Glomerular podocytes: A study of mechanical properties and mechano-chemical signaling

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ABSTRACT

Kidney glomeruli function as filters, allowing the passage of small solutes and waste products into the urinary tract, while retaining essential proteins and macromolecules in the blood stream. These structures are under constant mechanical stress due to fluid pressure, driving filtration across the barrier. We mechanically stimulated adherent wildtype podocytes using the methods of magnetic tweezer and twisting as well as cell stretching. Attaching collagen IV-coated or poly-L-lysine-coated magnetic beads to cell receptors allowed for the determination of cellular stiffness. Angiotensin II-treated podocytes showed slightly higher stiffness than untreated cells, the cell fluidity (i.e. internal dynamics) remained similar, and showed an increase with force. The bead detachment (a measure of the binding strength) was higher in angiotensin II-treated compared to untreated podocytes. Magnetic twisting confirmed that angiotensin II treatment of podocytes increases and CDTA treatment decreases cell stiffness. However, treatment with both angiotensin II and CDTA increased the cell stiffness only slightly compared to solely CDTA-treated cells. Exposing podocytes to cyclic, uniaxial stretch showed an earlier onset of ERK$^{1/2}$ phosphorylation compared to MEF (control) cells. These results indicate that angiotensin II might free intracellularly stored calcium and affects actomyosin contraction, and that mechanical stimulation influences cell signaling.

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1. Introduction

Chronic kidney disease (CKD) is among the leading health problems worldwide and options for treatment are limited to dialysis and kidney transplantation. Only recently, CKD as well as proteinuria have been attributed to the dysfunction of the glomeruli and the damage and loss of podocytes [1]. Glomerular podocytes are highly specialized epithelial cells with a complex cytoarchitecture that cover the outer layer of the glomerular basement membrane. Podocytes consist of cell bodies, major processes, and most prominently of foot processes of ~12 μm length and ~200 nm width which culminate between adjacent cells [2]. Specialized structures known as slit diaphragms function as modified adherens junctions connecting the podocyte foot processes. Since blood filtration is accomplished through a membrane comprising of three layers: endothelial cells, the glomerular basement membrane, and podocytes as an outer layer, podocytes represent the weak spot of the glomeruli. To date these cells are the focus to treat CKD disease and proteinuria.

Podocytes react in a stereotypic pattern to various damaging events, e.g. effacement (= loss) of foot processes results in reduced filtration and leakage of proteins (proteinuria) [3–5]. Foot processes are shaped by the actin cytoskeleton and adhere to the glomerular basement membrane. Mutation or loss of actin-associated proteins, focal adhesion proteins, and extracellular matrix (ECM) proteins of podocytes cause glomerular disease and renal failure in humans and transgenic mice [6]. Proteins essential for podocytes' viscoelastic and signaling properties include actin, myosin, α3/β1 integrin, ERK$^{1/2}$, Cas^{130}, and collagen IVα3, α4, α5 [7]. However, the molecular mechanisms that govern formation, maintenance, or effacement of foot processes are largely unknown.

It has previously been reported by several research groups that the polypeptide angiotensin II (Ang II) influences podocytes on many levels: the glomerular function (that can lead to CKD and proteinuria), the internal calcium release, and the cytoskeleton [8,9]. Other researchers described the effect of physiological hydrostatic pressure or external stimulus (stretch) on podocytes which react to Ang II treatment with a change in cell proliferation and pERK$^{1/2}$ signaling as well as with an increase in $[Ca^{2+}]_i$ [6,10,11]. The aim of this study is to analyze podocyte mechanics. Using

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various biophysical methods, we elucidated the complex interplay of cell stiffness, fluidity, and binding strength upon angiotensin (AT1) receptor stimulation by Ang II and calcium depletion by CDTA as well as the influence of mechanical stimulation on cell signaling.

