Interaction of Angiotensin II and Mechanical Stretch on Vascular Endothelial Growth Factor Production by Human Mesangial Cells

GABRIELLA GRUDEN,* STEPHEN THOMAS,* DAVINA BURT,* WUDIN ZHOU,†
GARY CHUSNEY,* LUIGI GNUDI,* and GIANCARLO VIBERTI*

*Unit for Metabolic Medicine, Department of Endocrinology, Diabetes and Internal Medicine; and
†Department of Nephrology and Transplantation, Division of Medicine, GKT School of Medicine, King’s College London, Guy’s Hospital Campus, London, United Kingdom.

Abstract. The antiproteinuric effect of angiotensin-converting enzyme inhibitors underscores the importance of a hemodynamic injury and the renin-angiotensin system in the proteinuria of various glomerular diseases. Vascular endothelial growth factor (VEGF), a potent promoter of vascular permeability, is induced in mesangial cells by both mechanical stretch and TGF-β1. This study investigates the effect of TGF-β blockade, angiotensin II (AngII), and the interaction between AngII and stretch on human mesangial cell VEGF production. Exposure to AngII (1 μM) induced a significant increase in VEGF mRNA and protein levels (1.5 ± 0.1 and 1.7 ± 0.3, respectively, fold increase over control, P < 0.05). The AngII receptor (AT1) antagonist Losartan (10 μM) prevented AngII-induced, but not stretch-induced, VEGF protein secretion (AngII 1.7 ± 0.3, AngII + Losartan 1.0 ± 0.1, P < 0.05; stretch 2.4 ± 0.4, stretch + Losartan 2.6 ± 0.5). Stretch-induced VEGF production was also unaffected by the addition of an anti-TGF-β neutralizing antibody (stretch 2.85 ± 0.82 versus stretch + anti-TGF-β 2.84 ± 0.01, fold increase over control). Simultaneous exposure to both AngII and stretch for 12 h had an additive effect on VEGF production (AngII 1.6 ± 0.1, stretch 2.6 ± 0.27, AngII + stretch 3.1 ± 0.35). Conversely, preexposure to stretch magnified AngII-induced VEGF protein secretion (unstretched + AngII 1.3 ± 0.0, stretched + AngII 1.9 ± 0.1, P < 0.01) with a parallel 1.5-fold increase in AT1 receptor levels. AngII and stretch can both independently induce VEGF production; in addition, mechanical stretch upregulates the AT1 receptor, enhancing the cellular response to AngII.

An increased glomerular permeability to protein is a key pathophysiologic feature of diabetic and other progressive glomerulopathies (1). The underlying mechanisms remain unclear, even though the antiproteinuric and renoprotective effects, in both human and animal studies, of BP-lowering drugs and particularly angiotensin-converting enzyme (ACE) inhibitors (2–4) strongly suggests the importance of the hemodynamic insult and implicates, in particular, the renin-angiotensin system (RAS).

Mesangial cells are in apposition to and in continuum with the glomerular capillaries and thus are a primary target for the mechanical insult induced by increased glomerular capillary pressure. The mesangium plays a crucial role in the glomerular trafficking of plasma protein, and their deposition and accumulation within the mesangium is believed to exert a local toxic effect, which may eventually lead to the development of sclerotic lesions, a typical feature of chronic progressive glomerular disease (5).

We have recently reported that mechanical stretching of cultured human mesangial cells, using an in vitro stretch model to mimic a hemodynamic insult, stimulates vascular endothelial growth factor (VEGF) gene expression and protein secretion (6). VEGF is a potent promoter of vascular permeability (7,8), induces proteinuria in rats (9), and has been implicated in the pathogenesis of the proteinuria of minimal change nephrotic syndrome (8). Thus, VEGF induction by a mechanical insult, secondary to glomerular pressure, may contribute to abnormal glomerular permeability.

