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Angiotensin II activates the Smad pathway during epithelial mesenchymal transdifferentiation

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Epithelial to mesenchymal transdifferentiation is a novel mechanism that promotes renal fibrosis and here we investigated whether known causes of renal fibrosis (angiotensin II and transforming growth factor β, TGFβ) act through this pathway. We infused angiotensin II into rats for 1 day and found that it activated the Smad pathway which persisted for up to 2 weeks in chronically infused rats. Renal TGF-β mRNA expression was increased at 3 days and its protein at 2 weeks suggesting Smad pathway activation occurred earlier than TGF-β upregulation. In cultured human tubuloepithelial cells, angiotensin II caused a rapid activation of Smad signaling independent of TGF-β however, Smad-dependent transcription after 1 day was TGF-β mediated. Two weeks of angiotensin II infusion activated genes associated with epithelial mesenchymal transdifferentiation. Stimulation with angiotensin II for 3 days caused transdifferentiation of the cultured epithelial cells by TGF-β-mediated processes; however, early changes were independent of endogenous TGF-β. Smad7 overexpression, which blocks Smad2/3 activation, diminished angiotensin II-induced epithelial mesenchymal transdifferentiation. Our results show that angiotensin II activates the Smad signaling system by TGF-β-independent processes, *in vivo* and *in vitro*, causing renal fibrosis.

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Activation of local renin–angiotensin system has been found in many kidney diseases. Angiotensin II (AngII), the main peptide of the renin–angiotensin system, contributes to the progression of renal damage.[1] This peptide activates tubuloepithelial cells, interstitial fibroblasts, and glomerular cells, regulating cell growth and extracellular matrix synthesis.[1–3] Many studies have demonstrated that AngII participates in renal fibrosis through endogenous production of growth factors.[1–4] The relation between AngII and transforming growth factor-β (TGF-β) in renal fibrosis is already known.[3] TGF-β transmits signals to the nuclei through the activation of the Smad pathway.[5,6] TGF-β binds to type II receptor, which activates the type I receptor kinase, which in turn phosphorylates the receptor-regulated Smads (R-Smads), Smad2 and Smad3, at C-terminal serines. The R-Smads then dissociate from the receptor complex to form a heterotrimeric complex with Smad4. These complexes translocate to the nucleus and function as transcriptional regulators of target genes. The inhibitory Smad7 binds to activated type I receptor, thereby preventing Smad2/3 phosphorylation, or recruits the ubiquitine ligases Smurf1 and Smurf2 to induce proteasomal degradation.[5,6] In renal cells, Smads participate in TGF-β-induced epithelial–mesenchymal transdifferentiation (EMT) and fibrosis.[6,7] The angiotensin type I receptor (AT_1) blockade diminishes Smad pathway activation in myocardial infarction in rats and in an experimental model of renal damage.[8,9] We have described that in vascular smooth muscle cells (VSMCs), AngII activates the Smad signaling pathway independently of TGF-β and linked to vascular fibrosis.[10] However, there are no studies investigating whether AngII activates the Smad signaling pathway in the kidney and its potential role in EMT.

Tubulointerstitial fibrosis is a final common pathway to end-stage chronic kidney diseases, and its severity correlates with renal prognosis. Many evidences suggest that renal tubuloepithelial cells can undergo EMT to become matrix-producing fibroblasts under pathologic conditions and, therefore, participate in the pathogenesis of chronic renal diseases. Cytokines, growth factors, adhesion molecules, and

changes in the extracellular matrix composition are involved in EMT.[11–13] TGF-β has been described as the most potent inducer of fibrosis and EMT.[5,7,11,14] In cultured tubulo-epithelial cells, AngII induces α-smooth muscle actin (α-SMA) expression and mesenchymal features.[15] Infusion of AngII into rats causes tubular injury associated with neoexpression of α-SMA and vimentin in renal interstitial cells;[16,17] therefore, it is reasonable to speculate that AngII could be involved in EMT in the kidney.

The aim of this work was to investigate whether AngII activates the Smad pathway in the kidney and its role in EMT, evaluating the involvement of endogenous TGF-β synthesis in these processes.

RESULTS

Systemic AngII infusion into rats activates the Smad pathway in the kidney

One of the initial steps of Smad activation is the phosphorylation of R-Smads.[5,6] We have investigated whether AngII *in vivo* could activate the Smad pathway in the kidney by evaluation of the time course evolution of R-Smad phosphorylation. In AngII-treated rats for 24 h total renal phosphorylated, Smad3 levels were significantly increased compared with control animals (western blot, Figure 1a), indicating a rapid Smad activation in the kidney. After 3 days, phosphorylated-Smad2/3 (p-Smad2/3) markedly appeared in the nucleus of tubuloepithelial cells (Figure 1b). Similar