2. Materials and methods

2.1. Cell culture, calcium, and angiotensin II

The immortalized mouse podocytes were a kind gift from Dr. J. Reiser, Miller School of Medicine, University of Miami [12]. Frozen

Fig. 1. Effect of angiotensin II on cell stiffness, fluidity, and binding strength using the magnetic tweezer. (A) Cell stiffness and (B) cell fluidity of untreated and with 100 nM angiotensin-treated podocytes were determined. (A, inset) shows the principle of a magnetic tweezer. The percentage of beads detached from the cells vs. pulling force are shown in (C). The adhesion strength was significantly lower at 100 nM angiotensin II-treated compared to untreated podocytes. Conditions: $n = 86$ of angiotensin II-treated cells, blue; and $n = 65$ of untreated cells, green; (mean value, ±S.E.). Note, that the x-axes in (A + B) are shown on a logarithmic and in (C) on a normal scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

podocytes were thawed at 37 °C and then incubated in RPMI 1640 (Biochrom), 10% FBS, 100 Units/mL Penicillin and 100 μg Streptomycin and γ-interferon (40 units/mL in the first two passages; 20 units/mL after the second passage; Gibco) at 33 °C. At ~80% confluency, the cells were passaged. For differentiation, the cells were incubated at 37 °C for 10 days in above medium without γ-interferon (modified [12]), and for proper cell attachment the flasks were collagen IV-coated (25 μg/mL; Sigma). Prior to experimentation, cells were serum-starved for 3 h. Magnetic tweezer and magnetic twisting measurements in the presence of 100 nM and 10 μM angiotensin II (Sigma), respectively, were performed 3 h after the compound addition. The calcium chelator CDTA (10 mM; Merck) was only used in magnetic twisting experiments.
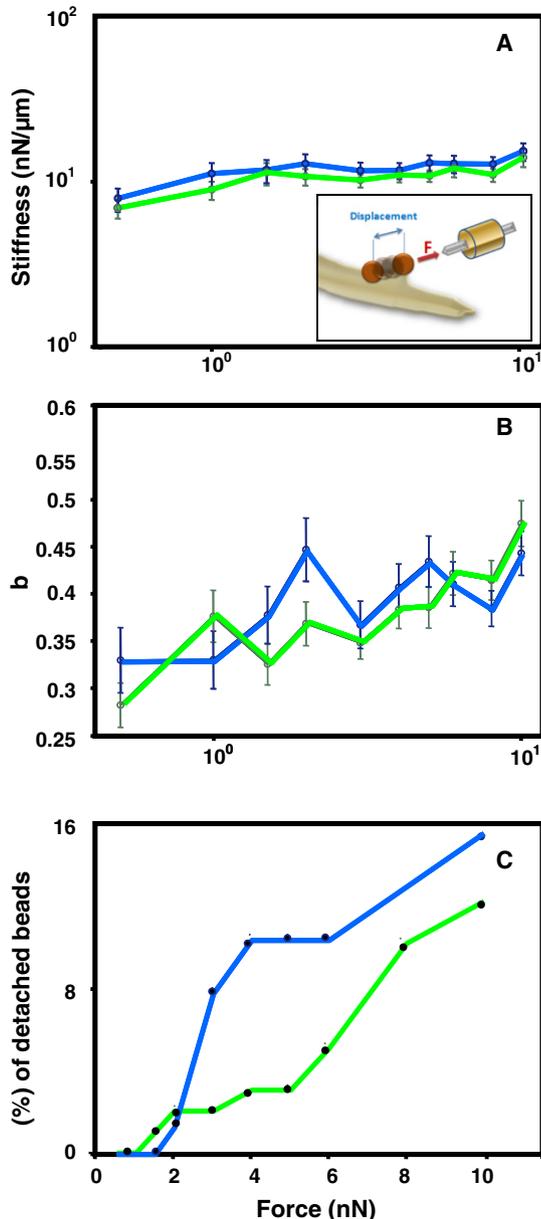
2.2. Magnetic tweezer

