

# Angiotensin II has acute effects on TRPC6 channels in podocytes of freshly isolated glomeruli

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**A key role for podocytes in the pathogenesis of proteinuric renal diseases has been established. Angiotensin II causes depolarization and increased intracellular calcium concentration in podocytes; members of the cation TRPC channels family, particularly TRPC6, are proposed as proteins responsible for calcium flux. Angiotensin II evokes calcium transient through TRPC channels and mutations in the gene encoding the TRPC6 channel result in the development of focal segmental glomerulosclerosis. Here we examined the effects of angiotensin II on intracellular calcium ion levels and endogenous channels in intact podocytes of freshly isolated decapsulated mouse glomeruli. An ion channel with distinct TRPC6 properties was identified in wild-type, but was absent in TRPC6 knockout mice. Single-channel electrophysiological analysis found that angiotensin II acutely activated native TRPC-like channels in both podocytes of freshly isolated glomeruli and TRPC6 channels transiently overexpressed in CHO cells; the effect was mediated by changes in the channel open probability. Angiotensin II evoked intracellular calcium transients in the wild-type podocytes, which was blunted in TRPC6 knockout glomeruli. Pan-TRPC inhibitors gadolinium and SKF 96365 reduced the response in wild-type glomerular epithelial cells, whereas the transient in TRPC6 knockout animals was not affected. Thus, angiotensin II-dependent activation of TRPC6 channels in podocytes may have a significant role in the development of kidney diseases.**

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Nephrotic syndrome is a group of kidney diseases characterized by heavy proteinuria, hypoalbuminemia, edema, and dyslipidemia. Urinary losses of macromolecules in nephrotic syndrome reflect a dysfunction of the highly permselective glomerular filtration barrier. In the past decade, genetic studies have led to the identification of proteins that have a crucial role in slit-diaphragm signaling and maintenance of podocyte integrity and functions.<sup>1</sup> Particularly, the gene encoding transient receptor potential canonical channel 6 (TRPC6) was identified as the basis for an autosomal dominant form of focal segmental glomerulosclerosis (FSGS).<sup>2,3</sup>

Interstitial angiotensin II (Ang II), a major bioactive product of the renin-angiotensin system, is found to be the key mediator of renal inflammation and fibrosis in progressive chronic nephropathies.<sup>4</sup> It was shown that the expression of Ang II and its receptor is increased in patients with progressive glomerulopathies.<sup>5</sup> It was also demonstrated that Ang II application increased intracellular calcium ( $[Ca^{2+}]_i$ ) in the podocytes.<sup>6-8</sup> As TRPC6 channel mutations were found in patients with FSGS, members of the TRPC family emerged as prime candidates for this increase in  $[Ca^{2+}]_i$ .

Ang II can act through two different types of receptors, AT<sub>1</sub> and AT<sub>2</sub>, which are both involved in regulation of intracellular signals in podocytes.<sup>9</sup> However, the majority of Ang II actions in the glomerulus are mediated by AT<sub>1</sub>. It was shown that increased AT<sub>1</sub> signaling in podocytes leads to proteinuria and FSGS.<sup>10</sup> Studies in models of chronic hypertension and protein-induced renal damages revealed that inhibition of AT receptors is effective against proteinuria.<sup>11</sup> AT<sub>1</sub> receptor antagonist candesartan ameliorates the peak level of proteinuria by preventing a reduction in the expression of slit-diaphragm functional molecules.<sup>12</sup> Human trials demonstrated that the inhibition of AT<sub>1</sub> receptors delayed disease progression in patients with diabetic kidney disease.<sup>13,14</sup>

Recent studies demonstrated that Ang II enhances albuminuria by activating TRPC6 channels.<sup>15</sup> Furthermore, Zhang *et al.*<sup>16</sup> showed that alteration of TRPC6 expression and Ca<sup>2+</sup> influx is involved in Ang II-induced apoptosis.

Besides, it was highlighted that the deleterious effects of Ang II on podocytes and its pathogenic role in glomerular diseases coincides with enhanced TRPC6 expression<sup>17</sup> and that Ang II activation of TRPC6 channels in rat podocytes requires the generation of reactive oxygen species.<sup>18</sup> However, the exact mechanisms of action of Ang II in intact glomeruli remain unclear. Furthermore, it is not clear whether this hormone mediates changes in the number of channels at the plasma membrane and/or channel gating.

We demonstrate here that Ang II upregulates TRPC6 activity in intact podocytes of freshly isolated glomeruli and that this channel's activation further results in extensive Ca<sup>2+</sup> flux in podocytes. For these experiments, recently developed single-channel analysis of TRPC channels<sup>19,20</sup> and calcium measurements<sup>21</sup> in their native setting, freshly isolated glomeruli, were performed. Transient over-expression of TRPC6 channels together with AT<sub>1</sub> receptor in Chinese hamster ovary (CHO) cells was also used to test the effects of Ang II. Altogether, these techniques were used to establish the effects of Ang II on TRPC channels in the podocytes of the glomeruli, and allowed hypothesizing that TRPC6 blockade and/or inhibition of AT receptors may be of therapeutic benefit in the treatment of the nephrotic syndrome and particularly FSGS.

