Activation of a local tissue angiotensin system in podocytes by mechanical strain

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HORMONES – CYTOKINES – SIGNALING

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Background. Glomerular capillary hypertension, a common denominator in various forms of progressive glomerular disease, results in mechanical distention of the capillary tuft, and subsequent injury of the overlying podocyte layer. The mechanisms by which elevated intraglomerular pressure is translated into a maladaptive podocyte response remain poorly understood. Angiotensin II plays a central role in the pathogenesis of chronic renal injury, largely through its actions on the subtype 1 receptor. Accordingly, we have tested the hypothesis that mechanical strain up-regulates local angiotensin II in podocytes, thereby resulting in a progressive reduction in podocyte number.

Methods. Conditionally immortalized mouse podocytes were subjected to cyclical stretch of 10% amplitude. Nonstretched podocytes served as controls. Angiotesin II levels were measured in whole cell lysates by competitive enzyme-linked immunosorbent assay (ELISA). Expression of angiotensin II receptors (AT₁R, AT₂R) was measured by quantitative polymerase chain reaction (PCR) and Western blot analysis. Apoptosis was measured by Hoechst staining. Immunostaining for AT₁R was performed in tissue sections from rats with 5/6 remnant kidney, consistent with our in vitro findings. Mechanical strain resulted in a 2.5-fold increase in apoptosis (P < 0.001 vs. nonstretched controls) in an angiotensin II–dependent fashion.

Results. Mechanical strain increased angiotensin II production in podocytes at 24, 48, and 72 hours (P < 0.05 vs. nonstretched controls). Stretching podocytes resulted in a fivefold increase in AT₁R mRNA expression at 24 hours and a twofold increase in protein levels vs. controls (P < 0.05), and also an increase in transforming growth hormone-β (TGF-β) mRNA expression. AT₁R staining was increased in a podocyte distribution in the 5/6 remnant kidney, consistent with our in vitro findings. Mechanical strain resulted in a 2.5-fold increase in apoptosis (P < 0.001 vs. nonstretched controls) in an angiotensin II–dependent fashion.

Conclusion. Mechanical strain leads to up-regulation of the AT₁R and increased angiotensin II production in conditionally immortalized podocytes. The resulting activation of a local tissue angiotensin system leads to an increase in podocyte apoptosis, mainly in an AT₁R-mediated fashion.

Podocytes are terminally differentiated epithelial cells lining the outer aspect of glomerular capillaries. Through an actin-based contractile apparatus, podocytes counterbalance the pressure within the underlying capillary, thereby preventing outward ballooning of the vessel and thus, preserving the normal architecture of the glomerular tuft [1]. Podocytes are vulnerable to many forms of injury, including both immune-mediated [2, 3] and nonimmune processes [4–9]. The subsequent loss of podocytes is central to the development of glomerulosclerosis. As glomeruli become irreversibly scarred, a decline in the functional nephron mass ensues, such that the remaining nephrons are subjected to elevated pressure gradient across the capillary wall (P₉c), which may exceed 60 mm Hg [10]. The heightened P₉c results in cellular strain [11, 12]. Therefore, independent of the nature of the inciting event, glomerular capillary hypertension perpetuates further damage to the podocyte and represents a final common pathway to glomerulosclerosis and end-stage renal failure [13]. Mesangial cells respond to mechanical stress by proliferating [14]. In contrast, we have recently shown that mechanical strain is anti-proliferative for podocytes [15]. The inability to effectively proliferate leads to a reduction in podocyte number (“podocytopenia”) in diabetic and nondiabetic renal diseases.

Angiotensin II has both hemodynamic and nonhemodynamic effects, and has been shown to be a critical mediator in progressive renal disease. Through its actions on the relative tone of the afferent and efferent arterioles, circulating angiotensin II is a critical determinant of P₉c. Inhibiting angiotensin II production and/or receptor binding ameliorates the loss of kidney function in chronic diabetic and nondiabetic renal diseases characterized by increased P₉c [16, 17]. This is likely due in part to a decrease in P₉c mediated by angiotensin II. However, local
tissue production of angiotensin II also mediates disease through a series of nonhemodynamic effects, including altered proliferation, apoptosis, and cellular hypertrophy [18]. In chronic disease states of the kidney associated with glomerular hypertension, intrarenal angiotensin II levels are dissociated from, and typically exceed, circulating levels [19]. That the beneficial effects of angiotensin II blockade on ameliorating disease are greater than what could be predicted by its hemodynamic effects alone further supports the notion of a pathogenic role for local production of angiotensin II [17, 20].