The principle of the magnetic tweezer device used was previously described in [13]. Single cells are deformed by generating a local magnetic field that attracts super-paramagnetic beads bound to the cells (Fig. 1A, inset). These beads (4.5 μm, Dynabeads M450; Invitrogen) at a concentration of 1×10^7 beads/mL are coated with collagen IV (100 μg/ml; Sigma) in PBS at 4 °C overnight. Beads are then washed three times in PBS and stored in this buffer at 4 °C. Before measurements, the beads are sonicated to avoid clustering. About 2×10^5 beads per 35 mm dish are added, and cells are incubated with the beads for 30 min at 5% CO_2 and 37 °C. Thereafter, the medium is exchanged with freshly, prewarmed medium to remove unbound beads. Measurements are performed on a heated inverted microscope stage at 40× magnification (NA 0.6) without CO_2. The measuring time was limited to 30 min per dish. When a force step with an amplitude ΔF was applied to a cell-bound bead, it moved with a displacement $d(t)$ towards the tweezer needle tip. The ratio $d(t)/\Delta F$ defines the creep response $J(t)$. $J(t)$ of the cells follows a power law: $J(t) = a(t/t_0)^b$, where the pre-factor a and the power law exponent b are both force-dependent, and the reference time t_0 is set to 1 s. The values for a and b are determined by a least-squares fit. The pre-factor a (units of μm/nN) characterizes the elastic cell properties and corresponds to compliance (i.e. inverse of stiffness). b reflects the dynamics of the force-bearing structures of the cell that are connected to the bead. Note, that a power law exponent of $b = 0$ indicates a purely elastic solid and $b = 1$ a purely viscous fluid. In cells, the power law exponent usually falls in the range between 0.1 and 0.5, whereby higher values have been linked to a higher turnover rate of cytoskeletal structures [14]. Higher b values are often associated with reduced cell stiffness.
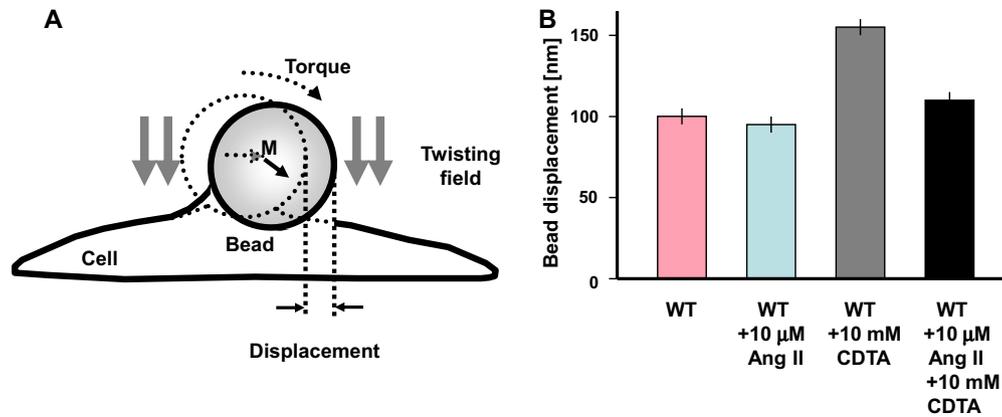
2.3. Bead detachment

The detachment of beads that are bound to cells for 30 min is measured during force application ranging between 0.5 and 10 nN. The percentage of all detached beads in relation to the detachment force is used as a measure for the bead binding strength to the cell.

### 2.4. Magnetic twisting cytometry with optical detection (OMTC)

Details of the magnetic twisting device are described in [15,16]. Controlled mechanical stresses are applied directly to cell surface receptors using ferromagnetic microbeads precoated with poly-L-lysine. The beads are prepared by gentle shaking for 2 h at room temperature in a 0.2 M sodium phosphate buffer at pH 7.4, then washed three times in PBS (80 mM Phosphate, 1.37 M NaCl, pH 7.4; BostonBioproducts) for at least 1 h prior to use. Adherent podocytes spread for 4 h on collagen IV-coated 35 mm ∅ dishes. Poly-L-lysine-coated beads were bound for 7 min to the cells in

