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Research Paper

Pharmacological Modulation of Epithelial Mesenchymal Transition Caused by Angiotensin II. Role of ROCK and MAPK Pathways

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Purpose. Tubulointerstitial fibrosis is a final common pathway to end-stage chronic kidney diseases, which are characterized by elevated renal angiotensin II (AngII) production. This peptide participates in kidney damage inducing fibrosis and epithelial mesenchymal transition (EMT). Our aim was to describe potential therapeutic targets in AngII-induced EMT, investigating the blockade of different intracellular pathways.
Methods. Studies were done in human tubular epithelial cells (HK2 cell line), evaluating changes in phenotype and EMT markers (Western blot and immunofluorescence).
Results. Treatment of HK2 cells with AngII for 3 days caused transdifferentiation into myofibroblast-like cells. The blockade of MAPKs cascade, using specific inhibitors of p38 (SB203580), extracellular signal-regulated kinase1/2 (ERK; PD98059) and Jun N-terminal kinase (JNK) (SP600125), diminished AngII-induced EMT. The blockade of RhoA/ROCK pathway, by transfection of a RhoA dominant-negative vector or by ROCK inhibition with Y-27632 or fasudil, inhibited EMT caused by AngII. Connective tissue growth factor (CTGF) is a downstream mediator of AngII-induced EMT. MAPKs and ROCK inhibitors blocked CTGF overexpression induced by AngII. HMG-CoA reductase inhibitors, although blocked AngII-mediated kinases activation, only partially diminished EMT and did not regulate CTGF.
Conclusions. These data suggest a potential therapeutic use of kinase inhibitors in renal fibrosis.

KEY WORDS: angiotensin; epithelial mesenchymal transition; kinase inhibitors; renal damage; statins.

INTRODUCTION

The incidence of renal diseases is growing in Western countries. Independently of the initial insult a common feature of renal diseases is the progression to tubulointerstitial fibrosis and end-stage kidney failure. Among the current clinical treatments, the blockade of angiotensin II (AngII) is one of the best pharmacological options with proven organ-protective effects (1). However, these drugs only slow the progression of the disease and novel therapeutic options are needed to regress renal fibrosis. The molecular mechanisms involved in renal fibrosis and its pharmacological modulation are very important fields of research in chronic kidney diseases.

Tubulointerstitial fibrosis is characterized by an excessive accumulation of extracellular matrix proteins, such as collagens, in part attributable to an elevated synthesis mainly by interstitial fibroblasts. Many evidences suggest that under pathological conditions renal tubuloepithelial cells can undergo epithelial mesenchymal transition (EMT) becoming matrix-producing fibroblasts, and therefore contribute to renal fibrosis and progression to end-stage kidney disease (2). EMT is characterized by a phenotypic conversion from epithelial cells to fibroblast-like morphology. During this process, there is an induction of mesenchymal markers, such as α-smooth muscle actin (α-SMA) and vimentin, and epithelial markers disappear, like E-cadherin that is essential for the structural integrity of renal epithelium (2). Most of the studies of EMT have focused on TGF-β responses. This growth factor participates in all of the steps of EMT (2). AngII shares many cellular responses with TGF-β (3, 4). In the kidney, AngII actively participates in renal fibrosis, in part mediated by TGFβ (5). Recently, we

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ABBREVIATIONS: AngII, angiotensin II; AT, angiotensin receptors; CTGF, connective tissue growth factor; EMT, epithelial mesenchymal transition; ERK, extracellular signal-regulated kinase1/2; FBS, fetal bovine serum; HK2, human tubular epithelial cell line; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; JNK, Jun N-terminal; MAPK, mitogen activated kinases; ROCK, rho-kinase; TGF-β, transforming growth factor-beta; VSMC, vascular smooth muscle cells.