Recent evidence suggests that angiotensin II may have direct biologic effects on podocytes. Angiotensin II mediates rearrangement of the actin cytoskeleton and inhibiting angiotensin II prevents redistribution of the structural protein zonula occludens-1 (ZO-1) in the slit diaphragm [21]. It has been demonstrated that in diabetic nephropathy, reduced levels of nephrin are restored to normal following angiotensin II blockade [22]. Furthermore, foot process broadening in experimental diabetic nephropathy was attenuated with angiotensin blockade [23]. Taken together, these findings argue that the beneficial effects of angiotensin II blockade on reducing proteinuria and preventing glomerulosclerosis may not simply be the consequence of diminished P_{GC} [24] but may also be due to direct effects on the podocyte.

Although podocyte loss is an early event in the pathway to glomerulosclerosis, the mechanisms by which increased P_{GC} is translated into maladaptive podocyte responses remain poorly understood. Accordingly, we have performed studies to test the hypothesis that mechanical strain up-regulates local angiotensin II in podocytes, thereby resulting in a progressive reduction in podocyte number.

METHODS
Cell culture

Experiments were primarily performed utilizing early passage (passages 6 to 11), growth-restricted, conditionally immortalized mouse podocytes (gift from Peter Mundel, M.D., Albert Einstein College of Medicine, Bronx, N.Y.). When grown under permissive conditions (in the presence of gamma-interferon at 33°C), cells proliferate and display characteristics of undifferentiated podocytes [25]. However, when grown under restrictive conditions (absence of gamma-interferon at 33°C), proliferation is markedly reduced and cells undergo cytoskeletal rearrangement with the formation of arborizing cellular processes, resembling the morphologic appearance of mature differentiated podocytes in vivo [25]. Cells were grown on collagen type I-coated plates in RPMI 1640 media containing 10% fetal bovine serum (FBS) (Summit Biotechnology, Ft. Collins, CO, USA), penicillin (100 U/mL), streptomycin (100 ug/mL), glutamine (2 mmol/L), sodium pyruvate (1 mmol/L) (Irvine Scientific, Santa Ana, CA, USA), HEPES buffer (10 mmol/L) (Sigma Chemical Co., St. Louis, MO, USA), and sodium bicarbonate (0.075%, Sigma Chemical Co.). Cells were grown under restrictive conditions for greater than 10 days at 37°C in 95% air/5% CO_{2}.

Additionally, for the studies of angiotensin II production in response to cyclical strain, primary cultured mouse podocytes were utilized as previously described [26]. Briefly stated, glomeruli were obtained from C57/bl mice by sieving [26] and grown on a vitrogen matrix. Podocytes were isolated and subcultured on collagen type I-coated plates in media containing 5% FBS, penicillin (100 U/mL), streptomycin (100 ug/mL), and glutamine (2 mmol/L) and grown at 37°C in 95% air/5% CO_{2}. Podocyte identity was confirmed based on typical cobblestone appearance and positive staining for the podocyte-specific markers synaptopodin, podocin (gifts from Peter Mundel), and WT-1 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), as well as the absence of mesangial and endothelial cell markers (alpha-smooth muscle actin and factor VIII, respectively).

Experimental design for inducing mechanical strain of cultured podocytes

Conditionally immortalized podocytes grown under restrictive conditions for a minimum of 10 days were seeded onto flexible 6-well plates coated with bovine collagen type I (Flexcell International Corporation, Hillsborough, NC, USA) at a density of ~90,000 cells per well, yielding an initial confluence of 20%. Cells were allowed to adhere and further differentiate for 72 hours, at which time culture plates were loaded onto a computer-assisted stretch apparatus (FlexerCell Strain Unit 3000T) as previously reported [15]. Intermittent negative pressure was applied to the biomembrane by a vacuum, resulting in cyclical stretch and relaxation of the adherent cell layer. Based on pilot studies, a regimen of 60 cycles of stretch and relaxation per minute with an amplitude of 10% biaxial surface elongation was uniformly applied across the membrane. Cells grown under identical conditions (within the same incubator as the Strain unit), but not exposed to stretch, served as controls.