Fig. 2. Effect of angiotensin II on cell stiffness in the presence and absence of calcium using a magnetic twisting device (A, image taken from [16] with permission). Cell stiffness (low displacement amplitude indicates high cell stiffness; mean value, ±S.E.) of untreated wildtype (WT) podocytes (n = 530, red), in presence of 10 μM angiotensin II (n = 293, blue), in presence of 10 mM CDTA (calcium chelator; n = 287 cells, gray), and in the presence of 10 μM angiotensin II and 10 mM CDTA (n = 144 cells, black) (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
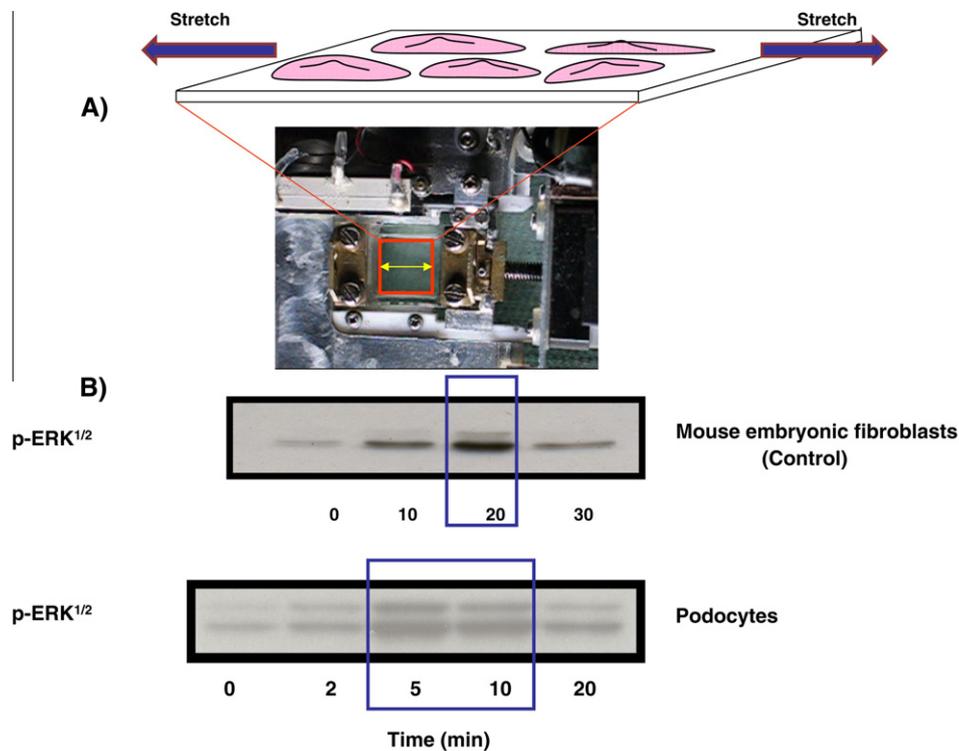
Fig. 3. Stretching of podocytes. Image and principle of the cell stretcher (A). Wildtype cells were seeded on fibronectin-coated silicone stretch insets, incubated for 72 h, and then stretched in serum-depleted medium at 1 Hz and 20% strain for up to 30 min (B). Cells were lysed and analyzed by Western blotting against phosphorylated ERK$^{1/2}$. MEFs were used as control cells.

serum -and growth factor free medium to, and unbound beads were washed off using PBS. Bound beads were magnetized horizontally using a 1000 Gauss field for 100 μs. A 30–50 Gauss oscillatory (0.5 Hz) magnetic field was then applied in the horizontal direction for 2 min to rotate the beads in place, thereby exerting a rotational shear force on the beads bound to the cells (Fig. 2A). The amplitude of the oscillatory bead displacements was extracted from images taken every 250 ms using a method described in [16], and the amplitude was averaged over the total measurement period. While forces are somewhat smaller compared to magnetic tweezer (force couples of up to ~1 nN), they are homogenously applied over a large area and on many cells [17]. In addition, a large number of cells are microscopically observed and measured at the same time.

2.5. Cell stretcher

Cell stretching experiments were carried out on a flexible silicone substrate, which can be stretched for mechanical stimulation (Fig. 3A). The cell stretcher was custom-built with a flexible stretch inset of 20 × 20 mm internal surface (B-bridge International). To seed the cells, the insets are coated with 25 μg/mL fibronectin in PBS for overnight at 4 °C. After coating, the insets are washed twice with PBS. Cells are seeded at least 24 h before stretching. The medium (RPMI 1640; Biochrom, 10% FBS, 100 Units/mL Penicillin and 100 μg Streptomycin) was exchanged for serum-depleted growth medium 12 h before stretching in all experiments. Stretching was performed in an incubator under normal conditions (37 °C, 5% CO_2, 95% humidity) for up to 30 min at 1 Hz and 20% strain.