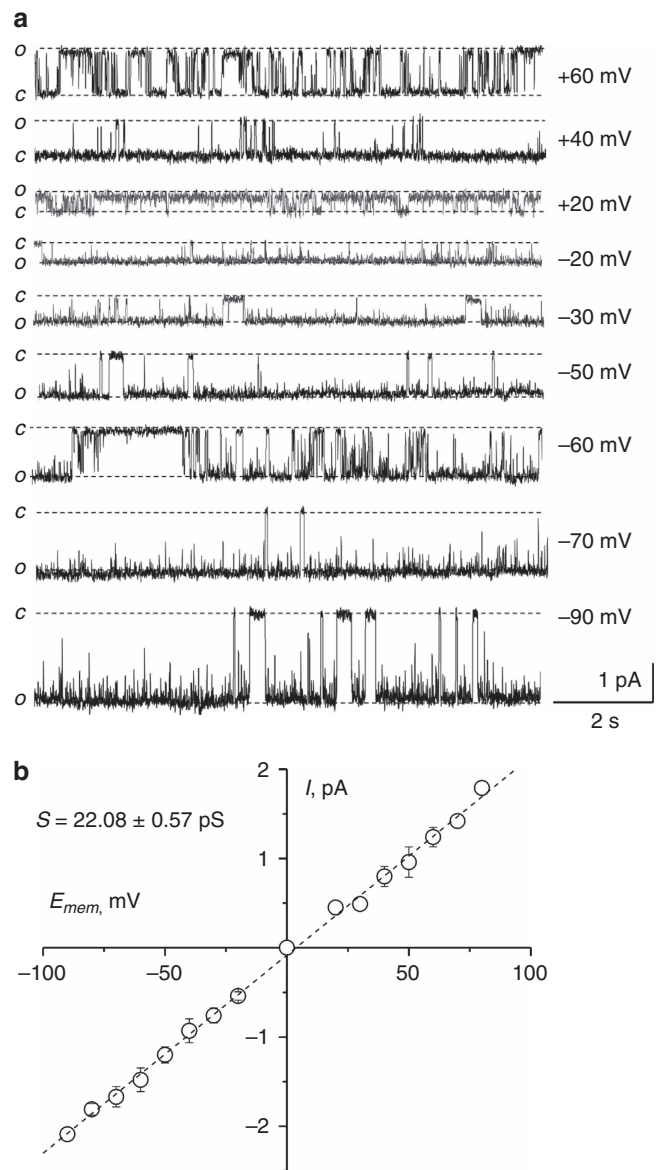
**RESULTS**

**TRPC6 channel recordings in the freshly isolated mouse glomeruli**

We have recently established a novel approach allowing us to perform single-channel analysis of native TRPC-like channels in the podocytes of freshly isolated glomeruli.<sup>19,20</sup> After the glomeruli are isolated from the kidneys of the mice, cell bodies of the podocytes appear in the light microscope as oval structures on the surface of the glomerular capillary loops. Single-channel analysis was used to assess TRPC activity in the podocytes in freshly isolated glomeruli of mice. TRPC channels typically show low levels of constitutive activity.<sup>22</sup> Figure 1a demonstrates the activity of a channel recorded in cell-attached configuration in symmetric chloride solutions at different potentials. The channel has distinct TRPC family properties, including reverse potential close to zero, kinetics, slight voltage dependency, and conductance of ~22 pS. The summarized current-voltage dependence for this channel is shown in Figure 1b. TRPC channel activity was also tested in the TRPC6 knockout mice; with the current solutions and conditions we were unable to record the activity of the channels similar to those recorded in the wild-type mice.

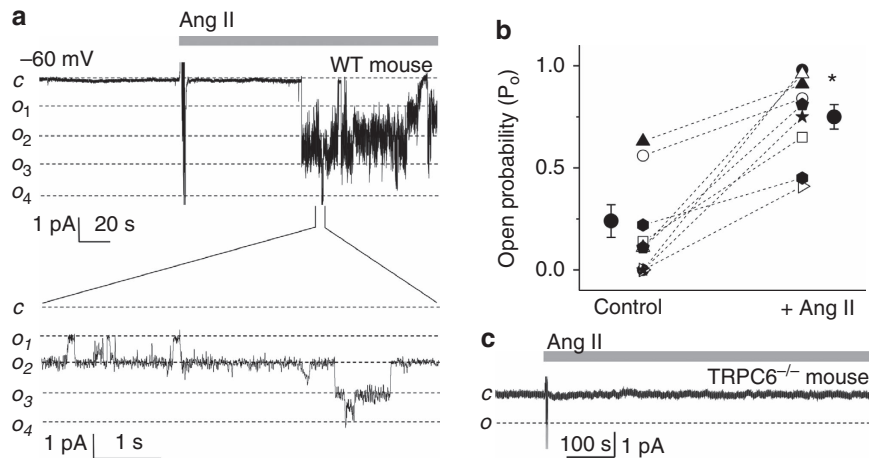
**Ang II activates TRPC6 channels in freshly isolated mouse glomeruli and in transfected CHO cells**

Figure 2a illustrates the time course of TRPC6 channel activity in the isolated glomerulus following the addition of Ang II (1 μmol/l). As summarized in Figure 2b, application of Ang II resulted in the acute increase in the channel open probability (P<sub>o</sub>) in this native preparation. Ilatovskaya *et al.*<sup>20</sup>



**Figure 1 | Native transient receptor potential canonical channel 6 (TRPC6) channels in the freshly isolated mouse glomeruli.** (a) Representative current traces from the podocytes of the freshly isolated wild-type mouse glomeruli. The activity of the identified TRPC6 channels is shown at different potentials. c and o denote closed and open states of the channels. (b) A summarized current-voltage dependency of the identified TRPC6 channel in the podocytes of the freshly isolated glomeruli. Conductance (S) is shown on the graph. Each point is the mean of at least six independent observations made on five animals.

and others<sup>3,7,22,23</sup> previously demonstrated that multiple members of the TRPC family are expressed in podocytes, but only TRPC6 is known as a cause of FSGS.<sup>2,3,24</sup> Thus, we tested an effect of Ang II specifically on the TRPC6 channel. For these experiments, we analyzed the activity of endogenous channels in response to treatment with Ang II in TRPC6 knockout mice. We did not observe any similar ion channel activity in the podocytes of the TRPC6<sup>-/-</sup> mice.