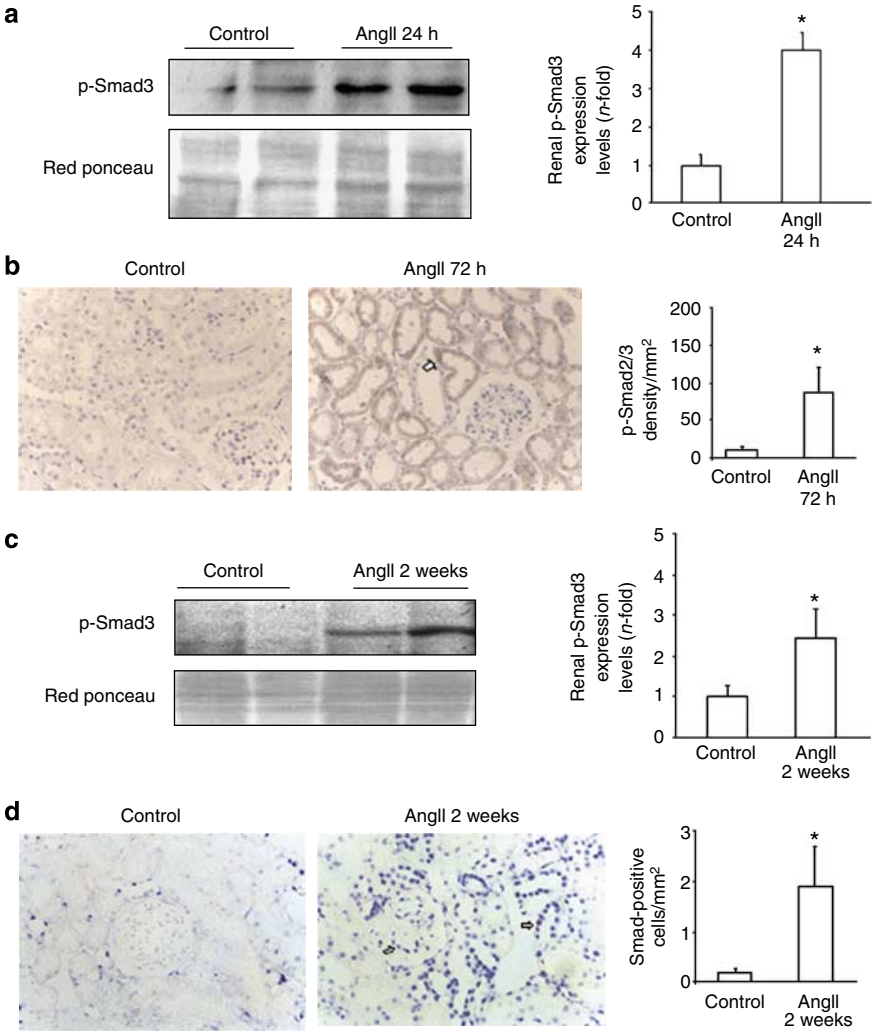

Figure 1 | AngII infusion activates the Smad pathway in the kidney. Rats were infused with AngII (100 ng/kg/min) from 24 h to 2 weeks and phosphorylated levels of Smad proteins were evaluated by (**a**, **c**) western blot and (**b**) immunohistochemistry. In western blot, phosphorylated-Smad3 (p-Smad3) levels were evaluated in renal protein extracts. Panels (**a**) and (**c**) show a representative experiment on the right panel and data as mean ± s.e.m. of 4–6 animals per group on the left. *$P<0.05$ vs control. Panel (**b**) shows a representative experiment of pSmad2/3 immunohistochemistry on the right and the quantification of data as mean ± s.e.m. of 4–6 animals per group on the left. (**d**) By Southwestern histochemistry, active Smad complexes, shown by nuclear blue staining (marked by arrows), were detected in AngII-infused rats for 2 weeks. The figure shows a representative experiment of three animals studied in each group on the right panel and data as mean ± s.e.m. of 4–6 animals per group on the left. *$P<0.05$ vs control. Original magnification × 200.

results were found with the antibody that recognizes only p-Smad3 (not shown). The Smad pathway remained activated in chronic AngII-infused rats, as shown by western blot (Figure 1c) and by Southwestern histochemistry, a technique that allows to detect Smad–DNA binding activity in paraffin-embedded tissues.[18] In AngII-infused rats for 2 weeks, active Smad complexes were observed in some glomerular and tubuloepithelial cells, whereas only a few positive cells were found in control animals (Figure 1d).

AngII-induced Smad activation occurs earlier than TGF-β production. To investigate whether Smad activation was directly induced by AngII or mediated by endogenous TGF-β synthesis, we have evaluated TGF-β mRNA and protein levels at different time points. AngII did not increase renal TGF-β mRNA levels after 24 h, but this gene was upregulated at 72 h and remained elevated after 2 weeks (real time PCR, Figure 2a). TGF-β is synthesized as an inactive protein, which is anchored to the cell membrane before activation.[6] In cultured cells, AngII increases TGF-β mRNA expression, protein production, and activation of latent TGF-β, the latter regulated by a thrombospondin-1 mediated process.[19] In rats that have been AngII-treated for 72 h, throsmbospondin-1 mRNA levels were not upregulated compared with controls (real-time PCR, not shown). Moreover, in these animals, there was no increase in TGF-β protein levels, which were dramatically overexpressed after 2 weeks of AngII infusion

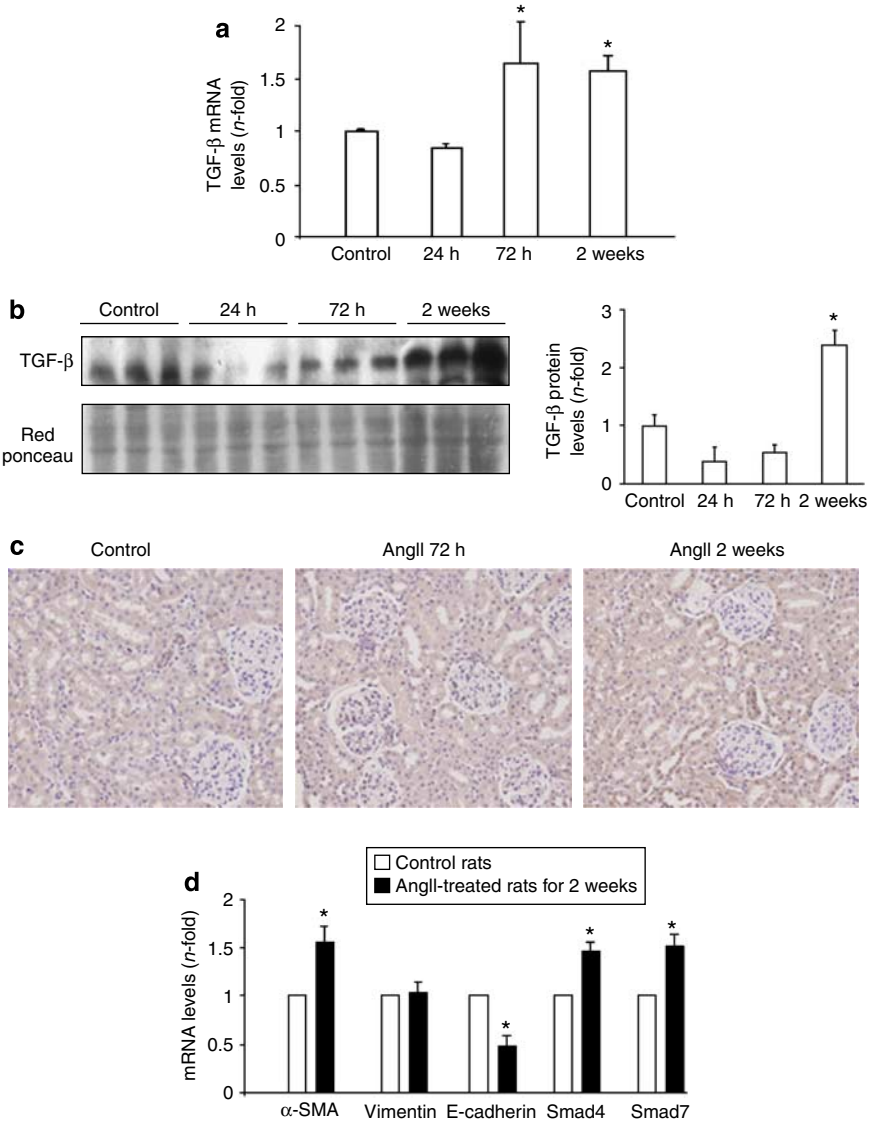

Figure 2 | Renal TGF-β expression is upregulated in chronic, but not acute, AngII infusion. Renal gene and protein expression levels of TGF-β were evaluated at different time points of AngII infusion. Panel (**a**) shows data of real-time PCR experiments as mean ± s.e.m. of 6–8 animals per group. *$P<0.05$ vs control. Panel (**b**) shows a representative experiment evaluated by western blot on the right panel and data as mean ± s.e.m. of 4–6 animals per group on the left. *$P<0.05$ vs control. Panel (**c**) shows a representative experiment of TGF-β immunohistochemistry. (**d**) Renal gene expression of EMT markers and Smad proteins in AngII-infused rats for 2 weeks. The figure shows data of real-time PCR experiments of Smad4, Smad7, E-cadherin, α-SMA, and vimentin gene expressions as mean ± s.e.m. of 6–8 animals per group. *$P<0.05$ vs control.