Measuring angiotensin II levels

We were concerned that residual angiotensin II present in FCS in the cell culture media may potentially interfere with enzyme-linked immunosorbent assay (ELISA) measurements, necessitating growth under serum-free conditions. Because conditionally immortalized mouse podocytes grown under restrictive conditions do not survive under serum-free conditions, primary cultured mouse podocytes were utilized for these experiments. Early passage primary mouse podocytes were seeded on
6-well collagen type I-coated flexible plates at 60,000 cells per well and allowed to adhere overnight. Cells were then washed with Hank’s balanced salt solution (Irvine Scientific) twice and grown in serum-free media for 48 hours. Cyclical stretch of 10% amplitude was initiated and cells harvested at 24, 48, and 72 hours by trypsin/collagenase digestion (37°C for 10 minutes). Cells were washed with ice-cold phosphate-buffered saline (PBS) twice, centrifuged at 1400 rpm for 5 minutes at 4°C, and suspended in 200 uL of extraction buffer containing 20 mmol/L Tris HCl (pH = 7.40), 10 mmol/L ethylenediaminetraacetic acid (EDTA), and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Cells were lysed by pulse sonication on ice (12 cycles) and centrifuged at 15,000 rpm for 10 minutes at 4°C. Protein concentrations were measured by BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and samples adjusted to a final concentration of 0.5 ug/uL. Angiotensin II levels in stretched and control podocytes were measured by competitive ELISA (Peninsula Laboratories, Inc., San Carlos, CA, USA) according to the manufacturer’s instructions.

To determine whether stretch-induced increase in angiotensin II generation was dependent on angiotensin-converting enzyme (ACE) activity, podocytes were incubated in the presence of the ACE inhibitor captopril (10⁻⁶ mol/L) (Sigma Chemical Co.) or vehicle for 60 minutes prior to the initiation of mechanical strain. Protein harvest and measurement of angiotensin II levels were carried out as previously outlined.

Measuring mRNA levels

Total RNA was harvested from stretched and static cells at 18, 24, 48, and 72 hours using the Trizol method (Sigma Chemical Co.) as previously reported [15]. Three micrograms of total RNA was reverse transcribed into cDNA using the oligo(dT) method (Gibco BRL, Superscript First Strand Synthesis System, Life Technologies, Inc., Rockville, MD, USA) in 20 uL reaction volume. One microliter of reaction volume was used for conventional polymerase chain reaction (PCR) in a reaction volume of 50 uL, containing 1.5 mmol/L MgCl₂. Each PCR cycle involved an annealing step of 1 minute, followed by a replication step of 2 minutes. The following primer sequences and conditions (annealing temperature, cycle number) were utilized: angiotensin II type 1 receptor (AT₁R): 5'-GGAAACAGCTTGGTGCTG-3', 3'-TTCTTCCGAATACCTTAAGTC-5' (55°C, ×35); angiotensin II type 2 receptor (AT₂R): 5'-TATGCTCAGTGGTCTGCG-3', 3'-CGAGGTTCGTTTCTCTCTC T-5' (60°C, ×39).

To determine the effects of mechanical strain on AT₁R and AT₂R mRNA expression, real-time PCR was performed, based on a quantitative colorimetric assay as recently described [27]. PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60 seconds for 1 minute. A sample of stock cDNA (synthesized from podocyte RNA as above) was serially diluted in tenfold steps and a standard curve generated based on threshold cycle (Ct) values using Sequence Detector 1.7 (Applied Biosystems, Foster City, CA, USA). Quantification of mRNA was determined based on Ct value, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expressed as the magnitude of change relative to controls. Primer and TaqMan probes were designed by Primer Express 1.0 (Applied Biosystems) and ordered (Invitrogen Life Sciences, Carlsbad, CA, USA, and Syntegen, Houston, TX, USA, respectively) with sequences as follows: AT₁R primers: 5'-CCATGTCCACCGATGAA-3', 3'-ACGACCACCGTTTACAGT-5'; AT₁R probe: 5'-TC TCGCTCAGGCGACG-3'; AT₂R primers: 5'-CCG AGAACAGGAAATCAAGTATGAAC-3', 3'- AGA ACGCCAGGAAACCG-5'; AT₂R probe: 5'- AAATTCATTAATCCACCAGCTTICAGTGATAG TGCC-3'.

Substitution of cDNA with water was used as a negative control to exclude contamination of reagents or the reaction mixture with genomic DNA.