Angiotensin II (AngII) affects the level of glomerular hemodynamic activity that may contribute to the progression of renal disease (11,12). In particular, AngII can directly induce mesangial cell TGF-β1 production, extracellular matrix deposition (13), and increased glomerular permeability to protein (14,15). However, it is unknown whether AngII stimulates mesangial cell VEGF production, a potentially important mechanism by which it may modulate glomerular permselectivity.
In cardiac myocytes, stretch-induced effects are partially mediated via AngII release (16). Furthermore, stretch and AngII act synergistically in inducing cardiac myocyte proliferation (17) and additively in inducing mesangial cell TGF-β1 expression (18). In turn, TGF-β1 can induce VEGF synthesis in human mesangial cells (19). The hemodynamic and nonhemodynamic actions of AngII may thus combine at the cellular level promoting glomerular injury. To date, there is no information in human mesangial cells on the role of AngII as a potential mediator of stretch-induced effects or on its interaction with stretch. Similarly, it is unclear whether TGF-β1 may be important as a mediator of stretch-induced VEGF production. The present study was designed to test whether AngII induces VEGF in human mesangial cells, to investigate the interactions between AngII and mechanical stretch on VEGF production, and to examine the role of TGF-β1 in stretch-induced VEGF production.

Materials and Methods

Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. RPMI 1640 culture medium and fetal calf serum were obtained from Life Technologies-BRL (Paisley, United Kingdom). Flex I and Flex II plates were from Flexcell Corp. (McKeesport, PA). The AngII antagonist Losartan-DuP753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-(1H-tetrazole-5-yl)biphenyl-4-hydroxymethyl] imidazole was kindly supplied by Dr. Ronald D. Smith (DuPont Company Merck & Co., Inc., West Point, PA). Monoclonal antihuman VEGF and pan-neutralizing anti-TGF-β antibodies were obtained from R&D Systems (Minneapolis, MN), and rabbit polyclonal antihuman VEGF antibody was from Serotec (Oxford, United Kingdom). Polyclonal rabbit anti-AT1 was purchased from Insight Biotechnology (Wembley, Middlesex, United Kingdom). The reverse transcription system was purchased from Promega (Southampton, United Kingdom), oligonucleotide primers were from Os威尔 (Southampton, United Kingdom), and AmpliTaq was from Perkin Elmer (Warrington, PA).

Cell Culture

Human mesangial cells were isolated as described previously (20). Briefly, normal renal cortex was taken from the opposite tumor-free pole of nephrectomy specimens, removed for localized, capsulated grade 1 hypernephromas. Intact glomeruli were collected by serial sieving of cortical homogenates. Tissue was analyzed by light microscopy and by immunofluorescence to exclude the presence of tumor cells and glomerular abnormalities (21). Cells obtained from three separate kidneys were used in this study. After digestion with collagenase (type IV, 750 U/ml), the isolated glomeruli were seeded in culture flasks. Following the outgrowth of mesangial cells, the glomeruli were removed by washing with phosphate-buffered saline and the cells were cultured in RPMI 1640, supplemented with insulin-transferrin-selenium and l-glutamine and containing 20% fetal calf serum, 7 mM glucose, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO2 incubator at 37°C. Mesangial cells were harvested using 0.25% trypsin and 0.5% ethylenediaminetetra-acetic acid. The cells were stellate or fusiform in appearance, grew in multilayers, formed hillocks in long-term culture, and stained for α-smooth muscle actin by direct immunofluorescence. Cells did not stain for cytokeratin, factor VIII, common leukocyte antigen (Dako, High Wycombe, United Kingdom) and Thy-1 (Serootech), excluding contamination of epithelial cells, endothelial cells, lympho-monocytes, and human fibroblasts (22). Studies were performed between passages 4 and 7, while the cells retain all the morphologic and immunofluorescent features described above.

Application of Mechanical Stretch to Cultured Cells

Mesangial cells were seeded in equal number (12,000/cm2) onto six-well collagen-coated silicone elastomer-base culture plates (Flex I plates) and control plates (Flex II plates). After 3 to 5 d, they were serum-deprived and incubated in insulin-free medium for 48 h, and then subjected to repeated stretch/relaxation cycles by mechanical deformation using a stress unit. The stress unit is a modification of the unit initially described by Barnes and coworkers (23) and consists of a vacuum unit and a baseplate. A vacuum was cyclically applied (60 cycles/min) to the rubber-base plates via the baseplate, which was placed in a humidified incubator with 5% CO2 at 37°C. Vacuum pressures sufficient to induce an average 10% uniaxial elongation in the culture surface, sufficient to induce VEGF (6), were applied. 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 (Flex II plates) in parallel.

