation) for various time periods and assessed nephrin mRNA and protein by quantitative real-time RT-PCR, immunoblotting, and immunofluorescence. Exposure to stretch induced a significant 50% reduction in nephrin mRNA levels at 48 h with no significant differences at 12 and 24 h (Fig. 1A). The light cycler analysis showed one single product, and amplification of total RNA gave no product, excluding contamination with genomic DNA. Comparable results were obtained when HPRT rather than GAPDH was used as housekeeping gene (54.16 ± 4.6 and 54.2 ± 8.56% decrease stretch vs. control at 48 h using HPRT and GAPDH, respectively), indicating that GAPDH was not regulated in our system.

Consistently, densitometric analysis of immunoblots for nephrin protein demonstrated a significant 48% decrease in nephrin protein expression in stretched cells compared with control cells at 72 h (Fig. 1D). Total protein extracts from mouse glomeruli, used as positive control, showed a band of identical molecular mass (180 kDa), confirming the specificity of the anti-nephrin antibody (Fig. 1B). A diminution in nephrin protein expression was also seen in immunofluorescence anal-

---

**Fig. 1. Stretch induces nephrin downregulation.** Both nephrin mRNA and protein expression were studied in human cultured podocytes by real-time RT-PCR and immunoblotting as described in MATERIALS AND METHODS. **A:** nephrin mRNA expression in podocytes after 12, 24, and 48 h of stretch. Results were corrected for GAPDH and are expressed as percent decrease relative to control. Values are means ± SE; n = 3. *P < 0.05, stretch at 48 h vs. control. **B:** representative immunofluorescent image of cultured podocytes stained with a specific anti-nephrin antibody (1). Incubation with anti-nephrin antibody was omitted to show specificity, and cells were counterstained with 4′,6-diamidino-2-phenylindole (2). The representative immunoblot shows nephrin expression in protein extracts from isolated mouse glomeruli (a), used as positive control; cultured podocytes (b), breast cells (c), and cultured podocytes incubated only with the secondary antibody (d). Arrow indicates the 180-kDa band corresponding to nephrin. **C:** representative images of immunofluorescence staining for nephrin in podocytes exposed to stretch (2) for 72 h and in control cells (1). Magnification, ×400. **D:** nephrin protein expression in podocytes exposed to stretch for 72 h and in control cells. A representative immunoblot and results of densitometric analysis, corrected for tubulin and expressed as percent change relative to control, are shown. Values are means ± SE; n = 5. *P < 0.05, stretch vs. control.
ysis (Fig. 1C). Although our cultured podocytes formed cell-to-cell junctions in basal conditions (Fig. 1B), the structure of the flexible culture plates prevented analysis of cell-to-cell junctions in stretched cells. No major changes were observed in subcellular localization of nephrin when a permeabilization step was included before immunofluorescence (data not shown).

**Stretch-Induced Nephrin Downregulation is Not Due to Podocyte Apoptosis and/or Reduced Proliferation Rate**

To exclude the possibility that nephrin loss was secondary to stretch-induced apoptosis, we tested whether stretching of podocytes induced changes in the cytoplasmatic levels of histone-associated DNA fragments, a marker of apoptotic cells. We found that podocyte exposure to stretch for 72 h induced only a modest and nonsignificant increase in cytoplasmatic histone-associated DNA fragments (Fig. 2A). Furthermore, mechanical stretch did not significantly alter the number of podocytes after 48 and 72 h of exposure (Fig. 2B).
stretch was completely abolished by the addition of rosiglitazone (BRL 49653; 0.1 μM) (Fig. 5, A and B). To assess whether rosiglitazone interferes with stretch-induced activation of the angiotensin II-AT1 system, we exposed podocytes to stretch for 24 and 48 h in either the presence or absence of rosiglitazone (0.1 μM) and then studied both angiotensin II secretion and AT1 receptor expression. We found that the increase in AT1 protein expression induced by stretch at 24 h was completely abolished by rosiglitazone (Fig. 5C), whereas the rise in angiotensin II secretion at 48 h was unaltered (Fig. 5D). Together these data support the hypothesis that prevention of the activation of the angiotensin II-AT1 system by stretch is a possible mechanism of rosiglitazone protective effect on nephrin.