Studies have shown that transforming growth factor-β (TGF-β) causes glomerulosclerosis in many forms of renal disease. To determine the effects of mechanical strain on TGF-β mRNA expression, RNA se Protection Assay was performed as we have previously reported per manufacturer’s instructions. A commercially available DNA template (BD Biosciences Pharmingen, San Diego, CA, USA) for the TGF-β isoforms β1, β2, β3, and the housekeeping gene GAPDH was used to generate a P-32 uridine triphosphate (UTP)-labeled single-stranded antisense mRNA probe. Following RNA se digestion, radioactivity of the probe was quantitated by scintillation counter (Beckman LS7500) and the probe was diluted to target concentration. 5 ug of RNA (from stretched and control podocytes) were hybridized with the diluted probe overnight at 56°C. Following RNA se digestion of single-stranded fragments, the remaining double-stranded hybridized product was precipitated, washed, and solubilized in loading buffer. Samples were separated on a polyacrylamide QuickPoint gel (Novex, San Diego, CA, USA) and quantitative analysis performed by phosphoimaging with densitometry. Results were standardized to GAPDH and expressed as degree of change relative to controls.

Western blot analysis

The protein levels of AT₁R and AT₂R were measured in control and stretched podocytes at 18, 24, 48, and 72 hours by Western blot analysis as previously described.
with primary antibody overnight (4°C) to reduce background, membranes were incubated for 30 minutes in 5% nonfat dried milk to a polyvinylidine difluoride (PVDF) membrane (Immobilon-P) by electroblotting at 350 mA for 75 minutes. After blocking for 5 minutes, five micrograms of reduced protein sample was then loaded per lane on an 8% SDS-polyacrylamide gel and subsequently transferred to a periconcentrated by centrifugation (1400 rpm for 5 minutes at 4°C), washed twice with ice-cold PBS, and then suspended in lysis buffer containing 1% Triton, 10% glycerol, 20 mmol/L HEPES, 100 mM NaCl, and protease inhibitor cocktail (Roche Molecular Biochemicals). Following an overnight freeze-thaw cycle, lysates were cleared by centrifugation at 17,000g for 10 minutes at 4°C and protein concentration determined by BCA Protein Assay Kit (Pierce) according to the manufacturer’s directions. Reducing buffer was added to each protein extract and samples boiled for 5 minutes. Five micrograms of reduced protein sample was then loaded per lane on an 8% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidine difluoride (PVDF) membrane (Immobilon-P) by electroblotting at 350 mA for 75 minutes. After blocking for 30 minutes in 5% nonfat dried milk to reduce background, membranes were incubated with primary antibody overnight (4°C) as follows: AT1R (1:200 dilution) (Santa Cruz Biotechnologies), AT2R (1:200) (Santa Cruz Biotechnologies), tubulin (1:2000 dilution) (NeoMarkers, Fremont CA, USA). Incubation of membranes with blocking peptide in tenfold excess of primary antibody was performed as negative controls to ensure antibody specificity. Following three wash cycles with Tris-buffered saline with 0.1% Tween (TBST), membranes were incubated with either an antinouse or anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:2000 dilution) (Promega, Madison, WI, USA) for 60 minutes at room temperature. The chromagen 5-bromo-4-chloro-3-indoly phosphate/nitro blue tetrazolium (Sigma Chemical Co.) was used for detection of the resultant bands. Densitometric quantitation was performed using ImagePro-Plus software (Media Cybernetics, Silver Spring, MD, USA) and results corrected to tubulin levels, used as a housekeeper, to correct for any potential errors in loading.

**Tissue levels of AT1R in experimental models of glomerular capillary hypertension**

In order to ensure that the cell culture results also occurred in vivo, the 5/6 remnant kidney disease model of glomerular capillary hypertension [28] was created in Sprague-Dawley rats by performing unilateral nephrectomy and infarction of the upper and lower poles of the remaining kidney through ligation of the posterior and anterior branches of the renal arteries. Animals were sacrificed on day 35 and the remnant kidney was harvested. Biopsies were fixed in formalin and immunostaining performed as previously described [27] for AT1R. Tissue from age-matched sham-operated rats served as controls. Briefly, tissue sections were deparaf-finized in Histoclear (National Diagnostics, Atlanta, GA, USA), rehydrated with ethanol, and treated with hydrogen peroxide to neutralize endogenous peroxidase. Antigen retrieval was performed by boiling in citric acid for 10 minutes and background staining reduced by applying avidin-biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA, USA) per manufacturer’s instructions. Tissue sections were incubated with the primary AT1R antibody overnight (1:500 dilution) (Santa Cruz Biotechnologies) at 4°C, then biotinylated secondary antibody (1:100 dilution) (Promega) for 60 minutes at room temperature, followed by ABC reagent (Vector Laboratories, Inc.) for 20 minutes at room temperature. Color development was achieved by incubating in diaminobenzidine (DAB) solution at 37°C for 10 minutes and counterstaining in methyl green for 2 minutes. Substitution of the primary antibody with an irrelevant rabbit IgG served as a negative control.