Cell Number Determination

Cells were harvested with 0.25% trypsin and 0.5% ethylenediaminetetra-acetic acid, and the cell number was determined by a Coulter Cell Counter (Coulter Electronics, Luton Beds, United Kingdom).

mRNA Analysis

Total RNA was isolated using a commercial preparation based on a guanidium and phenol extraction (Triazol) and reverse-transcribed (1 μg) according to standard protocols using AMV reverse transcriptase and poly(dT). PCR was performed with oligonucleotide primers complementary with sequences located in exons 2 to 3 and 5 to 7 (24). The primer for exon 2 to 3 was based on that described by Iijima et al. (19), whereas the primer for exon 5 to 7 was designed to amplify specifically the 165 isoform of human VEGF, which lacks exon 6 (6,24). A single PCR product of 317 bp was obtained, the identity of which was confirmed by digestion with the restriction enzyme HindIII (Promega) yielding two fragments of 181 and 136 bp as predicted from the known cDNA sequence for VEGF165 (24). Expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined in parallel to control for amount of RNA input and reverse transcription efficiency using primer sequences reported previously (25). VEGF and GAPDH mRNA levels were quantified by competitive reverse transcription-PCR, using deletion-mutated cDNA to control for PCR amplification efficiency and for use in quantitative analysis (26). Competitor cDNA with a 50-bp deletion were generated by PCR as described previously (6,27), and PCR products were resolved in a 3% Nu-Sieve agarose gel containing ethidium bromide, analyzed by an image system (Eagle Eye System, Stratagene, Cambridge, United Kingdom), and quantified using densitometry analysis software (QGEL; Stratagene).

Protein Measurement

Culture supernatants from all experimental conditions were collected, centrifuged to remove cell debris, and stored at −70°C for analysis. VEGF protein concentration was measured by an in-house two-site immunoenzymometric assay, using a mouse monoclonal and a rabbit polyclonal antihuman VEGF165 (range, 1 to 40 pM; intra-assay coefficient of variation, 5.3%) (6). For each experiment, VEGF protein levels were determined within a single assay run. Ninety-six-
well microtiter plates were coated overnight at 4°C with a mouse monoclonal anti-VEGF antibody as the capture antibody. The plates were blocked with bovine serum albumin, after which the samples were added and incubated for 5 h. After washing, a rabbit polyclonal antihuman VEGF165 as the detection antibody was added and incubated overnight. Immunocomplexes were detected by alkaline phosphatase-conjugated goat-anti-rabbit IgG and revealed by 3,3′,5,5′-tetramethylbenzidine dihydrochloride substrate. The reaction was stopped with H2SO4 and the absorbance was measured at 450/690 nm. The assay also detects the VEGF 121 isoform, but no cross reactivity was detected with human platelet-derived growth factor, human TGF-β1, and bovine VEGF. All protein results were adjusted for cell number.

**Western Blotting**

Immunoblotting of AT1 protein was performed according to Kijima et al. (28). Total proteins from mesangial cell lysates were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. The membranes were blocked in 5% skim milk in Tris-buffer-Tween, pH 7.6, incubated with a specific rabbit polyclonal antihuman AT1 antibody, extensively washed in Tris buffer-Tween, then incubated with horseradish peroxidase-linked anti-rabbit antibody. Detection was performed by enhanced chemiluminescence, and the band intensity was quantified by laser densitometry. Equal protein loading of each lane was verified with Ponceau staining of total proteins on the nitrocellulose membranes.

**Statistical Analyses**

Presented in the figure legends is the number of experiments, which were carried out in triplicate. All data are presented as mean ± SEM. t test was used for comparison between two groups. When more than two groups were studied, data were analyzed by ANOVA and, if significant, the Neuman–Keuls procedure was used for post hoc comparisons. Values for P < 0.05 were considered significant.