(Figure 2b and c). These data indicate that Smad activation (observed at 24 h) occurs before the increase in TGF-β synthesis (observed after 72 h), suggesting that AngII *in vivo* directly activates Smad pathway in the kidney.

Chronic infusion of AngII into rats induces EMT in the kidney. By means of real-time PCR, we have analyzed the renal expression of the mesechymal marker α-SMA at different time points of AngII infusion, and only after 2 weeks, α-SMA mRNA was upregulated. In these animals, vimentin was not modified and the epithelial marker E-cadherin was downregulated (Figure 2d). Renal gene expressions of Smad4 and Smad7 were also increased in AngII-infused rats compared with controls, indicating upregulation of Smads expression (Figure 2d). These data show that chronic infusion of AngII into rats causes renal fibrosis, associated with induction of EMT, overexpression of TGF-β, and activation of the Smad pathway.

AngII activates the Smad pathway in cultured human tubuloepithelial cells

To further demonstrate a direct effect of AngII in Smad pathway, *in vitro* studies were carried out in cultured human tubuloepithelial cells (HK2 cell line). In these cells, stimulation with AngII and TGF-β (1 ng/ml) induced a rapid phosphorylation of R-Smads at 15 min of incubation, demonstrated by a significant increase in nuclear levels of phosphorylated Smad2 and Smad3 (Figure 3a, western blot). By confocal microscopy, we have observed that, in growth-arrested HK2 cells, Smads proteins are located in the cytosol, as demonstrated using indirect immunofluorescency for Smad4 (Figure 3b). Treatment with AngII for 15 min increased total p-Smad2 staining and caused the nuclear translocation of p-Smad2 and Smad4 (Figure 3b).

In the nucleus, the R-Smad/Smad4 complex can activate transcription through direct binding to certain DNA sequences.[6] In tubuloepithelial cells, AngII increased DNA-binding activity to the CAGA box as early as at 10 min, with a maximal response at 15 min (3.5-fold, $P<0.05$ vs control, $n=3$ electrophoretic mobility shift assay experiments). The same was observed with TGF-β (Figure 4a). The specific Smad complexes were detected by competition assays with a 100-fold excess of unlabeled or mutant CAGA box (marked by arrows in Figure 4a). By supershift assays, we have observed that the antibodies against Smad2 and Smad4, alone or in combination, shifted the band to a higher molecular weight (Supershifted bands marked by arrows), supporting the involvement of Smad2 in AngII responses (Figure 4b).

We have investigated whether AngII regulates Smad-mediated gene expression by transient transfection with a luciferase Smad reporter plasmid (Smad/luc), which contains

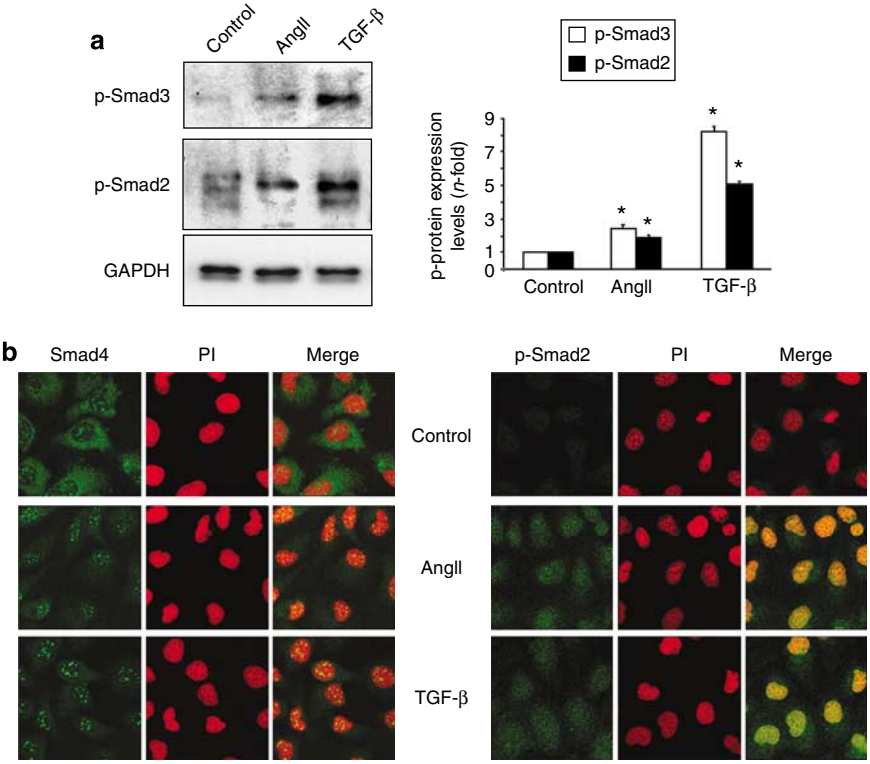

Figure 3 | AngII induces a rapid activation of Smad pathway in cultured human tubuloepithelial cells. Cells treated with 10^{-7} mol/l AngII or 1 ng/ml TGF-β for 15 min. (**a**) AngII induces Smad2/3 phosphorylation. Total protein levels of phosphorylated-Smad2 (p-Smad2) and p-Smad3 were quantified by western blot. The figure shows a representative experiment on the right panel and data as mean ± s.e.m. of three experiments on the left. *$P<0.05$ vs control. (**b**) AngII causes the nuclear translocation of Smad4 and p-Smad2. The evaluation of Smad4 and p-Smad2 was done by confocal microscopy with fluorescein isothiocyanate-labeled secondary antibodies (green staining). Nuclei were stained with propidium iodide (in red). In the merge of FITC and PI staining, the yellow staining indicates nuclear localization of Smads proteins. The results are representative of three independent experiments.