2.6. Western blotting

After stretching, adherent cells were washed with PBS, lysed with ice cold RIPA buffer containing 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% Triton × 100, 0.5% sodium deoxycholate, 0.1% SDS, a protease inhibitor cocktail, and boiled immediately in Laemmli buffer (BostonBioproducts). An aliquot of pure protein lysate was used for protein quantification in Bradford assays. Equal amounts of protein extracts (12–50 μg) were loaded on 10–15% Tris–Glycine SDS–Poly-acrylamide gels and separated at 130 V. Polyvinylidenefluoride (PVDF) membranes were equilibrated for 20 min in transfer buffer containing 25 mM Tris-base, 192 mM glycine, and 10% methanol, and the polyacrylamide gels were blotted at 100 V for 1 h. The blotted membranes were saturated with blocking buffer (5% dry milk powder, 0.1% Tween, TBS). The pERK[1/2] antibody (Thr202/Tyr204; New England Biolabs) was diluted in blocking buffer at the given ratios. The membrane was incubated for at least 1 h with the antibody solution, then washed in 0.1% Tween-20 (TBS), incubated with HRP–conjugated secondary antibody, and finally washed to remove the excess of the HRP conjugate. An ECL solution was used for chemoluminescence reaction, and detection was carried out in a dark room with chemiluminescence hypersensitive films. Stripping of membranes was performed in 0.6 mM Tris pH 6.8, 2% SDS, 0.7% (v/v) β-mercaptoethanol at 50 °C for 30 min.

3. Results

3.1. Angiotensin II influences cell stiffness, cytoskeletal dynamics, and adhesion strength

We used the magnetic tweezer method to apply forces of up to 10 nN to collagen IV-coated, super-paramagnetic beads. After a stepwise increase in force (creep measurement), the bead displacement followed a power law. The creep response, $J(t) = a(t/t_0)^b$ was determined for podocytes as the ratio of bead displacement $d(t)$ and the amplitude of the step force ΔF, which can be divided into an elastic response (cell elasticity or stiffness, $1/a$) and a frictional response (cytoskeletal fluidity, b) [18]. Podocytes treated with 100 nM angiotensin II were slightly stiffer compared to untreated cells, and the differential cell stiffness increased with increasing force (Fig. 1A). Both, angiotension II-treated and untreated podocytes displayed similar fluidity indicating equal internal actin dynamics. In both cases, the cytoskeletal fluidity increased with increasing forces imposed on the beads (Fig. 1B).

The binding strength between the beads and cells was determined by applying a force to the beads that increased over time from 0.5 to 10 nN in a staircase-like fashion. The force value at which a bead detached from the cell surface was measured. For forces above 2 nN, the fraction of beads that detached from the cell surface was significantly higher for angiotensin II-treated compared to untreated cells (Fig. 1C).

3.2. Angiotensin II and calcium affect cell stiffness

Prior to measurements using wildtype podocytes, we calibrated the magnetic twisting device to enable us to estimate the apparent cell stiffness. Here, poly-L-lysine-coated magnetic beads attached to podocytes were briefly magnetized in the horizontal direction before an oscillatory magnetic field was applied for 120 s. The bead displacement amplitude was taken as a measure of cell compliance (inverse of cell stiffness) [16,19]. The bead displacement amplitude of angiotensin II-treated and untreated podocytes in the presence or absence of calcium are shown in Fig. 2B. Bead displacements were small (on the order of 100 nm) and were significantly

($p < 0.05$) decreased in angiotensin II-treated (blue) compared to untreated wildtype (WT) cells (red), indicating that cell stiffness increased after angiotensin II treatment [20]. Removing calcium from the cell medium using 10 mM CDTA (gray) increased the amplitude by around 50% compared to untreated WT cells (red); and the addition of both 10 μM angiotensin II and 10 mM CDTA (black) resulted in a ~20% decrease in amplitude compared to purely CDTA-treated cells (gray). These results indicate that the removal of extracellular and probably free intracellular calcium (due to the chelation of CDTA) leads to a weakening of the actin cytoskeleton [21], whereas angiotensin II treatment leads to an increase of intracellular calcium and to a subsequent strengthening of the actin cytoskeleton [8,22]. Note, that in these experiments poly-L-lysine-coated magnetic beads were used instead of collagen IV-coated magnetic beads since calcium influences integrin-bead binding and integrin signaling.