**Measuring apoptosis**

Apoptosis of stretched and nonstretched podocytes was measured at 24 hours by Hoechst staining (Hoechst 33342) (Molecular Probes, Eugene, OR, USA) as previously reported [29]. Conditionally immortalized podocytes were grown in the presence of 1% fetal calf serum (FCS). One millimole per liter stock of Hoescht stain was added to each well in a 1:100 dilution 5 minutes prior to visualization by inverted fluorescent microscopy. Apoptosis was determined by nuclear condensation pattern and expressed as the percentage of apoptotic cells per high power field. A total of 10 high power fields in a pericentric distribution were quantitated per well and 2 wells per experimental condition studied.

**Statistical analysis**

All experiments described in this manuscript were performed a minimum of three times.

Statistical analysis on data obtained for angiotensin II measurements and apoptosis was performed using analysis of variance (ANOVA) with a Bonferroni-Dunn correction (Statview 5.0) (Abacus Concepts, Berkeley, CA, USA). A P value <0.05 was considered statistically significant.

**RESULTS**

**Mechanical strain increases angiotensin II levels in primary cultured podocytes**

Podocytes are known to possess the metabolic machinery necessary for autologous synthesis of angiotensin II [30]. Accordingly, we determined the effects of mechanical strain on local angiotensin II production by cultured podocytes, using a competitive ELISA. Primary culture mouse podocytes were subjected to either cyclical stretch...
of 10% amplitude or static conditions and protein harvested from total cell lysate at 24, 48, and 72 hours as outlined in the Methods section. As shown in Figure 1, there was a threefold increase in angiotensin II levels at 24 hours under stretch conditions and these changes were still persistent at 72 hours. Preincubation with the ACE inhibitor captopril (10^{-6} mol/L) did not block the stretch-induced increase in angiotensin II generation (data not shown).

**Conditionally immortalized podocytes express AT_{1R} and AT_{2R}**

We next wished to verify that conditionally immortalized mouse podocytes grown under restrictive conditions express receptors for angiotensin II. Conventional reverse transcription (RT)-PCR was performed to measure the mRNA expression of AT_{1R} and AT_{2R}. As shown in Figure 2, bands of 556 and 427 bp, the predicted size for AT_{1R} and AT_{2R}, respectively, were detected at 40 cycles. In contrast, no product was detected under control conditions. Immunoblotting studies confirmed the presence of AT_{1R} and AT_{2R} at the protein level in the conditionally immortalized podocyte cell line (data not shown).

**Mechanical strain increases AT_{1R} expression**

The effects of mechanical strain on angiotensin II receptor expression in podocytes was determined by quantitating mRNA and protein levels in cells exposed to stretch. Conditionally immortalized mouse podocytes grown under restrictive conditions were subjected to cyclical stretch of 10% amplitude. mRNA expression of AT_{1R} was measured by quantitative real-time PCR. As shown in Figure 3, there was a fivefold increase in AT_{1R} mRNA levels (corrected for the housekeeping gene GAPDH) in stretched cells, compared to static controls, at 24 hours. AT_{1R} mRNA expression normalized by 48 hours. Figure 4 shows the protein levels of AT_{1R} measured by Western blot analysis. Densitometric analysis confirmed a twofold increase in AT_{1R} protein levels at 18 to 24 hours, and the increase was sustained at 72 hours. In contrast, cyclical strain did not alter AT_{2R} mRNA expression or protein levels (data not shown).
Podocyte AT1R levels are increased in experimental model of glomerular capillary hypertension

We were next interested in confirming in vivo relevance of our in vitro findings in a disease model. The 5/6 remnant kidney model in the rat is known to be associated with glomerular capillary hypertension with resultant distention of the capillary tuft and strain of the overlying podocyte layer. Accordingly, tissue samples from day 35 remnant kidneys were obtained for AT1R immunostaining. As shown in Figure 5, when compared with tissue sections obtained from sham-operated controls, there was a significant increase in the intensity of AT1R staining in a podocyte distribution, consistent with the findings of the cell culture model described earlier.