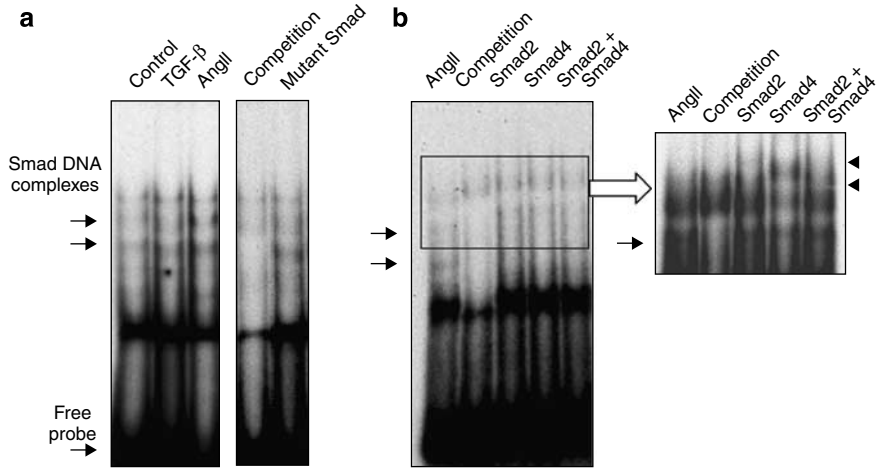

Figure 4 | AngII increases Smad DNA-binding activity in human tubuloepithelial cells. (**a**) HK2 cells were incubated with 10^{-7} mol/l AngII or 1 ng/ml TGF-β for 10 min. Smad activity was determined by electrophoretic mobility shift assay. Competition assays with a 100-fold excess of unlabeled or mutant CAGA box show specific Smad complexes (marked by arrows). (**b**) Identification of Smad complexes induced by AngII in HK2 cells. The nuclear extracts of AngII-treated cells were pre-incubated for 1 h with antibodies against the Smad2 and Smad4, alone or in combination, before incubation with labeled oligonucleotide. Protein complexes were resolved by electrophoresis (supershift). The enlarged picture corresponds to the area marked by upper arrow in (**b**). Supershifted bands are observed with anti-Smad2 and anti-Smad4 and with the combination of both antibodies (marked by arrowheads). Figure shows a representative electrophoretic mobility shift assay of three experiments.

four copies of the recognition site for the Smad sequence. After 24 h of incubation with AngII, the Smad promoter activity was increased (1.5-fold, $P<0.05$ vs control, $n=4$ experiments).

Mechanisms involved in AngII-induced Smad activation in cultured human tubuloepithelial cells

AngII rapidly activates Smad pathway by a TGF-β-independent mechanism. To block TGF-β, we used two different strategies: a neutralizing antibody against active TGF-β, which blocks AngII-induced extracellular matrix production,[4] and the SB431542 compound, an inhibitor of the activin receptor-like kinase 5.[20] In HK2 cells, the blockade of TGF-β did not modify AngII-induced Smad2/3 phosphorylation (Figure 5a) or their nuclear translocation (Figure 5b and not shown) observed after 15 min of incubation. Similar results were found in the regulation of Smad DNA-binding activity in cells treated with AngII for 15 min (not shown). These data show that early Smad activation by AngII is independent of endogenous TGF-β production or activation.

TGF-β mediates late activation of Smad pathway caused by AngII. The blockade of TGF-β diminished Smad-dependent transcription observed after 24 h of incubation with AngII (Figure 5c), suggesting that TGF-β is involved in long-term Smad activation.

Mitogen-activated protein kinase signaling mediates AngII-induced Smad activation. Previous studies in VSMCs have demonstrated a cross-talk between Smad and mitogen-activated protein kinase (MAPK) pathways,[10,21] but there are no studies in renal cells. The involvement of MAPKs cascade was evaluated using specific inhibitors of p38-MAPK (SB203580), extracellular signal-regulated kinase1/2 (ERK) (PD98059) and Jun N-terminal kinase (JNK) (SP600125) pathways.[22,23] We have found that in HK2 cells all three MAPK inhibitors (p38, ERK, and JNK) significantly diminished AngII-induced Smad2 phosphorylation (Figure 5d).

AngII causes EMT via AT_1 receptors in cultured human tubuloepithelial cells

Incubation with AngII for 3 days causes phenotypic conversion of HK2 cells. The transformed cells lost the typical cobblestone pattern of an epithelial monolayer and displayed a spindle-shaped, fibroblast-like morphology, assessed by phase contrast microscopy (Figure 6a). This effect was more marked after 5 days. AngII induced *de novo* protein expression of α-SMA and vimentin at 24 h and remaining elevated after 3 days. This was accompanied by the loss of the epithelial marker E-cadherin, essential for the structural integrity of renal epithelium (western blot; Figure 6b). In unstimulated tubuloepithelial cells, no staining for vimentin or α-SMA was observed by confocal microscopy. Treatment with AngII for 3 and 5 days induced vimentin and α-SMA-positive microfilaments in the cytoplasm, and E-cadherin immunostaining disappeared (Figure 6c). These data suggest that tubuloepithelial cells, stimulated with AngII, undergo a conversion process into myofibroblasts.

Pre-incubation of HK2 cells with the specific AT_1 antagonist valsartan blocked AngII-induced vimentin expression and changes in cell morphology (Figure 6d and e). These results suggest that AngII-induced EMT was mediated by AT_1 receptors.