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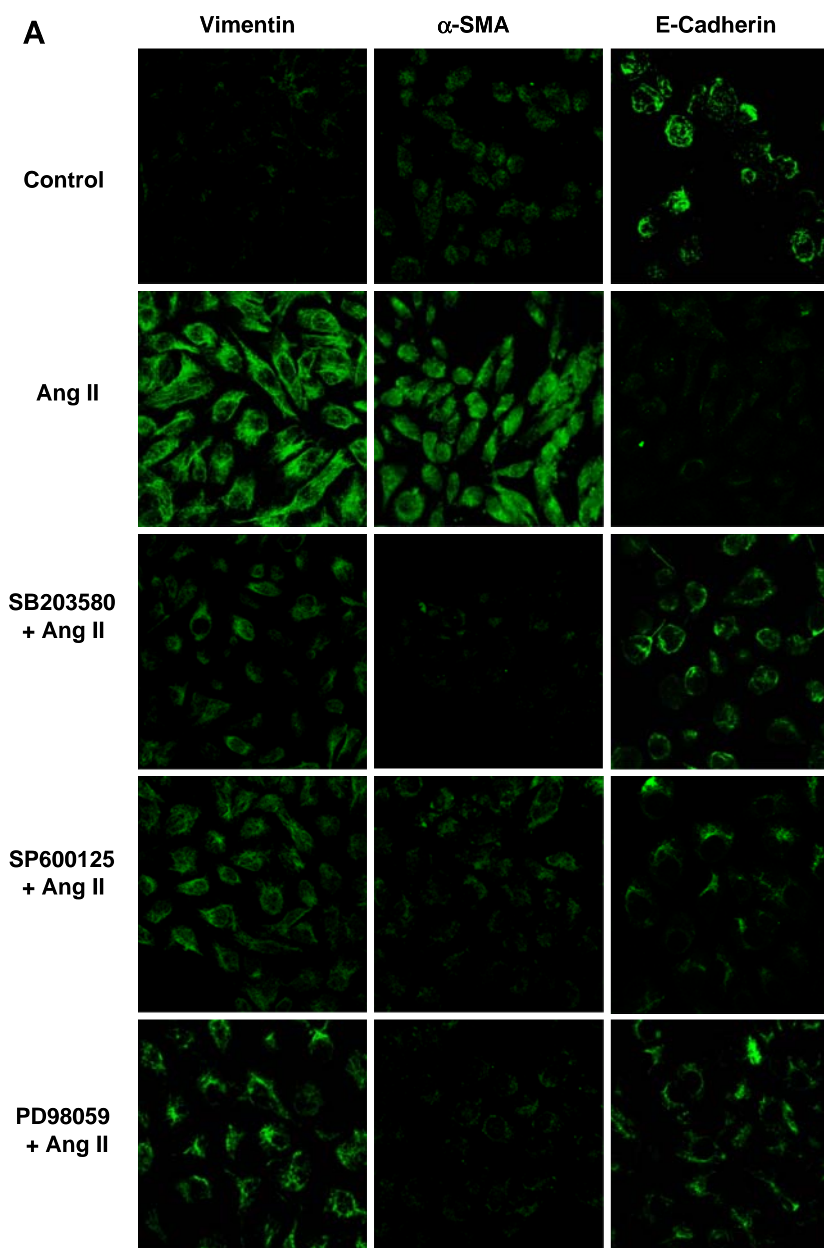
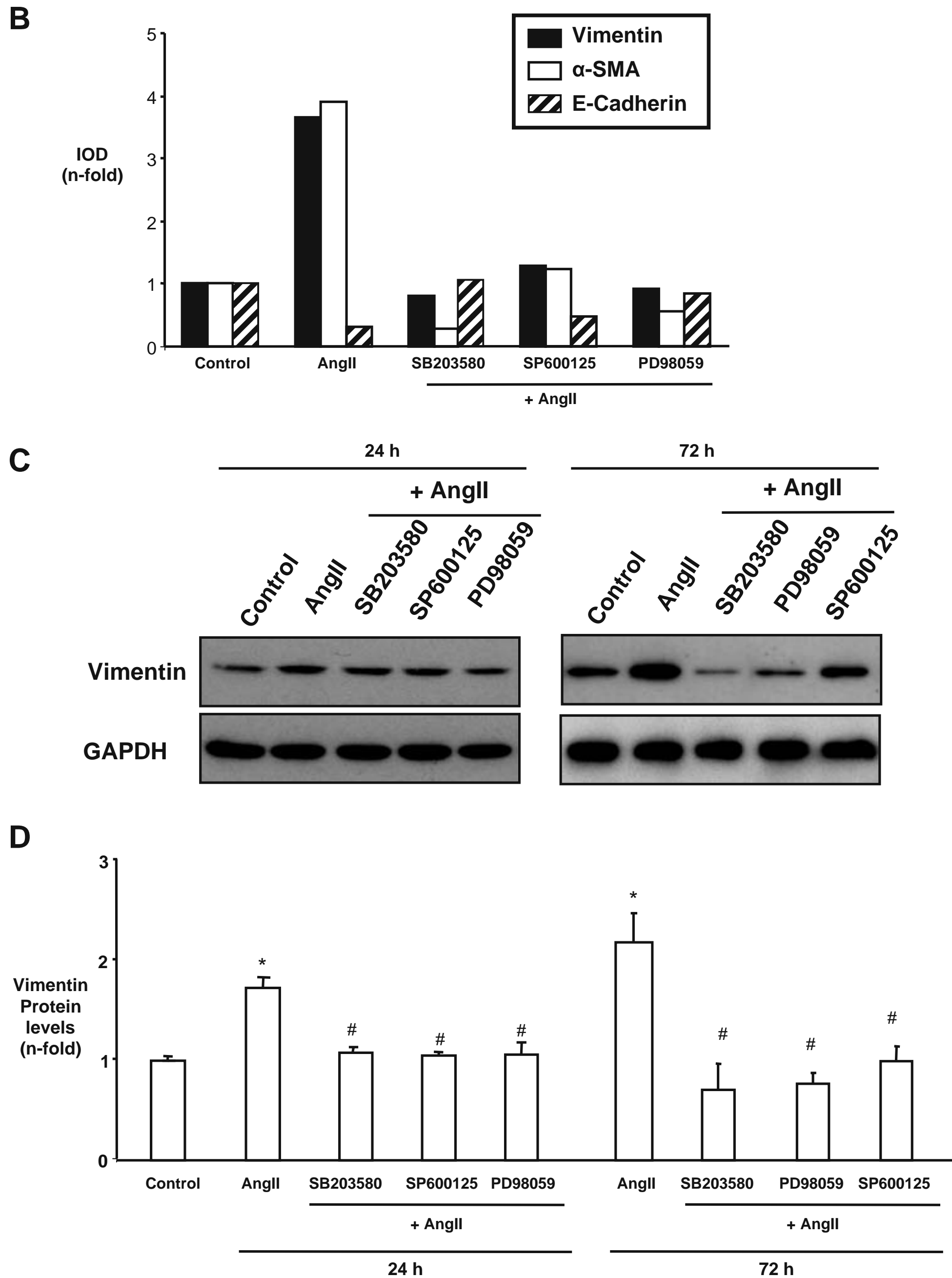


Fig. 1. MAPKs inhibitors diminish AngII-induced EMT in human tubuloepithelial cells. Cells were preincubated for 1 h with the following MAPKs inhibitors: SB203580 (p38 inhibitor, at 10^{-6} mol/l), PD98059 (ERK p42/44 inhibitor, at 10^{-5} mol/l) and SP600125 (JNK inhibitor, at 10^{-5} mol/l) and then treated with 10^{-7} mol/l AngII for 3 days. **A** Vimentin, α-SMA and E-Cadherin were detected by an indirect immunostaining using FICT-labeled secondary antibodies, and evaluated by confocal microscopy. Figure shows a representative experiment of three done. All three MAPKs inhibitors markedly diminished the phenotypic conversion caused by AngII; the cells present an epithelial morphology with positive E-cadherin staining, but a weak immunostaining for vimentin and α-SMA. **B** Quantification of immunofluorescence data expressed as integrated optical density (IOD) as described in Methods. Vimentin expression was quantified by Western blot after 24 and 72 h of incubation. **C** A representative Western blot and **D** data as mean ± SEM of three independent experiments. *P<0.05 *vs* control. #P<0.05 *vs* AngII.

have shown that AngII directly activates the Smad signaling system in the kidney and induces EMT through TGF-β/Smad pathway (6). Many studies have shown that AngII inhibitors diminish renal TGF-β overproduction and signaling activation (3, 4), showing that these drugs are one of the best options to block TGF-β in humans.