**Figure 2 | Angiotensin II (Ang II) activates transient receptor potential canonical channel 6 (TRPC6) channels in the podocytes of freshly isolated mouse glomeruli.** (a) Representative current traces of a TRPC6 channel from a cell-attached patch of a podocyte from a wild-type mouse glomerulus. A continuous current trace (upper row) and addition of Ang II (1  $\mu\text{mol/l}$ ) to the external bath solution are shown. All patches were held at a  $-60\text{ mV}$  during the course of experiment. The  $c$  and  $o_i$  denote closed and open current levels, respectively. (b) Summary graph for the channel's open probability before and after application of Ang II.  $*P < 0.01$  vs. before Ang II, the number of patches tested was 10. (c) A representative recording made on the podocytes of the freshly isolated glomerulus of a TRPC6 knockout mouse. No TRPC6 channel activity was recorded in any of the patches before or after application of Ang II. The total number of animals used was 11 wild-type and 7 TRPC6 $^{-/-}$ , respectively.

Furthermore, application of Ang II did not result in the activation of any kind of ion channels in podocytes of the TRPC6 $^{-/-}$  mice (Figure 2c). Occasionally, in both wild-type and TRPC6 knockout mice, we were able to record the background activity of other ion channels, distinctly different from the TRPC6 (data not shown). However, identification of these channels requires further studies, and none of them were activated by Ang II.

We further tested the involvement of TRPC6 channel in Ang II-mediated effects. For these experiments we over-expressed TRPC6 together with AT $_1$  receptor in CHO cells. Current-voltage dependence and representative current traces at different potentials recorded in the transfected CHO cells are shown in Figure 3; the conductance of the channels was  $24.0 \pm 1.1\text{ pS}$ . As shown in Figure 3a and summarized in Figure 3b, Ang II significantly increased TRPC6  $P_o$  in CHO cells, similar to native TRPC-like channels of the podocytes in the freshly isolated mouse glomeruli. Please note that Ang II washout resulted in fade away of the TRPC6 activity, whereas recurrent application of Ang II caused its restitution. Current-voltage relationship of the recorded channels is shown in Figure 3c. As a negative control we have used either untransfected CHO cells or cells transfected with the TRPC6 channel without AT $_1$  receptor. In both cases, we were unable to record the activation of the channels in response to Ang II (Figure 3d).

#### Ang II application in concentrations above 10 $\mu\text{mol/l}$ results in contraction of mouse glomeruli

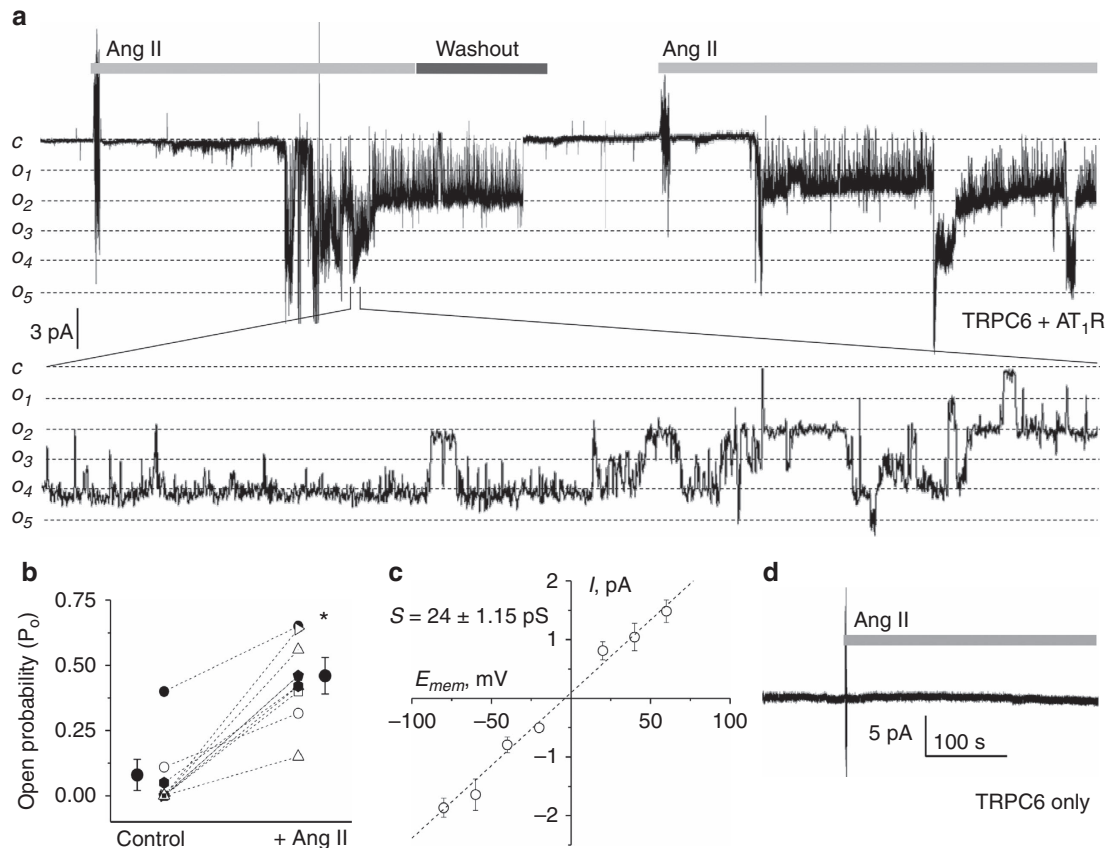
Different concentrations of Ang II in the range of 100 nmol/l to 25  $\mu\text{mol/l}$  were tested to establish the appropriate concentration for a detectable response both in patch-clamp experiments and in calcium-concentration measurements. Ang II

evoked the response at all studied concentrations (data not shown). However, the concentration of 1  $\mu\text{mol/l}$  was selected as the most suitable for the experimental procedures, as all the concentrations of Ang II above 10  $\mu\text{mol/l}$  resulted in contraction and subsequent relaxation of the glomeruli, which made electrophysiological studies impossible due to detachment of the patch pipette from the podocytes. Figure 4 summarizes the effect of 1  $\mu\text{mol/l}$  (Figure 4a) and 10  $\mu\text{mol/l}$  (Figure 4b) of Ang II on glomerular volume. Supplementary Video S1 online illustrates the absence of glomerular contraction in response to 1  $\mu\text{mol/l}$  of Ang II (first mark) and a significant visible contraction after addition of 10  $\mu\text{mol/l}$  of Ang II (second mark).