**DISCUSSION**

In the present study we have demonstrated that in human podocytes, mechanical stretch downregulates nephrin expression by 10.220.33.2 on October 15, 2017 http://ajprenal.physiology.org/ Downloaded from
ing a 10% cell elongation, showed a significant 50% reduction in nephrin protein expression as assessed by immunoblotting. Nephrin protein reduction was also observed by immunofluorescence; however, specific assessment of nephrin downregulation at cell-to-cell junctions could not be performed because flexible wells are not suitable for confocal microscopy analysis.

In stretched podocytes, nephrin downregulation is likely to occur via a transcriptional mechanism, given that a significant diminution in nephrin mRNA levels was also observed. However, the decline in nephrin protein expression was very rapid, and we cannot exclude the possibility that additional mechanisms of nephrin protein reduction, such as ubiquitination and shedding, may also take place. The degree of stretch-induced nephrin reduction was comparable to that reported in podocytes exposed in vitro to glycated albumin, TNF-α and puromycin (13, 14, 30). Furthermore, a nephrin downregulation of similar magnitude has been reported in vivo in the Dahl salt-sensitive rat, an animal model of glomerular hypertension (34). Studies performed on kidney biopsies from patients with DN have shown a slightly greater diminution of nephrin expression (5, 47); however, in diabetic patients, in addition to glomerular hypertension, other factors, such as glycated albumin (14) and oxidized LDL (11), can contribute to nephrin loss.

In our study we did not observe any significant change in proliferation/apoptosis in stretched podocytes that could explain the loss of nephrin we have reported. A previous study has shown stretch-induced apoptosis in podocytes; however, 1% FCS was used in the culture media in that study compared with 10% in the present study, and this may explain the higher degree of apoptosis noted in stretched cells. Furthermore, a less quantitative method was used to assess apoptosis (15).

Stretch-induced nephrin downregulation was mediated by angiotensin II acting via an autocrine mechanism. Indeed, we found that 1) podocyte stretching induced both angiotensin II secretion and AT1 receptor overexpression, 2) podocyte exposure to angiotensin II reduced nephrin protein expression, and 3) both the AT1 receptor antagonist candesartan and a specific anti-angiotensin II antibody completely abolished stretch-induced nephrin downregulation.

The ability of podocytes to synthesize angiotensin II is not surprising given that functional expression of all the key components of the renin-angiotensin system has been demonstrated in cultured podocytes (15, 32). Furthermore, exposure of podocytes to high glucose enhances angiotensin II levels by increasing renin activity, and in early DN there is glomerular upregulation of the (pro)renin receptor with a podocyte distribution (16).

Exposure of cultured podocytes to exogenous angiotensin II induced a significant decrease in nephrin expression. A rapid and transient loss of nephrin due to shedding was previously reported in response to angiotensin II (14), but our results suggest that angiotensin II can also affect nephrin protein expression. The effect of angiotensin II on nephrin is likely to occur via the AT1 receptor, since Suzuki et al. (44) have shown that the AT1 receptor mediates nephrin mRNA decline both in vitro and in vivo, whereas activation of the AT2 receptor enhances nephrin gene expression. The opposite action of the two receptors may explain the bimodal effect of prolonged angiotensin II infusion on nephrin that was recently described in vivo in rats (27); however, in vitro in podocytes, the effect of the AT1 receptor appears to prevail over that of the AT2 receptor (44). The mechanism whereby angiotensin II affects nephrin expression remains elusive; however, angiotensin II binding to the AT1 receptor induces activation of signaling molecules, such as protein kinase C (PKC) and AKT, which have been involved in suppression of the nephrin gene (11, 45, 50).

Interestingly, a significant reduction in nephrin protein expression was observed at a very low dose of angiotensin II, comparable to that we measured in the supernatant of stretched podocytes, and this is consistent with the hypothesis that endogenous angiotensin II, released in response to stretch, is the mediator of stretch-induced nephrin downregulation. It is noteworthy that this physiological concentration reduced nephrin expression without inducing podocytes apoptosis, whereas supraphysiological doses of angiotensin II caused an increase in apoptosis, which may also contribute to nephrin loss.