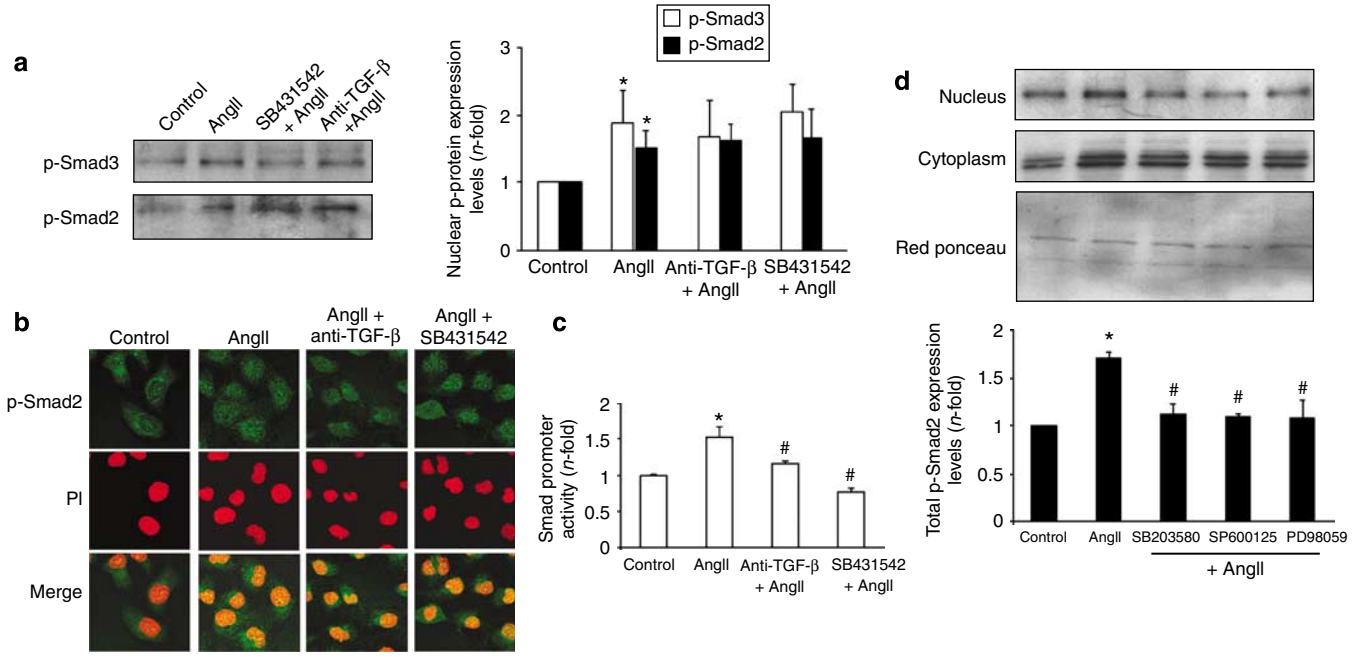

Figure 5 | AngII activates early Smad pathway independently of TGF-β in human tubuloepithelial cells. TGF-β was blocked by pretreatment with 10 μg/ml TGF-β neutralizing antibody or 10^{-5} mol/l SB431542 for 1 h, before AngII (10^{-7} mol/l) stimulation for 15 min. (**a**) Nuclear protein levels of phosphorylated-Smad2/3 were quantified by western blot. The figure shows a representative experiment on the right panel and data as mean ± s.e.m. of three experiments on the left. (**b**) The p-Smad2 was located by indirect immunofluorescence with a fluorescein isothiocyanate-labeled secondary antibody (green staining). The figure is representative of three experiments. (**c**) AngII-induced long-term Smad activation is mediated by TGF-β. HK2 cells were transfected with Smad/luc promoter and TK-renilla. After 24 h serum-starvation, TGF-β was blocked and cells were stimulated with AngII for 24 h. Then luciferase/renilla activity was measured. Data of mean ± s.e.m. of four experiments done in triplicate is shown. (**d**) MAPK activation is involved in AngII-induced Smad activation. HK2 cells were pretreated with 10^{-6} mol/l SB203580 (p38 inhibitor), 10^{-5} mol/l PD98059 (ERK p42/44 inhibitor), or 10^{-5} mol/l SP600125 (JNK inhibitor) for 1 h, before treatment with 10^{-7} mol/l AngII for 15 min. Nuclear and cytosolic fractions were extracted and levels of p-Smad2 were evaluated by western blot. Red ponceau staining was used as loading control. Panel (**d**) shows a representative experiment in the top panel and data of mean ± s.e.m. of three experiments in bottom panel. $^{*}P<0.05$ vs control. $^{\#}P<0.05$ vs AngII.

Role of endogenous TGF-β on AngII-induced EMT

TGF-β is a mediator of AngII-induced fibrosis. The effects of AngII and TGF-β at 24 h on the regulation of EMT markers were similar (Figure 6). We therefore tested whether early AngII-induced EMT was TGF-β independent by evaluating vimentin expression. In HK2 cells, vimentin induction caused by AngII after 18 and 24 h of incubation was not diminished by the endogenous blockade of TGF-β (Figure 7a), suggesting that the initial induction of EMT is independent of TGF-β. However, TGF-β blockers significantly diminished AngII-induced vimentin expression after 3 days, as well as the phenotypic conversion to fibroblast-like morphology (Figure 7b and c), suggesting that TGF-β contributes to the progression of the EMT process caused by AngII.

AngII-induced EMT is mediated by Smad activation

To block Smad actions, cells were transiently transfected with a Smad7 expression vector that inhibits TGF-β/Smad-mediated transcriptional effects by interfering with receptor-mediated activation of R-Smad.[14] HK2 cells transfected with Smad7 markedly diminished EMT caused by AngII and TGF-β. Overexpression of Smad7 inhibited vimentin upregulation caused by both AngII and TGF-β after 24 h, compared with cells transfected with an empty vector, pcDNA3B (western blot; Figure 8a). By immunofluorescence, we observed that Smad7 overexpression impaired AngII-mediated changes in cell shape and induction of α-SMA staining after 3 days (Figure 8b), as observed with TGF-β, whereas cells transfected with pcDNA3B undergo EMT in response to both stimuli.

DISCUSSION

We have evaluated the effect of AngII on the Smad signaling pathway in the kidney. Our *in vivo* studies using the model of systemic infusion of AngII showed the activation of the Smad pathway as early as 24 h and remained elevated until 2 weeks. AngII-treated rats presented increased renal levels of phosphorylated Smad2/3 proteins and active Smad complexes, mainly in tubuloepithelial cells and in some glomerular cells. In cultured human tubuloepithelial cells, AngII caused a rapid activation of Smad pathway (observed at 15 min of incubation), showed by increased Smad2/3 phosphorylation, nuclear translocation of phosphorylated Smad2 and Smad4, and increased DNA-binding activity to CAGA-box oligonucleotide. Our data demonstrate that AngII