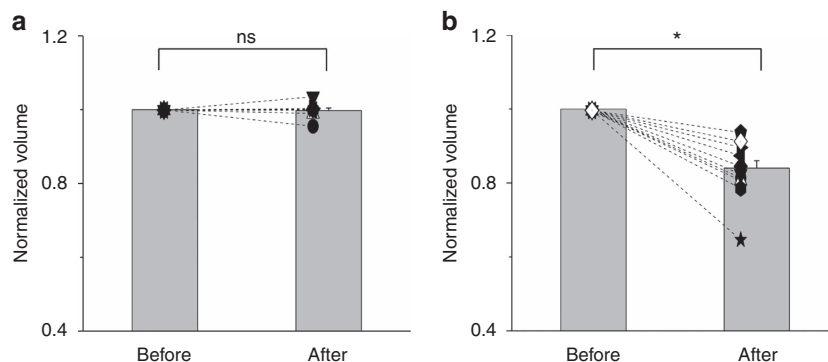
#### Intracellular calcium response is impaired in the podocytes of the TRPC6 $^{-/-}$ mice compared with wild-type animals

We tested the intracellular calcium response of the podocytes of the wild-type and TRPC6 $^{-/-}$  mouse glomeruli by the ratiometric confocal measurement with Fluo4/FuraRed fluorescent dyes. Figure 5a shows typical confocal images of the wild-type and TRPC6 knockout mouse glomeruli in the calcium-free solution and after changing the solution to the one containing 2 mmol/l CaCl $_2$ ; increase in the fluorescence intensity evoked by the calcium influx from the extracellular space can be clearly seen. Supplementary Video S2 online shows a rotating confocal three-dimensional reconstruction from a z-stack image collection (18 z-slices) of the glomerulus stained with Fluo4 and FuraRed. Fluorescent podocytes can be clearly seen on the surface of the glomerulus close to the glass.

Figure 5b summarizes the intracellular calcium response evoked by the extracellular calcium-concentration change; the transient was significantly higher in the wild-type



**Figure 3 | Angiotensin II (Ang II) activates transient receptor potential canonical channel 6 (TRPC6) channels in transfected Chinese hamster ovary (CHO) cells.** Shown is a representative current trace from the cells transfected with AT<sub>1</sub> receptor and TRPC6 before and after application of 1 μmol/l Ang II and following washout and second Ang II application (total length 30 min) with an expanded region (10 s) showing the activity of the channel on an expanded scale (a) and a graph summarizing the channels' open probability before and after application of Ang II (b). \**P* < 0.001 vs. before Ang II. Cumulative current–voltage dependence of the recorded TRPC6 channel calculated from six independent patches is shown on (c). The number of independent experiments testing the effects of Ang II was eight. (d) A representative current trace illustrating the absence of the TRPC6 activity before or after application of Ang II in CHO cells transfected with the TRPC6 channel only.

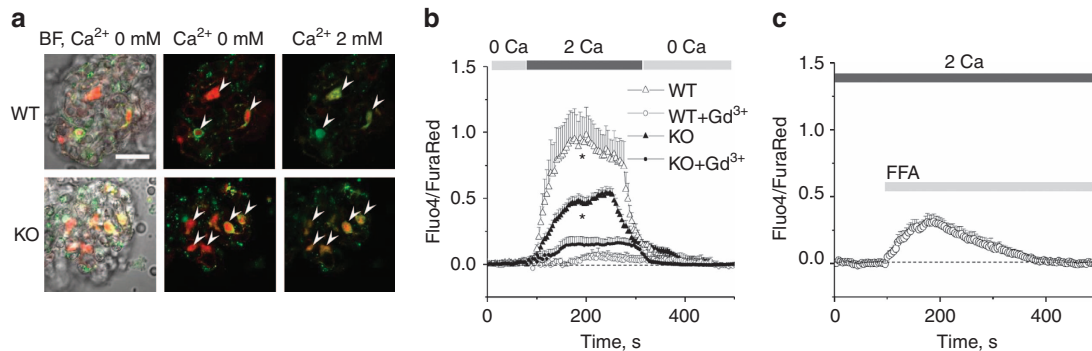


**Figure 4 | Angiotensin II application results in the changes of the glomerular volume.** The graph summarizes the effect of angiotensin II (Ang II) in concentration 1 (a) and 10 μmol/l (b) on the glomerular volume. \* denotes *P* < 0.05 vs. before application of Ang II. The summary was calculated from 12 independent glomeruli from five different wild-type mice. ns, not significant.

podocytes compared with the podocytes from the TRPC6<sup>-/-</sup> animals. Therefore, this observed difference in the increase of the intracellular calcium concentration conforms to the expected blunted calcium influx in cells deficient for the

TRPC6 channels. In all of the experiments, calcium levels returned to the basal level after the solution was changed for the calcium-free one. In order to estimate the contribution of the TRPC channels into calcium influx in the podocytes, we