In addition to inducing angiotensin II release, podocyte exposure to stretch also caused a significant increase in the expression of the AT1 receptor at 24 h. Surprisingly, we found that the expression of the AT1 receptor was decreased after 48-h exposure to stretch. Angiotensin II binding to AT1 is known to induce AT1 downregulation through both inhibition of AT1 synthesis and enhanced AT1 degradation (37); therefore, the significant increase in angiotensin II levels at 24 h may explain AT1 receptor downregulation at 48 h.

Stretch-induced angiotensin II secretion and/or AT1 expression has been demonstrated in other cell types, such as cardiomyocytes (41) and mesangial cells (22), whereas previous data in podocytes were conflicting, since Durvasala et al. (15) reported induction of both angiotensin II production and AT1 expression in response to stretch, whereas Liebau et al. (32) found that angiotensin II was not altered by mechanical stress.
in podocytes. In cardiomyocytes, stretch directly activates the AT1 receptor without the need for angiotensin II to bind the receptor (52); however, this does not appear to be the case in podocytes given that not only candesartan but also a specific anti-angiotensin II antibody completely prevented stretch-induced nephrin downregulation.

These in vitro findings may be of clinical relevance, because they suggest that the beneficial effect of ARBs in reducing both nephrin and protein loss in diabetic nephropathy may be related not only to their positive effect on glomerular capillary pressure but also to their ability to interfere with angiotensin II activity as a mediator of hemodynamic stress-induced effects on podocytes.

In our study, stretch-induced nephrin downregulation was also prevented by rosiglitazone. This is in keeping with a recent report in an animal model of Heymann nephritis (6) showing that the PPAR-γ ligand pioglitazone prevents nephrin downregulation with efficacy comparable to that of candesartan. In vitro studies have shown that in vascular smooth muscle cells, PPAR-γ ligands induce AT1 downregulation (43), and that in adipocytes, ARBs enhance PPAR-γ activity (42), providing evidence for a cross talk between the PPAR-γ and the angiotensin II-AT1 system. This raises the possibility that rosiglitazone prevents stretch-induced nephrin loss by interfering with the angiotensin II-AT1 system, and according to this hypothesis, we found that in podocytes, rosiglitazone inhibited AT1 upregulation in response stretch. However, additional mechanisms of prevention of nephrin loss, including the recently described regulation of the nephrin transcriptosome by PPAR-γ ligands (6), may also be implicated.

**Fig. 5.** Effect of rosiglitazone on stretch-AT1-induced nephrin downregulation. Podocytes were exposed to stretch in either the presence or absence of rosiglitazone (BRL; 0.1 μM). Nephrin mRNA at 48 h (A) and protein levels at 72 h (B) were assessed by real-time RT-PCR and immunoblotting, respectively. GAPDH and tubulin were used as internal controls, and results are expressed as percent change relative to control cells. Values are means ± SE; n = 3. *P < 0.05, stretch vs. control. C: AT1 protein expression was assessed by immunoblotting in podocytes exposed to stretch for 24 h in the presence or absence of BRL (0.1 μM). A representative immunoblot and results of densitometric analysis, corrected for tubulin, are shown. Values are means ± SE; n = 5. *P < 0.05, stretch vs. control. D: angiotensin II protein levels were measured in the supernatant of podocytes exposed to stretch for 48 h in the presence or absence of BRL (0.1 μM). Results were corrected for the number of cells and are expressed as percent change relative to control. Values are means ± SE; n = 5. *P < 0.05, stretch and stretch + BRL vs. control.
Rosiglitazone is currently in use in type 2 diabetic patients as an insulin-sensitizing agent and appears to ameliorate microalbuminuria in these patients (2). Furthermore, studies in experimental diabetes have shown a renoprotective and anti-proteinuric effect of PPAR-γ ligands that is independent of their insulin-sensitizing activity (15, 33, 51). Our observation that rosiglitazone can prevent nephrin loss in vitro by interfering with the angiotensin II-AT_1 system may suggest a novel mechanism for its antiproteinuric activity.

REFERENCES