Mechanical strain induces apoptosis in conditionally immortalized podocytes

To show biologic significance of mechanical strain-induced activation of a local angiotensin system in podocytes, the effects of mechanical strain on apoptosis were investigated. Conditionally immortalized mouse podocytes were grown under restrictive conditions in 1% FBS and subjected to cyclical stretch of 10% amplitude in the presence of saralasin (10^{-5} mol/L) (Sigma Chemical Co.), valsartan (10^{-5} mol/L) (Novartis Pharma AG, Basel, Switzerland), or vehicle. Nonstretched cells served as controls. As shown in Figure 6, mechanical strain caused a 2.5-fold increase in apoptosis compared to static controls (18.9% vs. 7.6%, respectively, $P < 0.001$). Blocking angiotensin II with either a nonspecific antagonist of angiotensin II receptor (saralasin) or a selective AT1R antagonist (valsartan) ameliorated the stretch-induced increase in apoptosis (11.3% and 11.8%, respectively, $P < 0.001$ vs. stretch alone), whereas stretching in the presence of vehicle did not significantly alter the rate of apoptosis (17.9%). These results show that stretch-induced podocyte apoptosis occurs mainly in an AT1R-dependent fashion.

Exogenous angiotensin II induces apoptosis in conditionally immortalized podocytes

To confirm that the observed stretch-induced increase in apoptosis was due to activation of local angiotensin production and not a result of low levels of FBS in the growth media, exogenous angiotensin II was added to cultured podocytes and apoptosis was measured. Conditionally immortalized podocytes grown under restrictive conditions were exposed to 1% FBS growth media containing exogenous angiotensin II (10^{-6} mol/L) or vehicle and apoptosis was measured at 24 hours. As shown in Figure 7, exogenous angiotensin II resulted in a near nine-fold increase in apoptosis when compared to podocytes treated with vehicle alone ($P < 0.001$).

Mechanical strain upregulates TGF-β mRNA expression in cultured podocytes

TGF-β is an important mediator of progressive renal injury in disease states associated with glomerular capillary hypertension through various mechanisms, including extracellular matrix accumulation, cellular hypertrophy, and altered proliferative capacity. TGF-β is known to mediate many of the deleterious downstream effects of angiotensin II and recently has been shown to induce podocyte apoptosis [31]. Therefore, it is feasible that angiotensin II–induced apoptosis of cultured podocytes may be mediated by TGF-β. Accordingly, the effects of mechanical strain on TGF-β mRNA expression in cultured podocytes was measured by RNA’s e Protection Assay. As shown in Figure 8, cyclical stretch resulted in a 29% and 39% increase in mRNA expression of TGF-β1 and TGF-β3 isoforms, respectively, at 24 hours, and these effects were sustained at 48 hours ($P < 0.05$ vs. nonstretched cells).

DISCUSSION

A reduction in podocyte number characterizes diabetic and nondiabetic nephropathies associated with glomerular capillary hypertension (increased intraglomerular pressure, $P_{gc}$), and has been causally linked to the development of glomerulosclerosis [14]. Although podocyte injury and loss occurs in experimental and human diseases characterized by increased $P_{gc}$, the mechanisms...
underlying these events are not well understood. In the current study, we show that when cultured podocytes are exposed to stretch, levels of angiotensin II and the AT$_1$R increase, and inhibiting this receptor significantly reduces stretch-induced podocyte apoptosis. We also show that immunostaining for AT$_1$R increases in podocytes in the remnant kidney model of increased P$_{gc}$.

Experimental and clinical studies have shown that reducing angiotensin II synthesis or blocking the AT$_1$R protects the kidney from progressive glomerulosclerosis in diabetic and nondiabetic glomerular diseases [16, 17, 20]. Moreover, inhibiting angiotensin II and/or the AT$_1$R significantly reduces proteinuria. Studies have shown that proteinuria characterizes injury to podocytes. However, it is not clear why podocytes are injured when P$_{gc}$ increases. In the current study, we exposed cultured murine podocytes to mechanical stretch, an in vitro model that we [15] have utilized to delineate the mechanisms of podocyte injury when P$_{gc}$ increases. Using the same model, Endlich et al [32] provided direct evidence that cultured podocytes exposed to mechanical stretch indeed are mechanosensitive, as evidenced by reorganization of the actin cytoskeleton in response to cyclical stretch.