**Figure 5 | Intracellular calcium response in the podocytes of the freshly isolated glomeruli of transient receptor potential canonical channel 6 (TRPC6) <sup>-/-</sup> mice compared with wild-type animals.** (a) Representative images illustrating the wild-type (upper row, WT) and TRPC6 <sup>-/-</sup> (bottom row) mouse glomeruli stained with Fluo4 (green pseudocolor) and FuraRed (red pseudocolor) in the calcium-free solution (bright field (BF) merged with fluorescence and fluorescence only) and after changing the solution to the calcium-containing solution (2 mmol/l Ca<sup>2+</sup>). The lower intensity green-colored fluorescence in the knockout podocytes after the solution change should be noted. Arrowheads denote the typical regions of interest in which the fluorescence signal intensity was recorded. Scale bar shown is 50  $\mu$ m. (b) Shown is the summarized intracellular calcium transient in the podocytes of the freshly isolated glomeruli of the wild-type and TRPC6 knockout mice in response to the extracellular solution change from calcium free (nominal 0 mmol/l Ca<sup>2+</sup>) to calcium containing (2 Ca<sup>2+</sup>) and back; traces recorded in the wild-type and TRPC6 <sup>-/-</sup> glomeruli in presence of 100  $\mu$ mol/l Gd<sup>3+</sup> are also shown. The calcium response is shown as the ratio of the signal from the fluorescent dyes Fluo4/FuraRed. The number of animals used was seven and nine (total number of region of interest was 89 and 106) for the knockout and wild-type mice, respectively. \**P* < 0.05. (c) The effect of flufenamic acid (FFA, 30  $\mu$ mol/l) on intracellular calcium concentration in podocytes of wild-type glomeruli.

performed ratiometric confocal calcium measurements with a pan-TRPC blocker GdCl<sub>3</sub>, which is also known to potentiate TRPC5 channels.<sup>25,26</sup> Figure 5b illustrates the effect of 1 min preincubation of the wild-type and TRPC6 <sup>-/-</sup> glomeruli with 100  $\mu$ mol/l of GdCl<sub>3</sub>; as was apparent from the calcium transient, Gd<sup>3+</sup> precluded the increase in the intracellular calcium levels stimulated by the solution change. In the TRPC6 <sup>-/-</sup> glomeruli, the same concentration of gadolinium resulted in a slightly less blunted decrease of the transient compared with control. Thus, although these data demonstrate that there are some compensatory changes in response to gadolinium, the data also show that TRPC6 is important for this response in wild-type animals. We have also tested flufenamic acid known to stimulate TRPC6 channels.<sup>25,27</sup> As seen from Figure 5c, flufenamic acid produced a fast and significant calcium transient in the podocytes.

#### The effect of SKF 96365 on Ang II-stimulated increase in the intracellular calcium in wild-type and TRPC6-deficient mouse podocytes

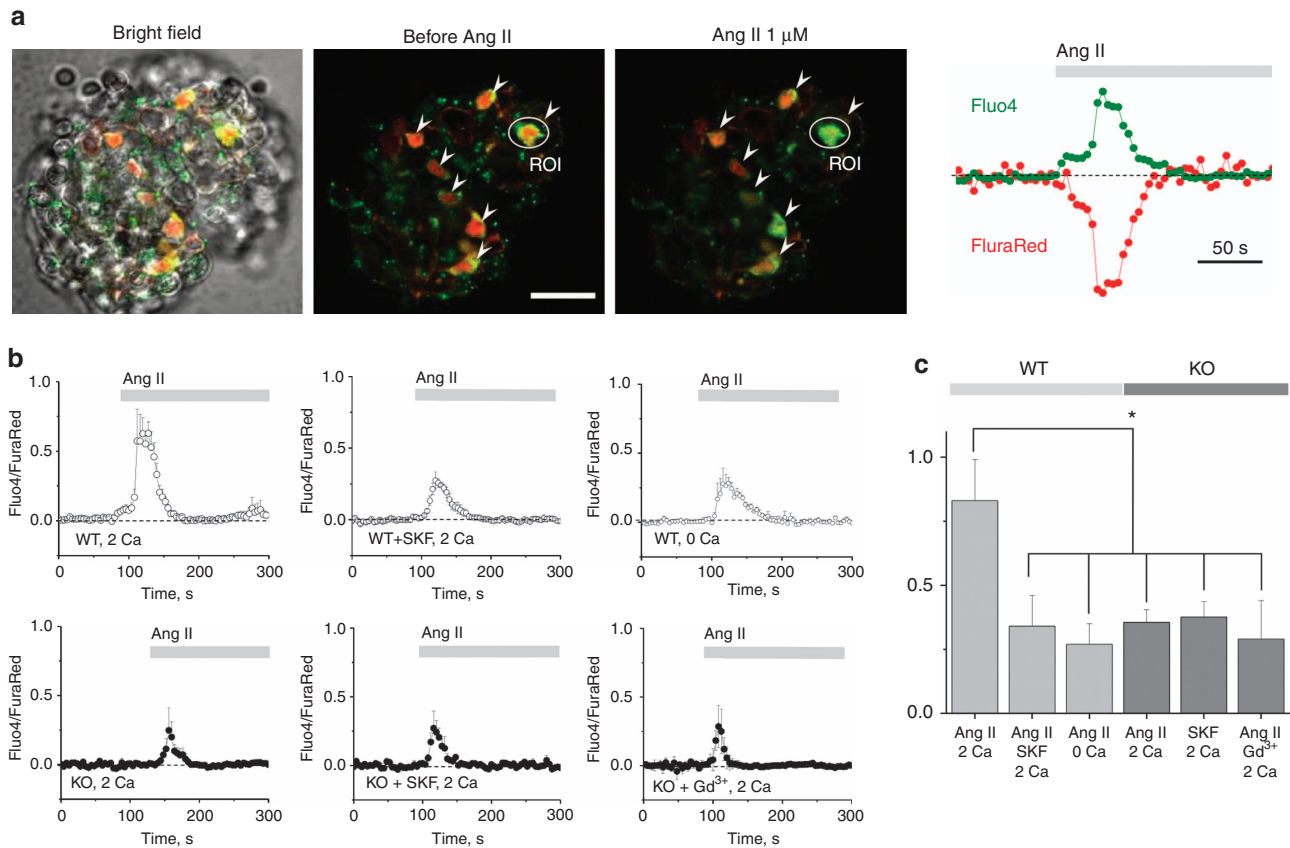
Figure 6a shows representative fluorescence images of the wild-type mouse glomeruli stained with Fluo4 and FuraRed before and after the application of 1  $\mu$ mol/l of Ang II. The right panel of Figure 6a demonstrates the responses of the Fluo4 and FuraRed signals separately. A region of interest denoted on Figure 6b was used to create separate intensity profiles recorded from Fluo4 and FuraRed.