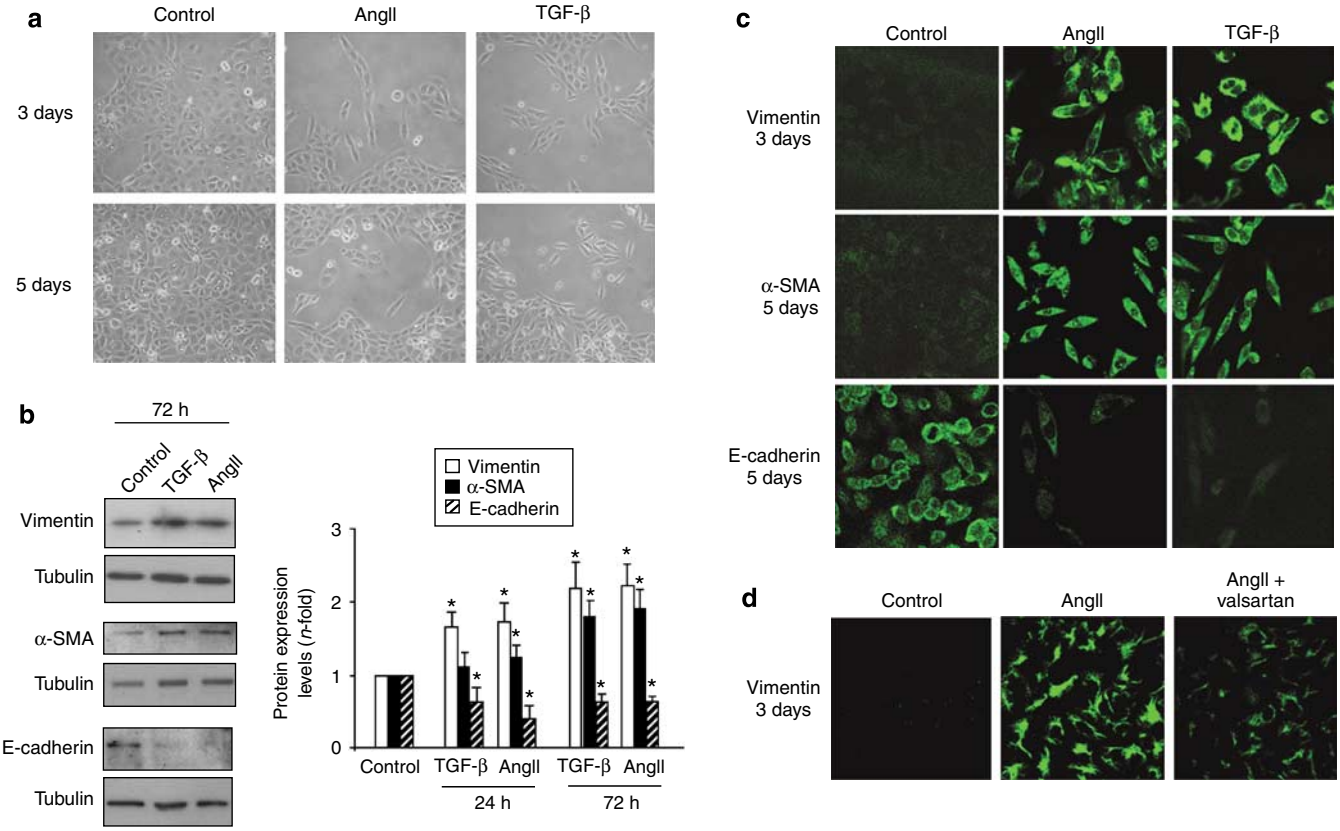

Figure 6 | AngII causes EMT in human tubuloepithelial cells. (**a**) Cells were stimulated with 10^{-7} mol/l AngII or 1 ng/ml of TGF-β1 for 3 and 5 days. Phase-contrast images were taken at ×200 original magnification. (**b**) AngII induces *de novo* expression of vimentin and α-SMA and the loss of E-cadherin. HK2 cells were treated with 10^{-7} mol/l AngII or 1 ng/ml of TGF-β1 for 24 and 72 h. Results of total protein expression were obtained from densitometric analysis and expressed as ratio protein/tubuline as *n*-fold over control. The figure shows a representative western blot in the left panel and data of total protein levels as mean ± s.e.m. of four independent experiments in the right panel. $^*P < 0.05$ vs control. (**c**) Confocal microscopy analysis. The vimentin, E-cadherin, and α-SMA were detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody. The figure is representative of three experiments. AngII induces vimentin expression via AT_1 receptor. Cells were pretreated with valsartan (10^{-6} mol/l) for 1 h and then treated with AngII for 24 h or 3 days. Figure (**d**) shows a representative confocal experiment of 3 done.

activates the Smad signaling in the kidney *in vivo* and in cultured human tubuloepithelial cells.

In the kidney, there is an interesting link between AngII and TGF-β. Angiotensin-converting enzyme inhibitors or AT receptor antagonists diminish renal expression of TGF-β and fibrosis.[3] In cultured renal cells, AngII stimulates TGF-β expression, and the blockade of TGF-β diminishes some AngII responses, including extracellular matrix regulation.[2,3] Infusion of AngII into rats also increased renal TGF-β synthesis.[24] However, we have found that AngII-induced Smad activation (observed at 24 h) occurs earlier than TGF-β upregulation (observed after 72 h in gene expression and later on in protein levels). These data suggest that in the kidney AngII causes a rapid activation of the Smad pathway by a TGF-β-independent mechanism. To further demonstrate whether the AngII effect was independent of endogenous TGF-β production, *in vitro* studies were done. Firstly, we have compared the time-course response of Smad activation and TGF-β induction. In tubuloepithelial cells, AngII only increases TGF-β production after 24 h of incubation (data not shown), whereas Smad activation was found after 15 minutes of AngII stimulation. Secondly, we blocked TGF-β using a neutralizing antibody against active TGF-β and an inhibitor of TGF-β receptor. The blockade of endogenous TGF-β did not alter nuclear translocation of phosphorylated Smad2 and Smad4 and Smad DNA-binding activity observed after 15 min of incubation with AngII, showing a TGF-β-independent Smad signaling activation. However, TGF-β mediates late activation of Smad pathway, because TGF-β blockers diminished AngII-induced Smad-dependent transcription noted after 24 h. Similar findings have been described in VSMCs. The early AngII-induced Smad activation is TGF-β independent,[10] whereas the late effect is mediated by TGF-β.[21]

There are several Smads proteins. Smad2 and Smad3 are specific mediators of TGF-β/activin pathways, whereas Smad1, Smad5, and Smad8 are involved in bone morphogenetic protein signaling.[6] Smad3 is critical for EMT in models of kidney injury and in aggressive carcinoma metastasis.[5,14] Smad3 and Smad2 have different roles in

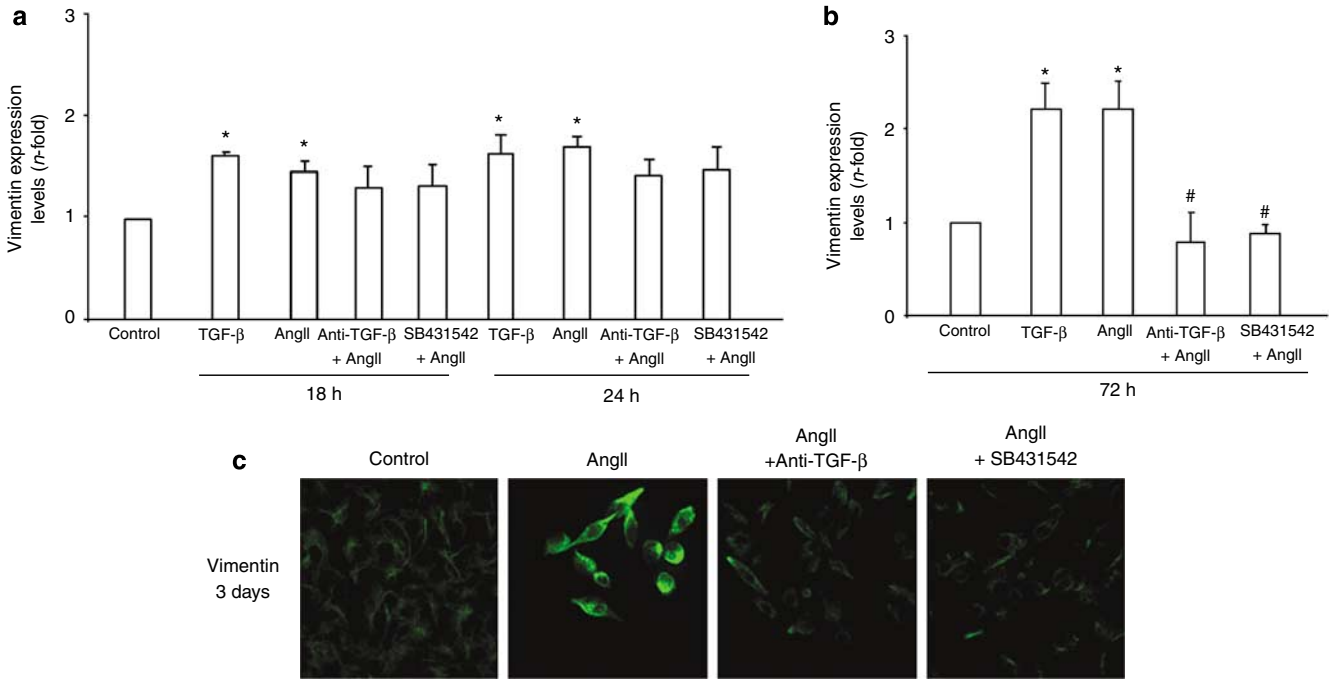

Figure 7 | AngII activates early EMT markers independently of TGF-β in tubuloepithelial cells. After TGF-β blockade, cells were stimulated for 18 and 24 h. (**a**) Vimentin expression as mean ± s.e.m. of three western blot experiments. TGF-β is involved in EMT and morphological changes observed after 3 days of AngII treatment. (**b**) Vimentin expression as mean ± s.e.m. of three western blot experiments. *$P<0.05$ vs control. #$P<0.05$ vs AngII. (**c**) Three representative inmunofluorescense experiments done.