AngII binds to specific receptors, AT_1 and AT_2, to activate cellular responses. AT_1 receptor mediates upregula-

Fig. 1. (continued)



tion of growth factors, extracellular matrix accumulation and EMT (5,6). The AT_1 signaling mechanisms are similar to those activated by cytokines, and include activation of protein kinases, as for example mitogen-activated protein kinase (MAPK) cascade and Rho-kinase (ROCK) (5). Several intracellular signaling systems are involved in EMT and renal fibrosis. Recent studies have demonstrated that the MAPK pathway regulates EMT caused by TGF-β (7, 8). Activation of small Rho GTPases is a key step in EMT (9). ROCK is a downstream target of RhoA involved in TGF-β-mediated EMT (10). For this reason, we have investigated the potential role of MAPK and RhoA/ROCK pathways in AngII-induced EMT.

Several clinical trials have demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) exert beneficial effects in patients at high risk of developing cardiovascular events (11). These drugs combine hypolipemic effect with other effects, such as antioxidant, anti-inflammatory, immunomodulatory, and antithrombotic (called "pleiotropic" effects) (11, 12). Their beneficial actions can be attributed to the inhibition of intracellular signaling pathways, including MAPK and RhoA/ROCK pathways (11–13). However, the role of statins in renal disease progression and in the regulation of renal fibrosis and EMT is not completely elucidated (14–17). In this work, we have investigated whether statins could directly modulate AngII-induced EMT, studying

the regulation of CTGF and the molecular mechanisms underlying this process, evaluating the role of the activation of Rho/ROCK and MAPK pathways. These experiments might help to unveil the mechanisms of renal damage perpetuation and suggest novel therapeutic strategies for the modulation of the pathobiology of renal injury.

MATERIALS AND METHODS

Cell Cultures

HK2 cells (human renal proximal tubuloepithelial cells) were grown in RPMI with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin, ITS (5 μg/ml) and hydrocortisone (36 ng/ml) in 5% CO_2 at 37°C. At 60–70% of confluence, cells were growth-arrested in serum-free medium for 24 h before the experiments.

Materials

AngII (from Fluka), at the dose of 10^{-7} mol/l, was added each day, and medium and all stimuli were replaced every 48 h. Cell culture reagents were obtained from Life Technologies, Inc. Atorvastatin was from Pfizer (Madrid, Spain) and simvastatin from Merck Sharp and Dome (Madrid). PD98059; ERK1/2 inhibitor, SB-203580; p38 MAPK inhibitor, and SP600125: JNK-1,-2,-3 inhibitor were from Stressgen Bioreagents Corp.(Victoria, British Columbia, Canada); Fasudil and Y-27632; ROCK inhibitors from Tocris Cookson (Bristol, UK), and the rest of compounds from Sigma-Aldrich. None of the inhibitors were toxic at the doses used (evaluated by cell viability assay MTS-PMS, Promega, not shown). The antibodies employed were: Smooth muscle α-actin (α-SMA) (Dako); Vimentin (BD Pharmingen), CTGF from Torrey Pines Biolabs (Houston, TX, USA), phospho-JNK1/2 from Stressgen Bioreagents Corp; Phospho-ERK1/2, ERK1/2, JNK1/2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), GAPDH (Calbiochem), peroxidase-conjugated secondary antibodies (Amersham). To block CTGF actions, we used a CTGF antisense oligonucleotide, constructed with a 16 mer derived from the starting translation site, which contained the initial ATG whose sequence is 5′-TACTGG CGGCGGTCAT-′3.

Transfection and DNA Constructs

HK2 cells, in 24 well-plates, were transiently transfected with FuGENE (Roche Molecular Biochemicals) and the reporter expression vectors for 18 h. The expression vectors containing cDNAs for constitutively active RhoA (pcDNA3-Q63L-RhoA) and wild type RhoA (pcDNA3-wtRhoA) gifts from Dr. Piero Crespo (Instituto de Investigaciones Biomédicas,