Typical acute transients evoked by Ang II (1  $\mu$ mol/l) under different conditions in the podocytes of the glomeruli isolated from wild-type and TRPC6 KO mice is shown on Figure 6a. The magnitude of the response to Ang II in podocytes isolated from wild-type mice was decreased by the

pretreatment with low concentrations of SKF 96365 (1  $\mu$ mol/l), which is a potent pan-TRPC inhibitor. The response to Ang II was significantly blunted in the podocytes of the TRPC6 <sup>-/-</sup> mice, whereas SKF 96365 did not affect this Ang II-evoked transient. We also tested the effect of 1  $\mu$ mol/l Ang II in the wild-type glomeruli in the absence of extracellular calcium. Podocytes still show an increase in calcium in response to Ang II in the absence of the extracellular calcium; thus, the transient recorded under these conditions most likely accounts for the release of calcium from the intracellular depot. The remaining response that we see after SKF 96365 incubation has the same amplitude as the transient recorded in the calcium-free solution. It can be concluded that the remaining response after SKF 96365 accounts for the depot depletion. This was further confirmed by testing the effect of Ang II in the TRPC6 <sup>-/-</sup> podocytes in presence of 100  $\mu$ mol/l of Gd<sup>3+</sup>; the data showed that the effect of Ang II remains unchanged when the sample was preincubated with Gd<sup>3+</sup>. Summarized responses to Ang II in wild-type and TRPC6 <sup>-/-</sup> mice are shown in Figure 6c and are represented by the maximum magnitude of the ratiometric transients. We further tested the effect of Ang II in the wild-type podocytes in presence of 10  $\mu$ mol/l MK801, which is a noncompetitive *N*-methyl-D-aspartate receptor antagonist, and found that the Ang II-mediated calcium transient is preserved when *N*-methyl-D-aspartate receptors are inhibited (Supplementary Figure S1 online).

#### DISCUSSION

Although many studies have shown an important role of TRPC channels in podocytes and mediation of calcium flux by Ang II, the regulation of these channels has not yet been investigated in freshly isolated glomeruli except for the



**Figure 6 | Angiotensin II (Ang II)-evoked calcium influx in the podocytes of the wild-type and TRPC6-deficient mouse glomeruli.** (a) Representative images of the wild-type mouse glomeruli stained with Fluo4 (green pseudocolor) and FuraRed (red pseudocolor) (shown as bright field merged with fluorescence and fluorescence only before and after application of Ang II). Regions of interest are marked with arrowheads. Right panel demonstrates separate intensity signals recorded from Fluo4 (green) and FuraRed (red) from a region of interest marked with a circle. Please note the typical increase and decrease in Fluo4 and FuraRed intensities, respectively. (b) Upper panel shows the representative calcium transients caused by the application of 1 μmol/l of Ang II in the podocytes of the wild-type mouse glomeruli, which were treated with vehicle (WT) or 1 μmol/l of the pan-TRPC channel blocker SKF 96365 (WT + SKF) in the presence of extracellular calcium, and with 1 μmol/l of Ang II in the calcium-free solution (WT, 0 Ca). Bottom panel illustrates the effects of Ang II on the calcium transients in the podocytes of the TRPC6<sup>-/-</sup> mouse glomeruli treated and not treated with SKF 96365 (KO and KO + SKF, respectively) and glomeruli pre-incubated with 100 μmol/l Gd<sup>3+</sup> (KO + Gd). (c) Graph demonstrating the summarized responses of podocytes of the wild-type or knockout mouse glomeruli treated or not treated with SKF 96365 or Gd<sup>3+</sup> to 1 μmol/l of Ang II in calcium-free or calcium-containing solution. The data were summarized from at least eight glomeruli for each column and the responses were recorded from at least six different animals for each group. Bar = 30 μm. \*P < 0.05 compared with application of Ang II in wild-type mice in calcium-containing buffer.

elegant study by Gloy *et al.*,<sup>6</sup> who demonstrated in the intact glomerulus that Ang II depolarizes podocytes directly by opening a Cl<sup>-</sup> conductance. The authors proposed that the activation of Cl<sup>-</sup> conductance is mediated by an AT<sub>1</sub> receptor and may be regulated by the intracellular Ca<sup>2+</sup> activity.<sup>6</sup> As it is difficult to directly transfer data obtained from cultured glomerular epithelial cells to the responses of podocytes *in vivo*, we have established an experimental approach that allowed us to study podocytes in freshly isolated intact glomeruli. Calcium imaging and single-channel analysis in the podocytes of intact glomeruli demonstrated that Ang II application results in calcium influx, which is regulated by the changes in the open probability of TRPC channels. Further, electrophysiological and confocal experiments in the podocytes of the wild-type and TRPC6<sup>-/-</sup> mice combined with the use of the pharma-

cological tools, TRPC blockers Gd<sup>3+</sup> and SKF 96365, TRPC6 activator flufenamic acid, N-methyl-D-aspartate blocker MK801, and studies in heterologous expression system confirmed that this effect is mediated by TRPC6 channels.