3.3. Cell stretcher and ERK[1/2] phosphorylation

Recent research has shown that angiotensin secretion is strongly induced by cyclic stretch in cardiovascular systems and that one of the major pathways induced by angiotensin II is the ERK[1/2] phosphorylation pathway [23]. Further, Naruse et al. [24] used cyclic, uniaxial stretch at a frequency of 1 Hz and at 20% strain and also reported phosphorylation of ERK[1/2] in endothelial cells. We therefore tested whether podocytes react to stretch with enhanced ERK[1/2] phosphorylation levels. The podocytes adhering to fibronectin-coated silicone stretch insets were cyclically stretched for up to 30 min, and ERK[1/2] phosphorylation was then examined by Western blotting. ERK[1/2] phosphorylation in podocytes was at its peak after 5–10 min compared to MEFs (control) at ~20 min (Fig. 3B). To analyze the reorientation of podocytes, they were stretched for 2 h at 1 Hz and 20% strain. Podocyte reorientation was not as obvious as reported for endothelial cells [24], but patches of realigned cells could be detected (data not shown). In summary, podocytes responded to uniaxial and cyclic stretch by ERK[1/2] phosphorylation and reorientation [25], however, further detailed work needs to be done.

4. Discussion

Mechanical tension transmitted between the extracellular matrix and the cytoskeleton plays a critical role in determining cell structure and function [26–29]. Since cell stiffness depends on multiple factors including the number and the combined bond elasticity of molecular interactions that transfer mechanical forces between cell and probe, we examined the influence of angiotensin II on podocyte mechanics in the presence and absence of calcium. The results of this study, however, are inconclusive to decide whether the slightly reduced stiffness or changed internal dynamics of untreated compared to angiotensin-treated podocytes was due to lower calcium release from intracellular stores to stimulate actomyosin. Hsu et al. [9] had previously reported that the stimulation of angiotensin II receptors on podocytes can lead to the release of calcium from intracellular stores in stably transfected podocytes expressing angiotensin receptor-rich AT1 and to a lesser degree in wildtype podocytes. Such calcium release can affect actomyosin interactions and cell stiffness.

The bead-binding strength (defined as the force that is necessary to detach a bead from the cell) showed significant differences between angiotensin II-treated and untreated podocytes. Similar to cell stiffness, this strength depends on the number of molecular interactions that transfer mechanical forces between the cell and bead, specifically between (i) the collagen IV-coated bead and integrins, (ii) integrins and the focal adhesion proteins, and (iii) focal

adhesion proteins and the cytoskeleton. Unlike cell stiffness, the binding strength also depends on the yielding force of those molecular interactions. The fraction of beads that detached over the entire force range remained higher in angiotensin II-treated cells, indicating that collagen IV-coated bead binding to integrins might either be weakened by Ang II treatment despite higher internal cell stiffness or reflects a different degree of integrin activation and signaling.

At this stage of our research we can only speculate whether $ERK^{1/2}$ phosphorylation upon cyclic stretch really depends on angiotensin II and/or increasing intracellular calcium concentration. However, the peaked increase of $ERK^{1/2}$ phosphorylation occurs much faster in podocytes than in MEF cells. Cyclic stretch experiments on primary human umbilical cord fibroblasts identified a similar time-limited increase in $ERK^{1/2}$ phosphorylation within the first few minutes (as found in podocytes) which could argue for a universal response to strain (data not shown).

In conclusion, AT1 receptor stimulation by angiotensin II probably leads to the release of intracellularly stored calcium which affects cell stiffness (i.e. actomyosin interaction), weakens the linkage of the integrin-focal adhesion-cytoskeleton, and affects signaling in podocytes. To support these assumptions and to elucidate mechano-chemical signaling in podocytes in more detail, further mechanical as well as biochemical experiments are needed.

Acknowledgments

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