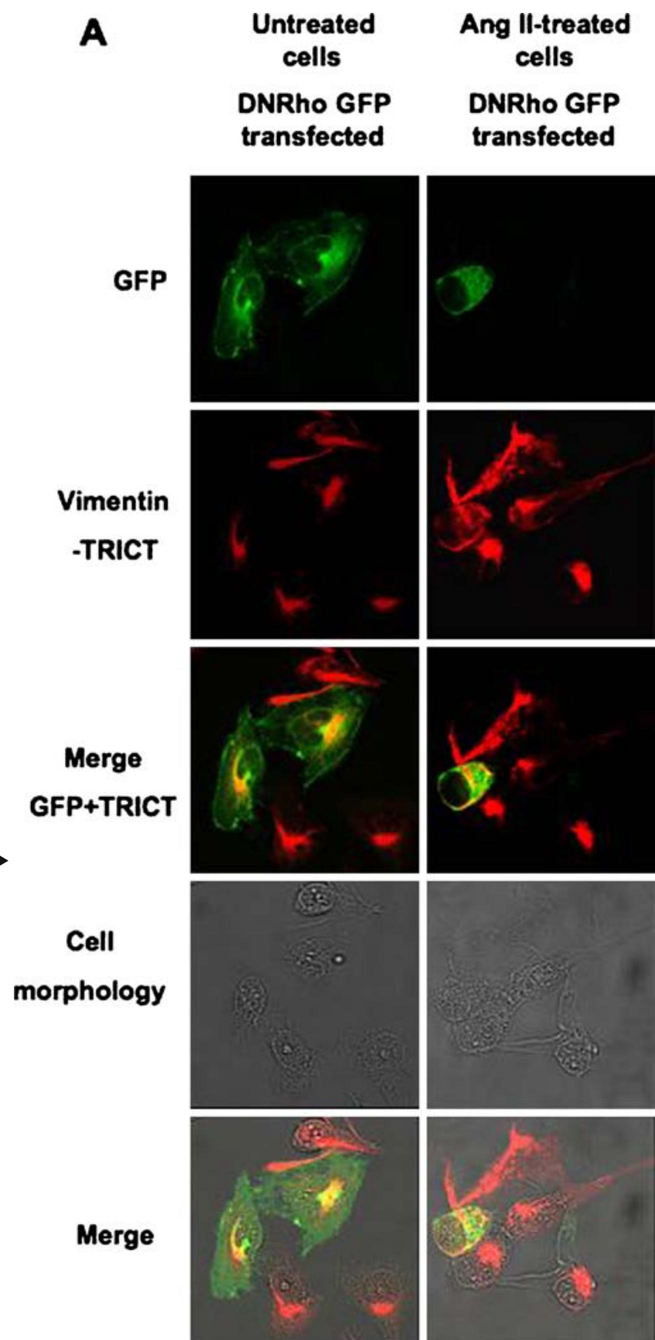


Fig. 2. The RhoA/ROCK pathway is involved in AngII-induced EMT in human tubuloepithelial cells. Cells were transiently transfected with **A** dominant negative isoform of RhoA (pcDNA3–GFP–N19RhoA) or **B** empty vector (pcDNA3–GFP) for 18 h and then treated with 10^{-7} mol/l AngII for 3 days. Immunocytochemistry shows green fluorescence in transfected cells (GFP-positive). Vimentin was detected by an indirect immunostaining using a TRICT-labeled secondary antibody (red staining). The colocalization of transfected cells and positive vimentin staining is shown by yellow staining (in merge GFP + TRICT). To better follow this data the morphology of the cells is shown. Merge: unstimulated cells present round shape, with a slight vimentin staining mainly in the nuclear membrane, showing no differences in cell shape and vimentin distribution between transfected (green) and non-transfected cells. **C** Cells were transiently transfected with a wild type of RhoA (WT–RhoA) and empty vector (pcDNA3B). Vimentin was detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody (green staining) after 48 h of transfection. The figures of confocal microscopy show a representative experiment of three done.

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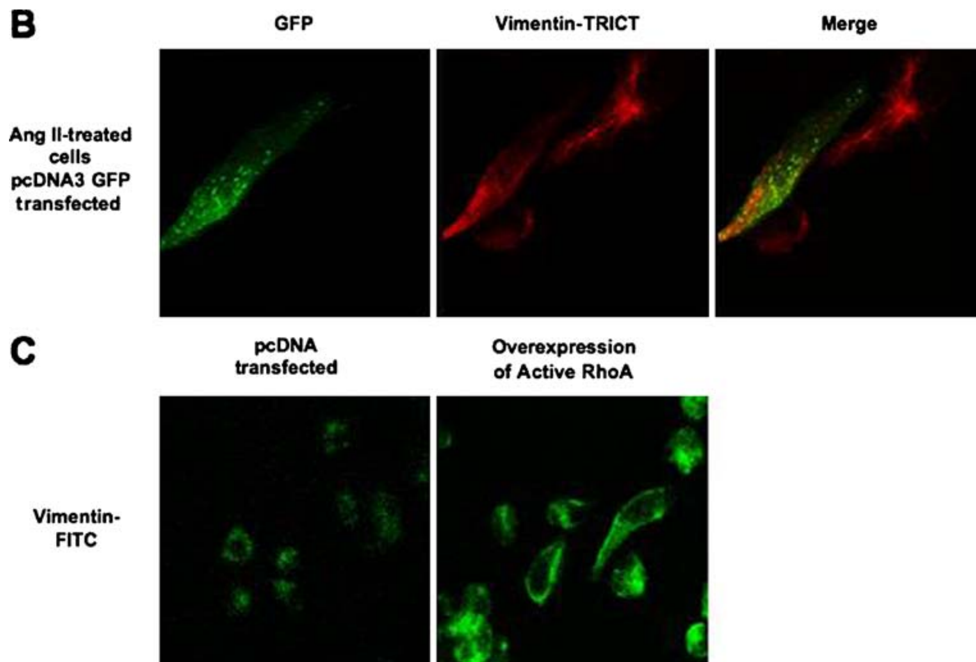

Madrid, Spain) and dominant-negative RhoA (pcDNA3-GFP-N19RhoA) and empty vector (pcDNA3B-GFP) from Dr. del Pozo (CNIC, Madrid, Spain). After transfection, cells were growth-arrested for 24 h before confocal microscopy experiments. In these experiments several differences in cell shape can be found compared to pharmacological studies, mainly due to the lower cell density necessary for a good efficacy of transfection and the different secondary antibody used.

Protein Studies

Cells were homogeneized in lysis buffer [170 mmol/l Tris HCl, 22% glycerol, 2,2% sodium dodecyl sulfate (SDS) with 0,1 mmol/l phenylmethylsulfonyl fluoride, NaF, dithiothreitol, ortovanadate and a protease inhibitor cocktail] and then separated by SDS-polyacrilamide gel electroforesis. CTGF, EMT markers and the phosphorylation levels of ERK and JNK levels were determined in total protein extracts by Western blot. Fifty micrograms of proteins were loaded in each lane. Protein content was determined by the BCA method (Pierce, Rockford, IL, USA). The efficacy of protein transfer to the membranes was assessed by Red Ponceau staining (not shown). Results of total protein expression were obtained from densitometric analysis and expressed as ratio protein/GAPDH or phosphorylated/total protein as *n*-fold over control.

For immunocytochemistry, cells growing in coverslips were fixed in merckofix (Merck), treated with 0.1% Triton-X100, incubated with primary antibodies followed by a FITC or TRIC-conjugated secondary antibody. The absence of primary antibody was the negative control. Samples were mounted in Mowiol 40–88 (Sigma) and examined by a laser scanning confocal microscope (Leika). The experiments were done with 3 different cell culture preparations. To validate the protein data obtained by Western blot and immunofluorescence, we have quantified the experiments of confocal microscopy using the Image-Pro plus 4.5.0.29 (Media Cybernetic Inc). The data are expressed as an arbitrary quantification of integrate optical density (IOD), calculated as average of density of fluorescence per area. These data are shown as n-fold of increase *vs* AngII of the representative experiment shown in the corresponding figure.

Quantification and Statistical Analysis

The autoradiographs were scanned using the GS-800 calibrated densitometer (Quantity One, Bio-Rad, Spain). Results are expressed as *n*-fold over control as mean ± SEM of experiments made. One-way ANOVA was used to test compare protein expression levels between groups. When statistical significance was found, Bonferroni post hoc comparison test was used to identify group differences. Differences were considered significant at $p<0.05$. Statistical analyses were conducted using the SPSS statistical software, version 11.0 (SPSS).

RESULTS

MAPKs Inhibitors Diminish AngII-Induced EMT in Human Tubuloepithelial Cells

In human tubuloepithelial cells (HK2 cell line) incubation with AngII for 3 days causes a phenotypic conversion from epithelial cells to myofibroblast-like cells, as described (6,18). The transformed cells lost the typical cobblestone pattern of an epithelial monolayer, as well as the expression

of the epithelial marker E-cadherin, and displayed a spindle-shape, fibroblast-like morphology associated with the induction of the mesenchymal markers, vimentin and α-SMA, which are not found in unstimulated epithelial cells (Fig. 1A).