Recent studies using TRPC6-deficient mice revealed that proteinuria is attenuated in TRPC6-deficient mice. Interestingly, elimination of TRPC6 has no effect on normal glomerular structure or function and does not affect blood pressure.<sup>15</sup> However, this could be potentially explained by compensatory mechanisms mediated by other TRPC family members expressed in podocytes.<sup>7,28</sup> For instance, recent studies identified that loss or inhibition of TRPC5 abrogates podocyte cytoskeletal remodeling and this channel is an important determinant of albuminuria.<sup>29</sup>

Nitschke *et al.*<sup>30</sup> demonstrated that podocytes in the intact glomerulus respond to Ang II with an increase of [Ca<sup>2+</sup>]<sub>i</sub> via

an AT<sub>1</sub> receptor. Interestingly, this effect was specific as neither bradykinin, nor arginine vasopressin, thrombin, or serotonin influenced [Ca<sup>2+</sup>]<sub>i</sub> in podocytes. The authors demonstrated that the Ang II-induced increase of [Ca<sup>2+</sup>]<sub>i</sub> was due to both a Ca<sup>2+</sup> release from the intracellular space and a Ca<sup>2+</sup> influx from the extracellular space.<sup>30</sup> Using available inhibitors, the authors attempted to identify a channel responsible for this extracellular calcium flux. However, neither the L-type Ca<sup>2+</sup> channel blocker nifedipine, nor an increase of the extracellular K<sup>+</sup> concentration changed [Ca<sup>2+</sup>]<sub>i</sub> in the glomeruli.<sup>30</sup>

Gd<sup>3+</sup> and SKF 96365 used in this study for identification of the source of Ca<sup>2+</sup> flux are not specifically selective for TRPC6 but rather are pan-TRPC inhibitors. However, our calcium measurement data obtained in the knockout animals in combination with electrophysiological observations provide definite conclusions about the channel responsible for the Ang II-mediated increase of [Ca<sup>2+</sup>]<sub>i</sub> in the podocytes of the intact glomeruli. Our data are in good agreement with recent data published by Anderson *et al.*<sup>18</sup> who reported that Ang II increases cationic currents in rat podocytes of an isolated glomerulus preparation and suggested that production of reactive oxygen species could permit activation of TRPC6 channels by G protein-coupled receptors and PLC-dependent cascades initiated by Ang II acting on AT<sub>1</sub> receptors in podocytes.<sup>18</sup> Role of NADPH oxidase Nox2 in this signaling mechanism was also proposed.<sup>31</sup> Nijenhuis *et al.*<sup>17</sup> demonstrated that Ang II regulates TRPC6 mRNA and protein levels in cultured podocytes and that Ang II infusion enhances glomerular TRPC6 expression *in vivo*, and that TRPC6 expression correlates with glomerular damage markers and glomerulosclerosis. The authors proposed that the deleterious effects of Ang II on podocytes and its pathogenic role in glomerular disease involved enhanced TRPC6 expression via the calcineurin/NFAT positive feedback signaling pathway.<sup>17</sup> Our studies demonstrate that Ang II has an acute effect on both increase of [Ca<sup>2+</sup>]<sub>i</sub> and TRPC6 activity. Furthermore, the effect on channel activity is mediated by changes in the channel open probability. However, we cannot exclude that both mechanisms mediate TRPC6 activity with rapid changes in gating properties and a long-term effect on the level of transcription and protein expression.

Furthermore, role of the actin cytoskeleton as a downstream signaling element involved in Ang II-mediated increase of [Ca<sup>2+</sup>]<sub>i</sub> was proposed. It was shown that Ang II caused a reactive oxygen species-dependent rearrangement of cortical F-actin and a migratory phenotype switch in cultured mouse podocytes with stable AT<sub>1</sub> receptor expression.<sup>32</sup> Small GTPases Rac1 and RhoA were identified as antagonistic regulators of actin remodeling in podocytes. The authors defined TRPC5 and TRPC6 as channels being downstream of the Ang II-evoked nonselective cationic conductance in podocytes.<sup>33</sup> The application of Ang II revealed three populations of channels with the corresponding conductances of 39, 68, and 80 pS. TRPC5 was identified

as the channel forming a molecular complex with Rac1, whereas TRPC6 was shown in a complex with RhoA.<sup>33</sup>

It is necessary to mention that apart from the Ang II-mediated calcium entry, which is now recognized to be an important part of the signal transduction events in the podocytes, there is a plethora of other processes in these cells that involve calcium influx through various calcium-conducting channels. For example, both TRPC5 and TRPC6 channels were identified to be antagonistic regulators of actin remodeling and cell motility in fibroblasts and kidney podocytes.<sup>29,33</sup> Functional N-methyl-D-aspartate receptors were also found to be expressed in the podocytes and to contribute to calcium entry in these cells.<sup>34–36</sup> However, our data are consistent with the idea that TRPC6 is the main channel responsible for Ang II-mediated calcium signaling.

Selective inhibition or activation of signaling pathways may be an effective means of modulating proteinuria. However, the cellular mechanisms of these processes are still uncertain and require further investigation. Successful specific therapy of glomerular diseases depends on concurrent targeting of multiple signaling pathways. The ability of podocytes to precisely regulate intracellular Ca<sup>2+</sup> levels has a central role in glomerular disease processes. Manipulating Ca<sup>2+</sup> levels by inhibiting TRPC channel activators may be a useful strategy for treating patients with FSGS and nephrotic syndrome.

## MATERIALS AND METHODS

### Animals

Animal use and welfare adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the IACUC of the Medical College of Wisconsin. For experiments, 8–12 week old male wild-type (c57BL/6) or TRPC6<sup>-/-</sup> mice were used. Mice were provided with food and water *ad libitum*.