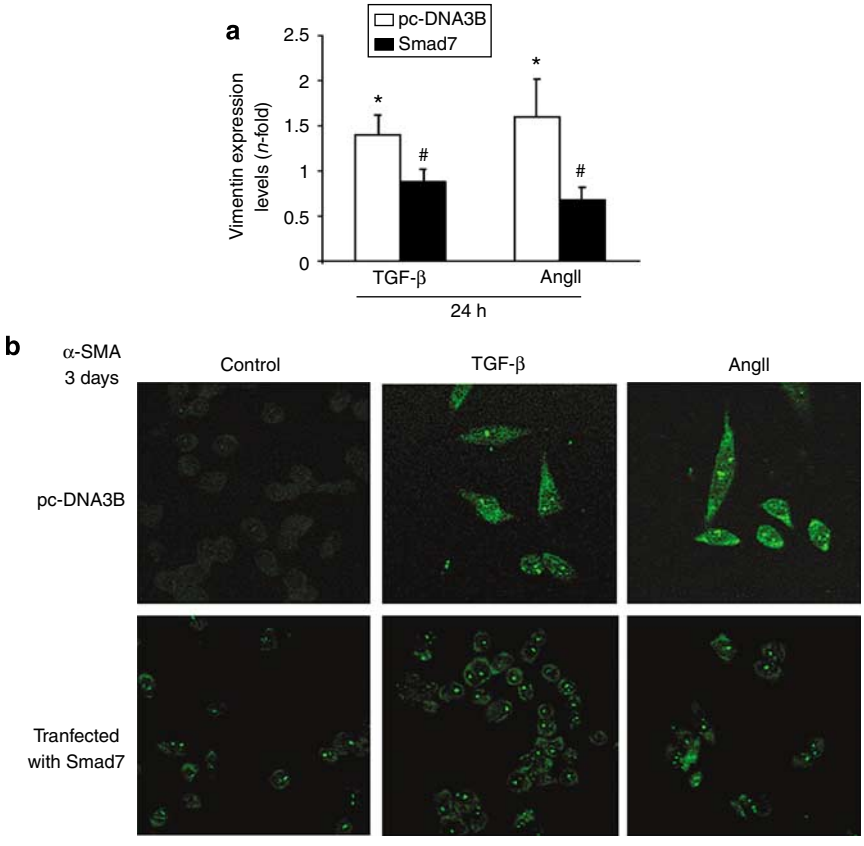

Figure 8 | Smad is involved in AngII-induced EMT. HK2 cells were transiently transfected with Smad7 expression vector or empty vector (pcDNA3B). Then, cells were stimulated with AngII or TGF-β for (**a**) 24 h and (**b**) 3 days (**b**). (**a**) Data of total vimentin protein levels as mean ± s.e.m. of three independent experiments. *$P<0.05$ vs control; #$P<0.05$ vs empty vector. (**b**) The α-SMA was detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody. The figure is representative of three experiments done.

TGF-β-induced EMT. The regulation of connective tissue growth factor (CTGF) and E-cadherin expression are Smad3 dependent, whereas MMP-2 is regulated by Smad2. Both Smad2 and Smad3 regulate α-SMA.[5] In VSMCs, Smad3 is involved in AngII-mediated vascular fibrosis.[21] Our data show that AngII activates Smad2/3 proteins in the kidney and cultured renal cells, showing a similar pathway than TGF-β. There is a cross-talk between MAPKs and Smad.[6] Overexpression of constitutively active members of the ras/MEK/ERK cascade promotes Smad3-dependent processes in kidney mesangial cells, while blocking the nuclear accumulation of Smads in epithelial cells.[25] In VSMC, AngII activates Smad pathway via MAPKs activation.[10,21] In HK2 cells, we have found that all three MAPK inhibitors (p38, ERK, and JNK) significantly diminished AngII-induced Smad2 phosphorylation. Future studies are needed to elucidate the interactions between Smad and MAPK signaling *in vivo* in the kidney.

Rats that have been AngII-infused for 2 weeks presented mild tubular damage, as described,[16,17] and induction of EMT, shown by α-SMA upregulation and downregulation of E-cadherin. Several data in experimental models of renal injury also indicate that AngII participates *in vivo* in the EMT process.[16] Evidences of EMT have also been reported in human diabetic and nondiabetic progressive nephropathies. In these renal pathologies, upregulation of renal renin–angiotensin system and tubular myofibroblast activation have been described.[18,26] We have demonstrated that, in cultured human tubuloepithelial cells, AngII causes suppression of E-cadherin and *de novo* vimentin and α-SMA expression, leading to the loss of epithelial cell adhesion and the change from epithelial to fibroblast-like morphology, suggesting a transitional phase in the dynamic phenomenon of EMT, confirming previous data.[15] TGF-β is a key factor in renal EMT, by initiating and regulating the entire process.[7,11,27] We have compared the effect of AngII with that of TGF-β. After 24 h of incubation, AngII induced vimentin and α-SMA production and suppression of E-cadherin, showing a similar early regulation of EMT markers to that observed with TGF-β. The presence of fibroblast-like cells was found at 3 days, with more positive cells at 5 days, but without differences between AngII and TGF-β, suggesting that both factors have a similar effect on EMT. We have further investigated whether TGF-β mediates AngII-induced EMT. In human tubuloepithelial cells, the induction of vimentin production at 18 h of incubation with AngII was not diminished by TGF-β blockers, showing that early EMT caused by AngII is TGF-β independent. However, the TGF-β blockade diminished the phenotypic conversion to fibroblast-like morphology and induction of vimentin after 3 days, showing that endogenous TGF-β production participates in long-term EMT. Systemic AngII infusion elicited changes in EMT markers after 2 weeks. At this time, Smad activation and TGF-β upregulation were also found, indicating that, in chronic infusion of AngII, the activation of Smad pathway by endogenous TGF-β could contribute to renal damage progression.

Transforming growth factor-β promotes EMT by several mechanisms, with Smad pathway being the most relevant. Our *in vitro* studies, blocking the Smad pathway by transient transfection with Smad7, which interfere with activation of Smad2 and Smad3, demonstrate that Smad pathway participates in AngII-induced EMT. Thus, in cultured tubuloepithelial cells, Smad7 overexpression prevented vimentin and α-SMA induction and the transition to fibroblast-like morphology caused by AngII. In different animal models, including unilateral ureteral obstruction and experimental hypertension, Smad7 overexpression attenuates renal fibrosis.[28] The same has been observed in animal models of peritoneal fibrosis induced by peritoneal dialysis, where Smad7 overexpression inhibited fibrosis.[29,30] Different therapies to interfere with TGF-β, including neutralizing antibodies, antisense oligonucleotides, and decorin, have been shown to diminish renal fibrosis. However, these treatments cannot be used in humans. Renin–angiotensin system blockers are commonly used in human renal diseases with proven end-organ protective effects.[3] The use of AngII blockers, besides inhibiting AngII actions, also interfere with TGF-β/Smad signaling, providing an important tool to hinder TGF-β and prevent the loss of functional renal tissue.