We have investigated the involvement of MAPKs cascade by a pharmacological approach, using specific inhibitors of p38 (SB203580), extracellular signal-regulated kinase1/2 (ERK; PD98059) and Jun N-terminal kinase (JNK; SP600125) (19, 20). We found that all three MAPKs inhibitors prevented the AngII-induced phenotypic conversion into myofibroblasts observed after 3 days of treatment, and markedly diminished the presence of vimentin and α-SMA-

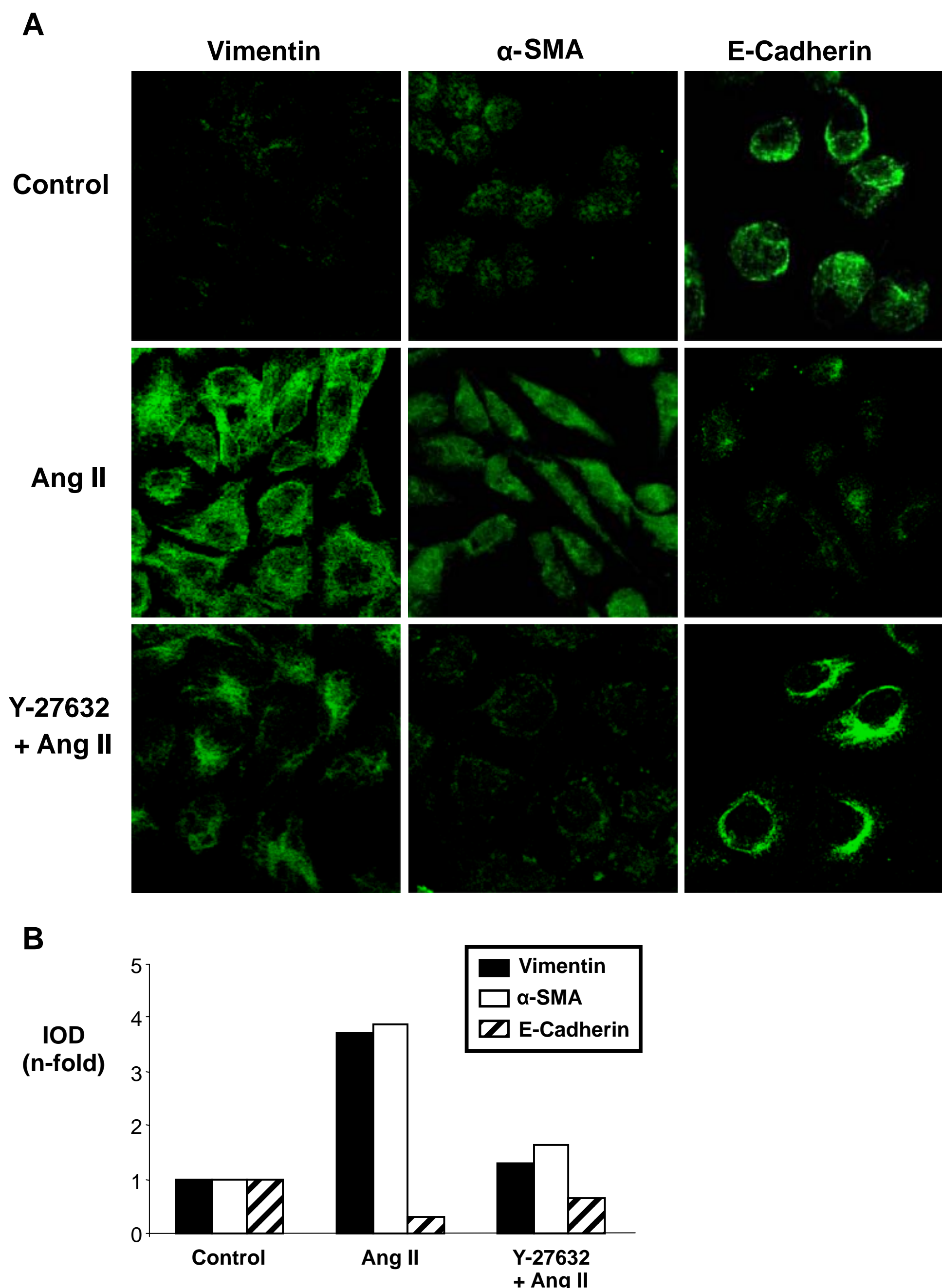

Fig. 3. ROCK inhibitors diminished AngII-induced EMT in human tubuloepithelial cells. Cells were preincubated for 1 h with the ROCK inhibitors Y-27632 or Fasudil (10^{-6} mol/l), and then treated with 10^{-7} mol/l AngII for 24 and 72 h. **A** Vimentin, α-SMA and E-Cadherin were detected by an indirect immunostaining using FICT-labeled secondary antibodies, and evaluated by confocal microscopy. The figures of confocal microscopy show a representative experiment of three done. **B** Quantification of immunofluorescence data expressed as integrated optical density (IOD) as described in "Materials." Vimentin expression was quantified by Western blot after 24 and 72 h of incubation. **C** A representative Western blot and **D** data as mean ± SEM of three independent experiments. *$P<0.05$ *vs* control. #$P<0.05$ *vs* AngII.