### Isolation of the mouse glomeruli

The kidneys of 8–12-week-old male mice were removed and then decapsulated; the cortex was isolated and minced as described previously for the rat kidney preparation.<sup>19,20</sup> The minced tissue was sequentially pushed through a steel sieve of 150- $\mu$ m mesh and then pipetted through a 106- $\mu$ m sieve. This tissue homogenate was then pipetted onto a 75- $\mu$ m sieve; the filtrate was then pushed through the 53- $\mu$ m sieve and the glomeruli were rinsed from both the 75- and the 53- $\mu$ m sieves. For experiments, isolated glomeruli were allowed to settle onto cover glass chips coated with poly-D-lysine.

### Electrophysiology

Cover glasses that contained glomeruli were placed into a perfusion chamber mounted on an inverted Nikon Ti-S microscope and superfused with a physiologic saline solution (pH 7.4). Single-channel current data were acquired as described previously.<sup>19,20</sup> After a high resistance seal was obtained, cell-attached recording was performed immediately. The membrane resistance was monitored regularly to ensure the quality of recording. For measurements of acute effect, only one experiment was performed per dish to avoid any possibility of examining cells whose properties may have been altered by extended exposure to Ang II. The recordings were made



in symmetric chloride solutions. The bath solution consisted of (in mM) 126 NaCl, 1 CaCl<sub>2</sub>, 10 HEPES, 2 MgCl<sub>2</sub>, and 10 glucose at pH 7.4. The pipette solution contained (in mM) 126 NaCl, 1.5 CaCl<sub>2</sub>, 10 HEPES, and 10 glucose at pH 7.4; plus, added directly before the patch-clamp experiments were 100 μmol/l niflumic acid or DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid disodium salt, to block Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels), 10 mmol/l TEA (N,N,N,N-tetraethylammonium chloride, to inhibit large-conductance Ca<sup>2+</sup>-dependent K<sup>+</sup> channel), 10 nmol/l iberiotoxin (to block Ca<sup>2+</sup>-activated K<sup>+</sup> channels), 10 μmol/l nifedipine (to block N-type Ca<sup>2+</sup> channels). During the patch-clamp measurements in the single-channel mode the activity of the ion channels was first monitored in response to the potential applied in steps of 10 or 20 mV in the range of -90 mV to +60 mV in order to estimate the channel's conductance and I-V relationship. After that the voltage was clamped at -60 mV, and the channels' activity was recorded for several minutes before the drugs were applied.

### Glomerulus contraction measurements

For the volume response measurements, freshly isolated mouse glomeruli were affixed to glass coverslips coated with poly-L-lysine. The glomerular responses were recorded using confocal microscopy (Nikon A1-R). Glomerular volume (*V*) was calculated at the maximum points before and after addition of the drugs from the surface area (*S*) of the glomerulus with the formula  $V = 3/4\pi(S/\pi)^{3/4}$  using the ImageJ software (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>) as previously described.<sup>39</sup>  $\Delta V$  was calculated as  $(V_{\text{final}} - V_{\text{initial}})/V_{\text{initial}}$ . At least ten glomeruli from three or more mice were studied under each condition. Control glomeruli were treated with equivalent volumes of buffer and no changes in the glomeruli volume were detected.

### cDNA constructs and cell culture

The wild-type CHO cells were obtained from the American Tissue and Culture Collection and cultured in Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum, 2 mmol/l glutamine, and 80 μg/ml gentamicin. The plasmid encoding the wild-type TRPC6 channel gene was previously described.<sup>40</sup> Plasmid encoding AT<sub>1</sub> receptor was obtained at Missouri S&T cDNA Resource Center (AGTR10TN01). Cell transfection with 1 μg of each plasmid encoding TRPC6 and AT<sub>1</sub> receptor and 0.5 μg of green fluorescent protein was performed 24–48 h before the experiments.

### Confocal laser-scanning fluorescence microscopy

Calcium imaging was performed with laser-scanning confocal microscope system Nikon A1-R. Images were collected in time series (xyt, 4 s per frame) with the Nikon imaging software (NIS Elements, Nikon Instruments Inc, Tokyo, Japan). Changes in intracellular Ca<sup>2+</sup> concentration were estimated according to a protocol described previously<sup>21</sup> from ratiometric fluorescence images of Fluo4 and FuraRed loaded glomeruli. Emitted light was collected by the objective lens Plan Apo ×60 oil DIC2. The glomeruli suspension was loaded with the dyes by adding Fluo4 AM and FuraRed AM (5 μmol/l of each; Invitrogen (Grand Island, NY)). Glomeruli were mounted on the poly-D-lysine covered glass in a registration chamber and washed for ~10 min with bath solution containing (in mmol/l): 145 NaCl, 4.5 KCl, 2 MgCl<sub>2</sub>, and 10 HEPES at pH 7.35. After stabilization of the fluorescence signal podocytes were identified on the basis of anatomic considerations, and fluorescence intensity ratios were recorded. Fluorescent signal was observed

only from cells on the surface of glomeruli in the area attached to the glass. In each experiment, 4–7 podocytes of at least one glomerulus were selected; experiments for every compound tested were repeated at least three times.

### Statistical analysis

Data are presented as mean ± s.e.m. The values of intracellular calcium ion concentration at every moment of time for individual cells were averaged by the number of regions registered in the experiment. Data are compared using the Wilcoxon signed-rank test and *P* < 0.05 is considered significant.

### DISCLOSURE

All the authors declared no competing interests.

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### SUPPLEMENTARY MATERIAL

**Figure S1.** Angiotensin II-mediated calcium transient is preserved when NMDA receptors are blocked.

**Video S1.** Illustration of the effects of 1 μM and 10 μM of angiotensin II on the wild type mouse glomeruli volume.

**Video S2.** 3D video of a mouse glomerulus stained with Fluo4 and FuraRed fluorescent calcium indicators.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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