The major finding in the current study was that exposing cultured podocytes to mechanical stretch caused a significant increase in the tissue levels of angiotensin II and its receptor, AT$_1$R. Both the mRNA expression and protein levels of the AT$_1$R were increased in stretched podocytes. In contrast, levels of AT$_2$R were unaffected by stretch. The increase in AT$_1$R was not restricted to only podocytes in culture, as we were able to demonstrate an increase in AT$_1$R immunostaining specifically in podocytes in the experimental remnant kidney model of glomerular capillary hypertension. This model has been shown to result in podocyte injury.

Fig. 5. Podocyte immunostaining for angiotensin II type 1 receptor (AT$_1$R) increases in remnant kidney. Immunostaining was performed for AT$_1$R in control (A and B) and remnant kidneys at day 35 (C). While AT$_1$R was detectable in an arteriole section from control kidney (A, arrowhead), minimal podocyte staining was noted. In contrast, AT$_1$R immunostaining was increased in the remnant kidney at day 35 in a podocyte distribution (C, arrows). The absence of staining with irrelevant rabbit IgG (not shown) confirms that AT$_1$R staining is specific.

Fig. 6. Mechanical strain induces apoptosis of cultured podocytes in an angiotensin II (ANG II)-dependent fashion. Apoptosis was measured by Hoechst staining, under static (A) and stretch conditions (B). The number of apoptotic cells (arrows) were quantitated per high power field under different experimental conditions and depicted graphically (C). Mechanical stretch (second column) resulted in a 2.5-fold increase in apoptosis compared to static controls (first column, P < 0.001). Blocking angiotensin II with 10$^{-5}$ mol/L saralasin (a nonspecific antagonist of angiotensin II receptor, third column) or 10$^{-5}$ mol/L valsartan (a selective AT$_1$R antagonist, fourth column) ameliorated stretch-induced apoptosis compared to stretch alone, #P < 0.001. In contrast, stretching podocytes in the presence of vehicle alone (fifth column) did not significantly reduce apoptosis.

Fig. 7. Exogenous angiotensin II increases apoptosis of cultured podocytes. Conditionally immortalized mouse podocytes grown in 1% fetal bovine serum (FBS) were exposed to exogenous angiotensin II (10$^{-6}$ mol/L) or vehicle. Apoptosis was measured at 24 hours by Hoechst staining. Angiotensin II increased podocyte apoptosis 8.5-fold compared to podocytes treated with vehicle alone (5.1% vs. 0.6%, respectively, P < 0.001).
Why focus on local angiotensin II and AT1R in podocytes? Angiotensin II is an octapeptide, which is generated from proteolytic cleavage of the decapeptide angiotensin I. Conversion of the inactive angiotensin I to the active angiotensin II may occur by various enzyme pathways, of which the ACE (made principally within the endothelial bed of the lung) is most important in the circulating pool. Under physiologic conditions, circulatory angiotensin II mediates vasoconstriction, sodium reabsorption, and aldosterone production [33]. Through its actions on the relative tone of the afferent and efferent arterioles, angiotensin II controls glomerular capillary pressures. Reducing Pgc by 50% and 90%, respectively, in cultured podocytes by 24 hours compared to the afferent and efferent arterioles, angiotensin II is also a critical determinant of glomerular capillary pressures. Reducing Pgc by inhibiting angiotensin II with an ACE-inhibitors and/or angiotensin receptor blockers protects the kidney from progressive glomerulosclerosis [16]. However, there is abundant evidence that local tissue production of angiotensin II also plays a critical role in mediating disease through a series of nonhemodynamic effects, including apoptosis, matrix accumulation, and aberrant proliferation [18]. In the present study, blocking ACE with the ACE inhibitor captopril did not abrogate the stretch-induced increase in angiotensin II generation. These results suggest that primarily non-ACE pathways may be involved in cleavage of angiotensin I into angiotensin II, consistent with what has been reported in the local production of angiotensin II in other tissue systems including ventricular myocytes [34, 35].