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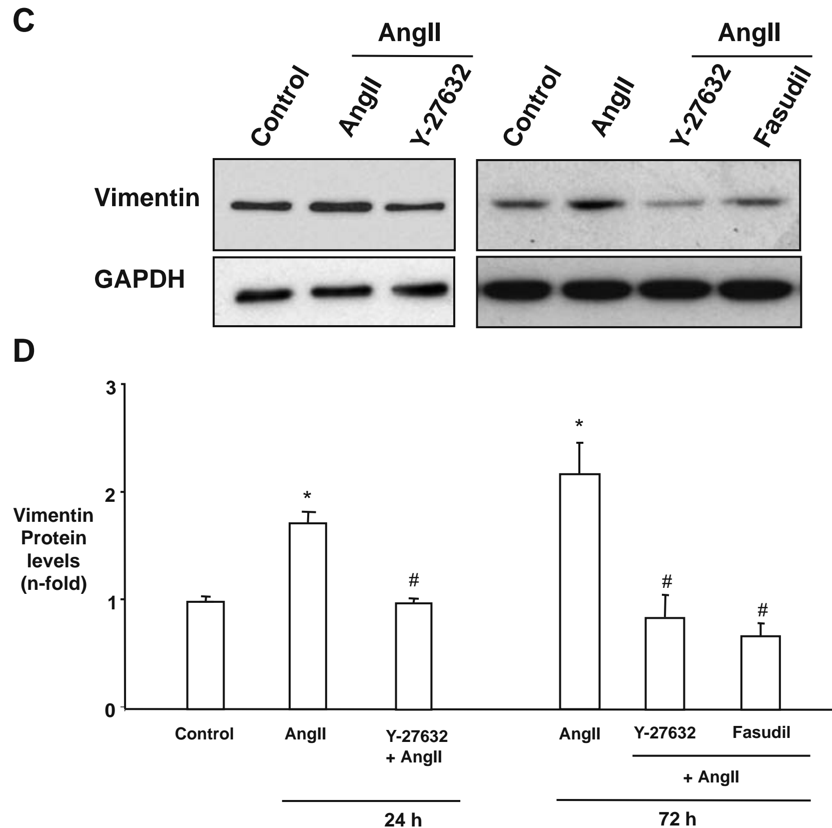


positive microfilaments in the cytoplasm of AngII-treated cells, as shown by confocal microscopy in Fig. 1A. Moreover, the loss of E-cadherin induced by AngII was recovered by the three MAPKs inhibitors (Fig. 1A). In Fig. 1B the quantification of immunofluorescence is shown. None of the inhibitors modified EMT markers in control cells (not shown). By Western blot we have further quantified the changes in EMT evaluating vimentin expression levels. AngII caused a rapid induction of vimentin observed at 24 h, which was remained elevated after 3 days. The three MAPKs inhibitors (p38, ERK and JNK) significantly diminished vimentin induction by AngII both at 24 h and 3 days (Fig. 1C and D). These data show the involvement of all three MAPKs in AngII-induced EMT.

The RhoA/ROCK Pathway Participates in AngII-Induced EMT in Human Tubuloepithelial Cells

The involvement of RhoA in EMT caused by AngII was evaluated by transient transfection of several expression vectors. Transient transfection of HK2 cells with a plasmid encoding a dominant negative RhoA isoform (DN–RhoA–GFP) inhibited morphological changes and vimentin induction caused by AngII at 3 days, and had no effect in unstimulated cells. By confocal microscopy, Fig 2A shows unstimulated samples of several cells transfected with DN-RhoA-GFP (green staining). These cells had an epithelial morphology and slight vimentin expression (red staining), presenting similar characteristics than untransfected control cells. In samples stimulated with AngII for 3 days, the non-transfected cells (with negative GFP staining) change their morphology to myofibroblast-like shape and showed a marked vimentin expression. However, DN–RhoA–GFP transfected cells (green staining) remained with a round shape characteristic of epithelial cells. The morphology and staining of all the different cells is more clear in the merge images (Fig. 2A). In cells transfected with the empty vector (GFP staining), stimulation with AngII elicited EMT, as observed in non-transfected cells of the same experiment (Fig. 2B). Moreover, overexpression of a plasmid encoding constitutively active form of RhoA induced a myofibroblast-like phenotype and vimentin expression, confirming that small G protein RhoA participates in EMT (Fig. 2C).

Rho-kinase is a downstrean target of RhoA. Selective pharmacological inhibition of the serine/threonine ROCK I and II, with Y-27632 and fasudil, significantly diminished AngII-induced vimentin immunostaining (Fig. 3A and B) and protein production, both at 24 and 72 h (Western blot; Fig. 3C and D) and restored E-cadherin expression, inhibiting the conversion into myofibroblasts (Fig. 3A and B).

Role of Endogenous CTGF on AngII-Induced EMT

CTGF is a potent profibrotic factor upregulated in renal diseases in association with scarring and sclerosis (21–23). CTGF is a mediator of AngII and TGF-β induced fibrosis and TGF-β-mediated EMT (21,24). We have investigated whether CTGF is a mediator of AngII-induced EMT in HK2

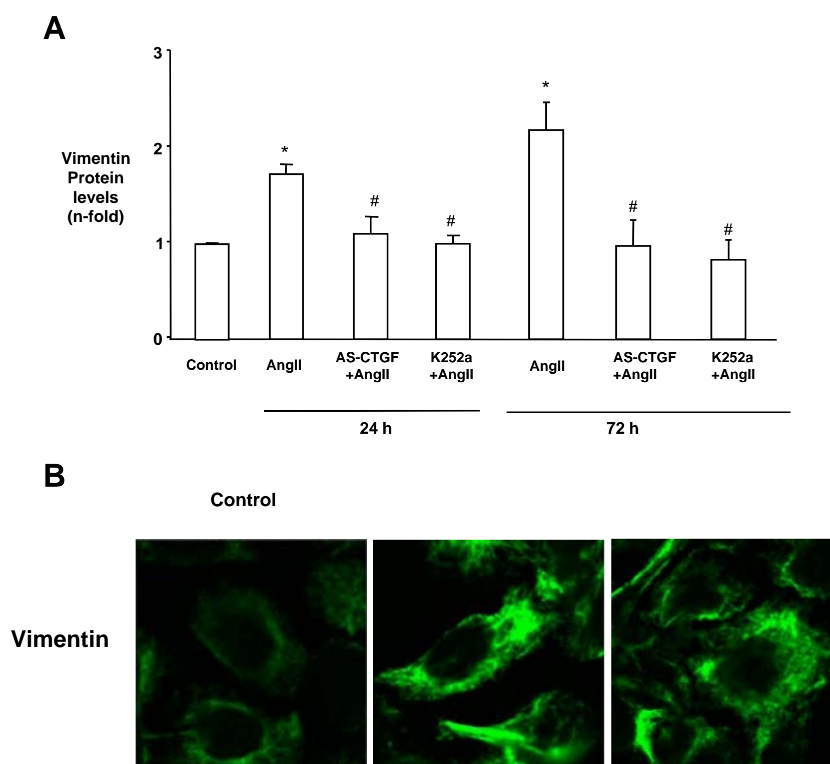


Fig. 4. AngII induces EMT *via* endogenous production of CTGF in human tubuloepithelial cells. CTGF was blocked by antisense oligonucleotide (20 μg/ml) or the CTGF receptor inhibitor K252a (10^{-5} mol/l). Then, cells were stimulated with AngII for 24 and 72 h. **A** Vimentin expression as mean ± SEM of three Western blot experiments. *$P<0.05$ *vs* control. #$P<0.05$ *vs* AngII. **B** A representative immunocytochemistry experiment of two done. Vimentin was evaluated after 3 days of incubation by an indirect immunostaining using a mouse FICT-labeled secondary antibody.

cells. CTGF was blocked by two methods: an antisense oligodeoxynucleotide, that inhibits its expression (24), and the K252a compound, a receptor tyrosine kinase inhibitor that inhibit internalization and sorting of CTGF receptor (25). The CTGF blockers diminished AngII-induced vimentin expression at 24 h and 3 days (Western blot and confocal microscopy, Fig. 4). These data suggest that CTGF is an early EMT mediator.