**Results**

**AngII Induces VEGF Gene Expression in Human Mesangial Cells**

Cells were serum- and insulin-deprived for 48 h before the experiment because VEGF mRNA expression is stimulated by low concentrations of fetal calf serum (19) and by insulin ([29]; our data in mesangial cells not shown). After preliminary experiments to establish the optimum concentration of AngII, VEGF165 RNA levels were measured after 4-, 6-, and 12-h exposure to either AngII (1 μM) or vehicle. VEGF165 mRNA was expressed in basal conditions and was induced by AngII after 6 h (1.5 ± 0.09-fold increase, P < 0.05) (Figure 1) with a return to baseline levels by 12 h.

**AngII Induces VEGF Protein Secretion via the AT1 Receptor**

To investigate the effect of AngII on VEGF protein production, serum- and insulin-deprived mesangial cells were exposed to increasing AngII concentrations (10⁻¹⁰, 10⁻⁸, 10⁻⁶, 10⁻⁴ M) for various time periods (6, 12, 24, and 48 h). Maximal VEGF protein production was seen at a dose of 1 μM. AngII-induced VEGF protein production increased by 12 h (1.74 ± 0.25-fold increase, P < 0.05) and progressively diminished toward baseline by 48 h (Figure 2, a and b). AT1 is the AngII receptor expressed by adult human mesangial cells and is responsible for all of the known actions of AngII (30,31). Supplementation with Losartan (10 μM), a selective and specific AT1 receptor antagonist (32), had no effect on basal VEGF secretion, but completely abolished AngII (1 μM, 12 h)-induced VEGF protein production (Figure 3a).

**Stretch-Induced VEGF Secretion Is Independent of AT1 Receptor**

Serum- and insulin-deprived mesangial cells were exposed to cyclical stretch (elongation 10%) for 12 h in the presence or in the absence of Losartan (10 μM). Stretch induced a significant 2.4-fold increase in VEGF secretion, which was unaffected by the addition of Losartan (stretch 2.40 ± 0.40 versus stretch + Losartan 2.62 ± 0.53, fold increase over control) (Figure 3b), indicating that stretch-induced VEGF protein production is independent of the AT1 receptor.

**Stretch-Induced VEGF Secretion Is Independent of TGF-β1**

Serum- and insulin-deprived mesangial cells were exposed to cyclical stretch (elongation 10%) for 12 h in the presence or in the absence of a specific pan-neutralizing anti-TGF-β antibody (20 μg/ml). The concentration of anti-TGF-β antibody used was chosen to ensure a condition of large antibody excess. No TGF-β activity was detectable in the supernatant in the presence of the neutralizing antibody when tested using the mink lung cells (data not shown). Stretch induced a significant 2.85-fold increase in VEGF secretion, which was unaltered by the inhibition of TGF-β activity (stretch 2.85 ± 0.82 versus stretch + anti-TGF-β1 2.84 ± 0.01, fold increase over control) (Figure 3c), indicating that VEGF protein production in response to stretch is independent of TGF-β1.
Simultaneous Exposure to AngII and Stretch Additively Enhance VEGF Production

To study the effect of combined exposure to AngII and stretch on human mesangial cell VEGF production, serum- and insulin-deprived mesangial cells were exposed to stretch (elongation 10%) and/or AngII (1 μM) for 12 h. AngII and stretch had a numerically additive effect on VEGF production (AngII 1.60 ± 0.13, stretch 2.59 ± 0.27, AngII + stretch 3.07 ± 0.35, fold increase over control) (Figure 4), but this was not statistically significant compared with stretch alone.

Preexposure to Stretch Augments the Cellular VEGF Response to AngII

Serum- and insulin-deprived mesangial cells were exposed to stretch (elongation 10%) for 12 h. The conditioned medium was removed, and after repeated washes in a phosphate buffer,
both prestretched and control unstretched cells were exposed for an additional 12 h to AngII (1 μM) or vehicle in fresh culture media. Cells preexposed to stretch and then exposed to vehicle for an additional 12 h showed a slight but nonsignificant rise in VEGF compared with unstretched cells exposed to vehicle (1.09 ± 0.06-fold increase, prestretch + vehicle versus unstretched + vehicle; \( P = \text{NS} \)). AngII induced a 1.33-fold increase in unstretched cells (unstretched + AngII versus unstretched + vehicle; \( P < 0.05 \)) and a twofold increase in cells preexposed to stretch (prestretched + AngII versus unstretched + vehicle, \( P < 0.001 \); prestretched + AngII versus unstretched + AngII, \( P < 0.01 \)) (Figure 5a).