Our results show that AngII activates the Smad signaling system by a TGF-β-independent process in the kidney, both *in vivo* and *in vitro*. We have also found that Smad proteins participate in AngII-induced EMT. This novel finding suggests that Smad activation could participate in AngII-mediated profibrogenic effects in renal diseases.

MATERIALS AND METHODS

Experimental studies

Systemic infusion of AngII (in saline) was done into male Wistar rats of 3 months of age (subcutaneous osmotic minipumps, Alza Corp., CA), at 100 ng/kg/min for different times (from 24 h to 2 weeks; $n = 4$–8 animals per group). A control group of saline-infused rats of the same age was also studied ($n = 8$ animals). The kidneys were perfused *in situ* with cold saline before removal. One kidney was fixed, embedded in paraffin, and used for immunohistochemistry, and the other kidney was snap-frozen in liquid nitrogen for RNA and protein studies (done in renal cortex). All experimental procedures were approved by the Animal Care and Use Committee of our Institution, according to the guidelines for ethical care of the European Community.

Cell cultures

HK2 cells were grown in RPMI with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin, ITS, and hydrocortisone in 5% CO_2 at 37°C. At 60–70% of confluence, the cells were growth-arrested in a serum-free medium for 24 h before the experiments. AngII (10^{-7} mol/l) was added each day, and the medium and all stimuli were replaced every 48 h. Cell culture reagents were obtained from Life Technologies Inc. and AngII was obtained from Bachem. The inhibitors used were PD98059, ERK1/2 inhibitor, SB203580, p38 MAPK inhibitor, and SP600125, JNK-1,-2,-3 inhibitor from Stressgen Bioreagents Corp. (Victoria, BC, Canada). None of the inhibitors were toxic at the doses used (evaluated by cell viability assay microculture tetrazoluin

assay, Promega). Antibodies employed were α-SMA (Sigma Chemical Co., St Louis, MO, USA); vimentin (BD Pharmingen, San Diego, CA, USA), E-cadherin (R&D Systems, Minneapolis, MN, USA), Smad2, Smad4, phosphorylated Smad2/3 (Sta. Cruz Biotechnology, Sta. Cruz, CA, USA), pSmad2 (Cell Signaling Technology, Danvers, MA, USA), pSmad3 kindly donated by Dr Leof (Mayo Clinic, Baltimore), TGF-β (ABCam, Cambridge, UK), and secondary antibodies (Amersham, Buckinghamshire, UK).

Protein studies

To quantify protein levels, western blot was done.[10,31] Protein content was determined by BCA method (Pierce Biotechnology, Rockford, IL, USA). The efficacy of protein loading and transfer to membranes was assessed by α-tubulin, GAPDH, and Ponceau S staining.

Immunocytochemistry was performed in cells growing in coverslips, fixed, treated with 0.1% Triton X-100, incubated with primary antibodies, followed by fluorescein isothiocyanate-conjugated antibodies.[10] Nuclei were stained with 1 μg/ml propidium iodide. Samples were mounted in Mowiol 40–88 and examined by a laser scanning confocal microscope (Leika).

Immunohistochemistry was done in paraffin-embedded renal sections.[18] Renal sections of 4 μm were deparaffinized, rehydrated, their endogenous peroxidase was blocked and incubated with primary antibody (overnight, 4°C), followed by incubation with the corresponding secondary antibody, and revealed by standard techniques. The specificity was checked by the omission of primary antibody and use of nonimmune sera (not shown).

Gene studies

Total RNA was isolated with Trizol (Gibco, Invitrogen, Carlsbad, CA, USA) and gene expression was analyzed by real-time PCR, performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Assay IDs used are TGF-β:Rn00572010_m1, thromsbospondin-1: Rn01513693_m1, Smad4;Rn00570593_m1, Smad7; Rn00578319_m1, α-SMA:Rn00570060_g1, E-cadherin:Rn00580109_m1, and vimentin:Rn0059738_m1. For normalized data, different approaches were done using several housekeeping genes, including GAPDH, Histone-3, and 18s ribosomal RNA expression (assay IDs: Rn99999916_m1 and Hs99999901_s). Each animal was evaluated independently by duplicate, and data were expressed as mean ± s.e.m. of eight animals per group as *n*-fold increase vs control group.

Analysis of Smad DNA-binding activity

Smad DNA-binding activity was determined in 6 μg nuclear extracts as described,[10] by binding with radioactive labeled consensus CAGA-box oligonucleotide (5′-TCGAGAGCCAGACAAAAAGCCA GACATTTAGCCAGACAC-3′, Sta. Cruz), and complexes were analyzed by electrophoretic mobility shift assay. Competition assays were done with a 100-fold excess of unlabeled or mutant oligonucleotide (5′-TCGAGAGCTAGATAAAAAGCTAGATATTAGCTAG ATAC-3).

Southwestern histochemistry was used for Smad detection as described,[18] including controls and the Smad binding consensus sequence (5′-GAGTATGTCTAGACTGACAATGTAC-3′).

Transfection, DNA constructs, and promoter studies

HK2 cells in fetal bovine serum were transiently transfected for 18 h with FuGENE (Roche Molecular Biochemicals, Indianapolis, IN, USA) and PcDNA3-FLAG-Smad7 expression vector (kindly donated by Dr Massagué, Memorial Sloan-Keternig Cancer Center, New York, USA) or empty vector (pcDNA3B). Cells were growth-arrested for 24 h before experiments. To demonstrate Smad7 transfection efficacy, an anti-FLAG antibody was used (not shown). Smad-dependent promoter activation was evaluated by transfection of Smad/luc (kindly donated by Dr Volgestein, Baltimore, MD, USA) and TK-renilla as internal control, as described.[10]

Statistical analysis

Histochemistry was quantified by image analysis using a KZ 300 imaging system 3.0. (Zeiss, Munchen-Hallbergmoos, Germany). For immunohistochemistry, the percentage of the stained area was calculated as the ratio of stained area vs total field area, and staining score is expressed as density/mm^2. For Southwestern histochemistry, positive cells were counted and expressed as number of Smad positive cells/mm^2. For each sample, the mean data was obtained by analysis of 20 different fields (× 200). These experiments were performed in two kidney sections per experimental animal to obtain a mean score for each of them. In all cases, evaluations were performed by two independent observers in a blinded fashion, and the mean score value was calculated for each rat.

The autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One, Bio-Rad, Hercules, CA, USA). Results are expressed as *n*-fold increase over control as mean ± s.e.m. Equality of variances was tested with Levene's test. Normally distributed continuous variables with equal variances were analyzed with analysis of variance, otherwise with Kruskall–Wallis test. A P value <0.05 was considered significant. Tests were done using the SPSS 11.5 software package.

DISCLOSURE

The authors declared no competing interests.

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