Previous studies have demonstrated that AngII upregulates CTGF *via* AT_1 receptors and activation of MAPK, PKC and ROCK pathways (5,26–28). Regarding MAPK cascade, we have found that in human cultured tubuloepitheial cells the three MAPKs inhibitors (p38, ERK and JNK) significantly diminished CTGF overproduction caused by AngII (Fig. 5). In fibroblasts, the inhibitors of ERK1/2 and JNK, but not p38/MAPK, decreased AngII-stimulated CTGF expression (27), while in mesangial cells only the p38 inhibitor SB203580 diminished AngII-induced CTGF production (26), showing a different response depending on the cell type. Pretreatment of HK2 cells with the selective ROCK inhibitor Y-27632 suppressed AngII-induced CTGF protein production (Fig. 5). These results suggest that in human tubuloepithelial cells AngII regulates CTGF *via* activation of three MAPKs (p38, ERK and JNK) and ROCK, showing a similar response to EMT regulation.

Effect of HMG–CoA Reductase Inhibitors on Angiotensin II Induced EMT in Cultured Human Tubuloepithelial Cells

HK2 cells were pretreated for 1 h with two statins: atorvastatin and simvastatin and the effect on EMT caused by AngII was evaluated after 3 days. By confocal microscopy, we have found that both statins, at the dose studied, only partially diminished the phenotypic conversion into myofibroblasts. Fig. 6A shows how several statin-treated cells remain with myofibroblast-like morphology. In these cells the induction of EMT markers (vimentin and α-SMA) and the loss of E-cadherin induced by AngII was only partially recovered by the statins. By Western blot we have observed that atorvastatin partially, but not significantly, diminished vimentin induction caused by AngII at 24 h (Fig. 6C).

As shown in Fig. 4, CTGF is a downstream mediator of AngII-induced EMT. We have recently shown that statins inhibited CTGF production caused by AngII in cultured vascular smooth muscle cells (VSMC) and in Wistar rat aorta (13). In cultured human tubuloepithelial cells atorvastatin did not inhibit CTGF production in AngII-treated cells (Fig. 6D), showing a different regulation between cell types. These data suggest that the partial inhibitory effect of statins on EMT regulation in tubuloepithelial cells could be due to the lack of effect on CTGF regulation.

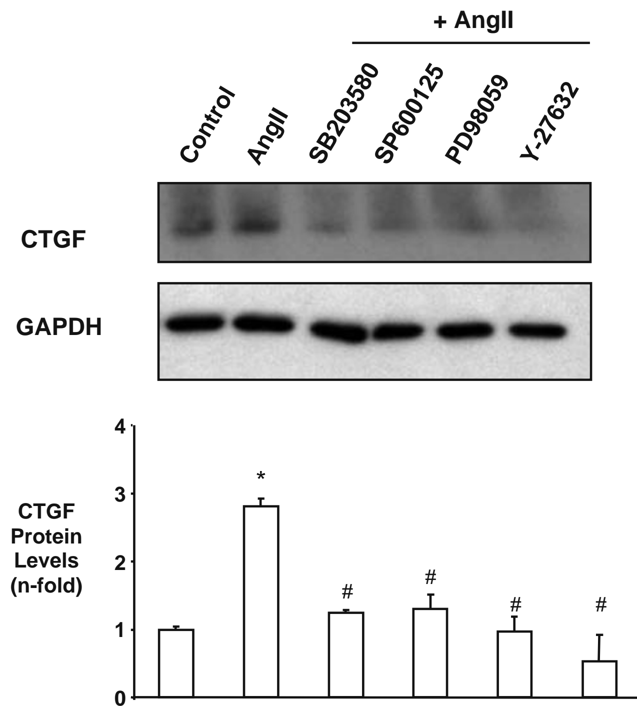


Fig. 5. AngII upregulates production of CTGF *via* MAPK and ROCK activation in human tubuloepithelial cells. Cells were pretreated for 1 h with 10^{-6} mol/l SB203580 (p38 inhibitor), 10^{-5} mol/l of PD98059 (ERK p42/44 inhibitor), SP600125 (JNK inhibitor), 10^{-5} mol/l Y-27632 (ROCK inhibitor), before treatment with 10^{-7} mol/l AngII for 24 h. Figure shows in *top panel* a representative Western blot and in bottom data of total CTGF production as mean ± SEM of four independent experiments. *$P<0.05$ *vs* control, #$P<0.05$ *vs* AngII-treated cells.

Atorvastatin Diminishes AngII-Induced MAPK Activation in Cultured Human Tubuloepithelial Cells

In renal cells AngII activates the MAPK pathway [(5,27) and references therein]. In HK2 cells, AngII triggered phosphorylation of all three MAPKs (Fig. 7), with a maximal response between 20 to 30 min. We further investigated whether statins could regulate several AngII-activated intracellular signaling pathways in HK2 cells, as described in VSMC (13). Preincubation with atorvastatin inhibited AngII-induced activation of JNK and ERK1/2 (Fig. 7), showing that in tubuloepithelial cells statins act at cellular level inhibiting AngII responses.

DISCUSSION

The investigation of the molecular mechanisms involved in renal fibrosis could lead to improve current clinical treatments for renal patients. Our *in vitro* data show that specific inhibition of MAPK and ROCK pathways are interesting options for the inhibition of EMT and renal fibrosis.