**Prestretch Amplifies the AngII effect by Induction of the AT1 Receptor**

To investigate the mechanism of the magnified AngII effect in prestretched cells, the AT1 receptor was studied in total protein extracts from mesangial cells. An upregulation in the AT1 receptor (1.5 ± 0.05-fold increase over control, \( P < 0.05 \)) was seen in cells prestretched for 12 h and then exposed to either AngII or vehicle for another 12 h (Figure 5b). In time-course experiments, it was found that the application of mechanical stretch for 24 h increased the AT1 receptor level by 1.8 ± 0.11-fold over control (\( P < 0.05 \)) with no change after 12 h (1.16 ± 0.3-fold increase over control).

**Discussion**

The present study demonstrates that AngII and stretch independently induce the production of VEGF, one of the most powerful promoters of vascular permeability, in human mesangial cells.

Exposure to AngII induced a significant increase in VEGF secretion, peaking after 12 h and returning to baseline by 48 h, with a temporally related increase in VEGF mRNA. There was a peak effect on VEGF secretion at an AngII dose of 1 μM, consistent with previous reports on AngII-induced TGF-β1

![Figure 4](image_url)

Figure 4. Effect of combined AngII and stretch on VEGF protein secretion. Serum- and insulin-deprived mesangial cells were exposed for 12 h to mechanical stretch and/or AngII (1 μM). VEGF protein levels were measured as described in Materials and Methods and expressed as fold increase versus control (\( n = 6 \)). *\( P < 0.05 \) control versus others.

![Figure 5](image_url)

Figure 5. Prestretching enhances VEGF response to AngII via upregulation of AT1 receptor. (A) Serum- and insulin-deprived mesangial cells were exposed for 12 h to mechanical stretch. The conditioned medium was removed, and after repeated washes in phosphate buffer, both prestretched cells and control unstretched cells were exposed for an additional 12 h to either AngII (1 μM) or vehicle in fresh culture media. VEGF protein levels were measured as described in Materials and Methods and expressed as fold increase versus control (\( n = 3 \)). *\( P < 0.05 \) AngII versus control; †\( P < 0.01 \) prestretched + AngII versus unstretched + AngII; **\( P < 0.001 \) prestretched + AngII versus others. (B) Cell lysates from 12-h prestretched and control mesangial cells were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, and the AT1 receptor was detected by immunoblotting using a rabbit polyclonal antihuman AT1 antibody. The arrow indicates the specific band for AT1 (\( n = 3 \); \( P < 0.05 \)). MWM, molecular weight marker.
expression in rat mesangial cells (13). At doses higher than this, there was a diminution in AngII-induced VEGF production, a phenomenon also reported for other in vitro AngII effects, such as mesangial cell proliferation, which may relate to toxicity at these high concentrations (33). Although a 1 μM AngII concentration is high compared with AngII plasma levels, local AngII levels in the kidney are reported to be significantly higher than those found in the circulation (34,35).

AngII induced VEGF protein secretion maximally by 12 h, a similar time course to that described for serum, platelet-derived growth factor, 12-0-tetradecanoylphorbol-13-acetate, and stretch-induced VEGF (6,19,36). After this there was a progressive reduction in AngII-induced VEGF production possible due to desensitization or downregulation of the AngII receptor (AT1) (37,38). This progressive reduction in response with time is a common feature of response to in vitro stimuli.

Several known AngII-induced effects, such as TGF-β1 expression and cell growth, are critically modulated by the presence of insulin and serum (39–41). Both serum and insulin are, however, potent inducers of mesangial cell VEGF production in their own right (19,29). The present study therefore was performed on serum- and insulin-deprived cells. This may in part explain the more modest effect of AngII on mesangial cell VEGF production compared with that reported in vascular smooth muscle cells (42) or observed for other AngII-induced cytokines (13,43).