Studies have shown that the AT1R is the predominant subtype in the kidney (>90%) and mediates both hemodynamic and nonhemodynamic effects of angiotensin II, including vasoconstriction, fibrosis, cellular hypertrophy, and a decrease in proliferation [36, 37]. Selective blockade of the AT1R has proven as effective as ACE inhibitors in retarding the progression of renal disease [38]. In contrast, AT2R expression is much higher during embryogenesis, present only in low levels in the healthy adult kidney. The physiologic effects of AT2R largely counteract those of the AT1R and include vasodilatation, natriuresis, and cell differentiation [36, 39].

Thus, to understand the potential role of increased angiotensin II and the AT1R in stretched podocytes, we studied the effects of AT1R inhibition, with either a nonselective (saralasin) or selective angiotensin receptor blocker (valsartan), on podocyte apoptosis. Apoptosis has been shown by Schiffer et al [40] to reduce podocyte number, thereby contributing to glomerulosclerosis. Our results showed that blocking the effects of angiotensin II significantly reduced stretch-induced apoptosis. A causal role for stretch-induced activation of a local angiotensin system in mediating apoptosis was further supported by the demonstration that the addition of exogenous angiotensin II to cultured podocytes was likewise able to induce apoptosis. The fact that saralasin and valsartan were equally efficacious suggests that apoptosis is occurring in an AT1R-dependent fashion. Indeed, studies by Ding et al [41] have also shown that angiotensin II induces apoptosis in cultured podocytes, and that this is largely mediated in an AT2R-dependent fashion.

TGF-β mediates a number of the downstream non-hemodynamic effects of angiotensin II as they relate to progressive tissue injury and scarring, and has recently been shown to play a role in mediating podocyte apoptosis. Therefore, the demonstration that mechanical strain also up-regulated mRNA expression of TGF-β1 and TGF-β3 may provide some clues into the mechanisms by which angiotensin II mediates podocyte apoptosis. However, angiotensin II blockade did not completely restore apoptosis rates to baseline, suggesting that mechanical strain may also be causing apoptosis through non-angiotensin II dependent pathways.

It should be noted that the demonstration in the present study of apoptosis resulting from mechanical strain is in contrast to what we have previously reported [15]. However, a number of differences exist in the current experimental design that may account for these findings. First, we used conditionally immortalized murine podocytes grown under restrictive conditions in the current study, whereas we used proliferating primary culture mouse podocytes in our prior studies. Second, the presence of survival factors in serum in our prior studies (conducted in 5% FCS) may have blunted a proapoptotic effect of stretch. Finally, the magnitude of stretch was greater in the current studies. Perfusion studies performed in isolated glomeruli have demonstrated that
glomerular volume increases approximately 16% in response to capillary hypertension in disease [42]. If one views the glomerulus as a simple sphere whose volume can be approximated by the equation \(4/3 \cdot \pi \cdot r^3\), and glomerular volume is increased by 16% in situations of increased \(P_{\text{m}}\), then surface area of this “sphere” (as measured by the equation \(4 \cdot \pi \cdot r^2\)) is increased \(\sim 10.4\%\). Based on these assumptions, our choice of 10% mechanical stretch for the current studies is justified and we believe approximates conditions in the in vivo setting.

Angiotensin II has been shown to have a number of other effects on podocytes. Angiotensin II mediates rearrangement of the actin cytoskeleton and inhibiting angiotensin II prevents redistribution of the structural protein ZO-1 in the slit diaphragm [21]. It has been demonstrated that in diabetic nephropathy, levels of nephrin in podocytes are reduced, but can be restored to normal following angiotensin II blockade [22]. Furthermore, foot process broadening in experimental diabetic nephropathy was attenuated with angiotensin blockade [23]. Angiotensin II also activated specific podocyte signaling [43]. Taken together, these findings argue that the beneficial effects of angiotensin II blockade on reducing proteinuria and preventing glomerulosclerosis may not simply be the consequence of diminished glomerular capillary pressure [24], but may also be due to direct effects on podocytes.

CONCLUSION

We have demonstrated that mechanical strain, the result of glomerular capillary hypertension, leads to up-regulation of the AT1R as well as increased production of the ligand angiotensin II in podocytes. The resulting activation of a local tissue angiotensin system leads to an increase in podocyte apoptosis, mainly in an AT1R-mediated fashion. The clinical consequences of stretch-induced apoptosis may include progressive podocyte loss and glomerulosclerosis in diseases associated with glomerular capillary hypertension.

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