The complete inhibition of AngII-induced VEGF production by Losartan indicates a specific AngII effect occurring via the AT1 receptor. Although two AngII receptors have been described in human tissues (AT1 and AT2), only the AT1 subtype is expressed on human mesangial cells, and its activation is responsible for all of the known biologic effects of AngII in human mesangial cells (30). Furthermore, AT1 blockade completely prevents the increase in glomerular permeability to proteins seen in rat kidneys perfused with AngII (15).

We have previously described that both tyrosine kinase and protein kinase C activation are important in stretch-induced VEGF production in human mesangial cells (6). In cardiac myocytes, stretch also activates these intracellular signaling pathways as well as inducing the local release of AngII, which then acts in an autocrine manner (9,44). Although human mesangial cells share similarities with cardiac myocytes, stretch-induced VEGF production is unlikely to be mediated via AngII, because it is independent of the AT1 receptor. Whether AngII has a role in other stretch-induced effects in this cell type remains uncertain.

We and others have previously demonstrated that stretch induces in mesangial cells the production of TGF-β1 (45,46), a known inducer of VEGF expression (19). However, the addition of a TGF-β neutralizing antibody did not affect stretch-induced VEGF secretion, indicating that the direct effect of stretch is independent of TGF-β1. This of course does not exclude a late, indirect effect whereby stretch-induced TGF-β1 may augment the VEGF response.

The simultaneous exposure to AngII and stretch had an additive effect on VEGF production, similar to that reported in stretch and AngII-induced TGF-β1 expression in rat mesangial cells (18). In addition, our results suggest that stretch may augment the effect of AngII on human mesangial cell VEGF production by upregulating the AT1 receptor. Preexposure to stretch for 12 h resulted in a threefold increase in AngII-induced VEGF production. This is unlikely to be due to a direct effect of stretch, because stretch-induced VEGF protein secretion peaks at 12 h, returning to baseline by 24 h with maximal VEGF mRNA levels at 6 h disappearing by 12 h (6). Because VEGF protein concentrations in the prestretching experiments were determined after 24 h, 12 h after the withdrawal of stretch, we would expect very little direct stretch-induced VEGF protein production. Indeed, there was a comparatively small and nonsignificant increase in VEGF protein after 24 h in prestretched cells compared with control unstretched cells.

The stretch-induced magnification of the VEGF response to AngII was likely to be mediated by the observed 1.5-fold upregulation of the AT1 receptor. This effect on the AT1 receptor was not seen after 12 h of stretch but only at 24 h, explaining perhaps why the simultaneous application of AngII and stretch only had an additive effect at 12 h on VEGF production.

These findings could have important pathophysiologic implications in clinical renal disease. AngII infusion increases protein excretion rate in animal studies (14), and both ACE inhibitors and AT1 receptor antagonists have powerful antioxidant and renoprotective actions in animal and human studies (2,3,4,10).

AngII exerts a powerful effect on renal hemodynamics and intraglomerular pressure, which may in turn affect VEGF production via mechanical stretch. We have previously shown that the degree of stretch required to induce VEGF would only occur in glomeruli exposed to high intracapillary pressure (6). AngII may induce VEGF via a nonhemodynamic mechanism by thus further exacerbating proteinuria.

In conclusion, the complex interaction between AngII and mechanical insult on VEGF reported here raises the interesting possibility that the preexistent intraglomerular pressure level modulates AngII effects on mesangial cells. Furthermore, it also suggests that the deleterious effect of high intraglomerular pressure results not only from a direct mechanical insult, but also from an enhanced response to AngII. The renoprotective effect of ACE inhibitors and AngII receptor antagonists may thus result from reductions of both the direct action of AngII and the hemodynamic insult.

Acknowledgments
This work was supported by British Diabetic Association Grant RG/95/0001151, by Novo Nordisk UK Grant 1998, and by the Special Trustees of Guy’s Hospital. Dr. Thomas was supported by a JDFI fellowship. Dr. Gruden is a fellow of the British Diabetic Association. We thank Mr. Remo E. Gruden for helpful technical assistance, Dr. Ronald D. Smith (DuPont Company Merck & Co., Inc) for the gift of DuP753, and Professor Steven Sacks for assistance with mesangial cell culture.

References