Several studies have shown that MAPK pathway is involved in EMT and fibrosis. AngII activates MAPK and through this pathway elicits many cellular responses (5,26). In cultured human tubuloepithelial cells we have found that specific inhibitors of all three MAPKs (p38, JNK and ERK1/2) prevented the phenotypic conversion of epithelial cells into myofibroblasts and the loss of E-cadherin observed after 3 days of treatment with AngII, and markedly diminished the induction of the EMT markers vimentin and α-SMA, observed by immunofluorescence and Western blot at 24 h and 3 days (Fig. 8). The MAPK pathway is involved in EMT, fibrosis and cell migration caused by TGF-β (29–31). In different cells, all three MAPKs, p38, ERK and JNK, participates in TGF-β -induced EMT, including in tubuloepithelial cell line NRK52E (32), showing a common intracellular mechanisms for TGF-β and AngII.

Studies done in human renal biopsies from different kidney diseases suggest that MAPK activation in resident and infiltrating cells can be involved in renal damage progression. ERK1/2 activation was associated with cellular proliferation and renal dysfunction (33). In human glomerulonephritis, p38 activation was observed in renal cells and infiltrating cells, correlated with renal dysfunction, proteinuria, inflammatory infiltration and proliferative lesions (34). In experimental models of renal injury activation of JNK have been found in podocytes, endothelial cells, macrophages, T cells and fibroblasts (35,36). In experimental models of renal injury MAPK inhibitors have shown beneficial effects. Treatment with JNK inhibitors reduced renal damage, collagen accumulation and apoptosis in the models of ureteral obstruction and ischemia reperfusion (35–37). Similar data were found in obstructed kidneys of JNK1 and JNK2 deficient mice (38). The pharmacological blockade of ERK1/2 prevented cellular proliferation in experimental glomerulonephritis (39). The effect of p38 inhibitors has been extensively studied. In hypertensive rats specific p38 inhibitors diminished proteinuria, sclerosis and interstitial macrophage migration, *via* suppression of NAD(P)H oxidase and enhanced NO bioavailability, showing end-organ protection regardless of overt antihypertensive action (40,41). In high-renin homozygous transgenic rats, p38 inhibition reduced both glomerular and tubulo-interstitial fibrosis and induction of α-SMA expression (42,43). In double transgenic rats for renin and angiotensinogen, p38 inhibition diminished renal expression of CTGF, TNF-α, IL-6, macrophages infiltration and fibrosis (44). Recently, it has been developed a novel strategy that inhibit p38 within proximal tubular cells, by using a renal-specific conjugate of the p38 inhibitor SB202190 and the carrier lysozyme. In the model of ischemia-reperfusion in rats this compound reduced intrarenal p38 phosphorylation and α-SMA protein expression (45). These data suggest that pharmacological inhibition of MAPK pathway could be an important therapeutic approach for renal diseases.

RhoA participates in some AngII responses, including vasoconstriction, premyofibril formation and cell hypertrophy (4,46). Several findings suggest that RhoA/ROCK pathway is implicated in the etiology of renal fibrosis. In our *in vitro* experiments, the transient transfection of a RhoA dominant negative vector or the use of two ROCK inhibitors (Y-27632 and Fasudil) clearly demonstrated that RhoA/ROCK pathway regulates AngII-mediated EMT (Fig. 8). The small G protein RhoA participates in TGF-β-mediated EMT (47,48). The mechanisms of this process involve RhoA degradation by recruitment of ubiquitin ligase Smurf1 (49). In experimental models of renal damage, such as unilateral ureteral obstruction, nephrectomized spontaneously hypertensive rats, L-NAME-treated and AngII infusion, ROCK inhibition improved glomerular and tubulointerstitial injury scores and fibrosis. In some of these models ROCK inhibition diminished gene

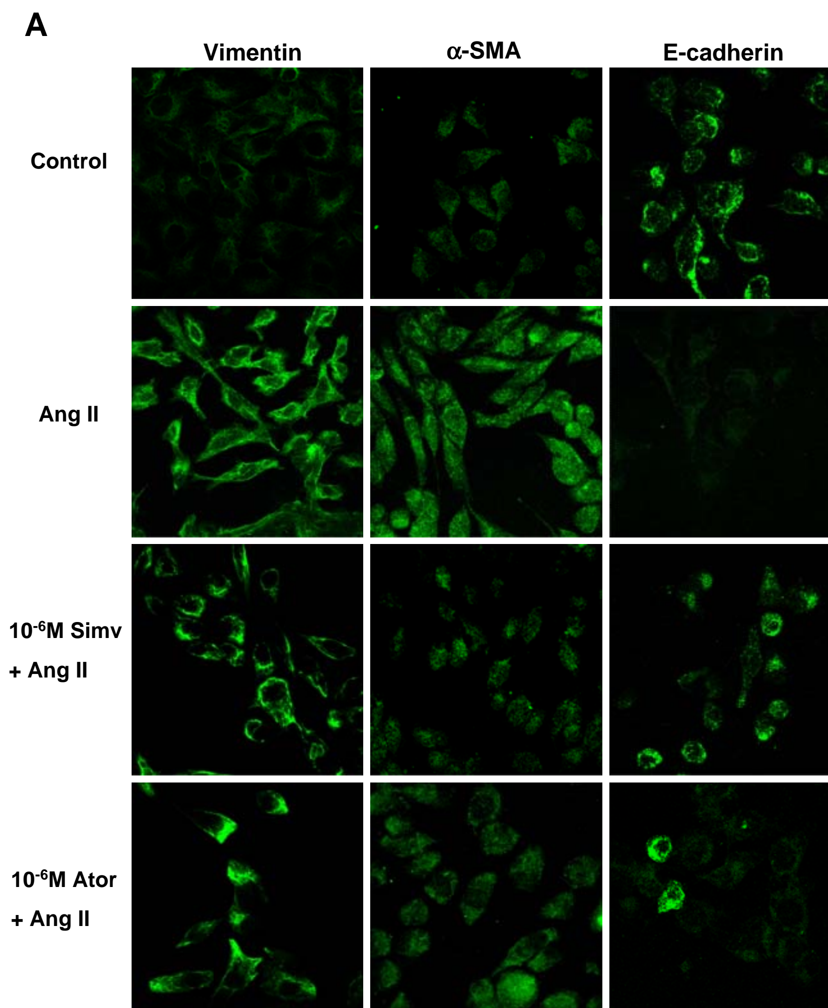
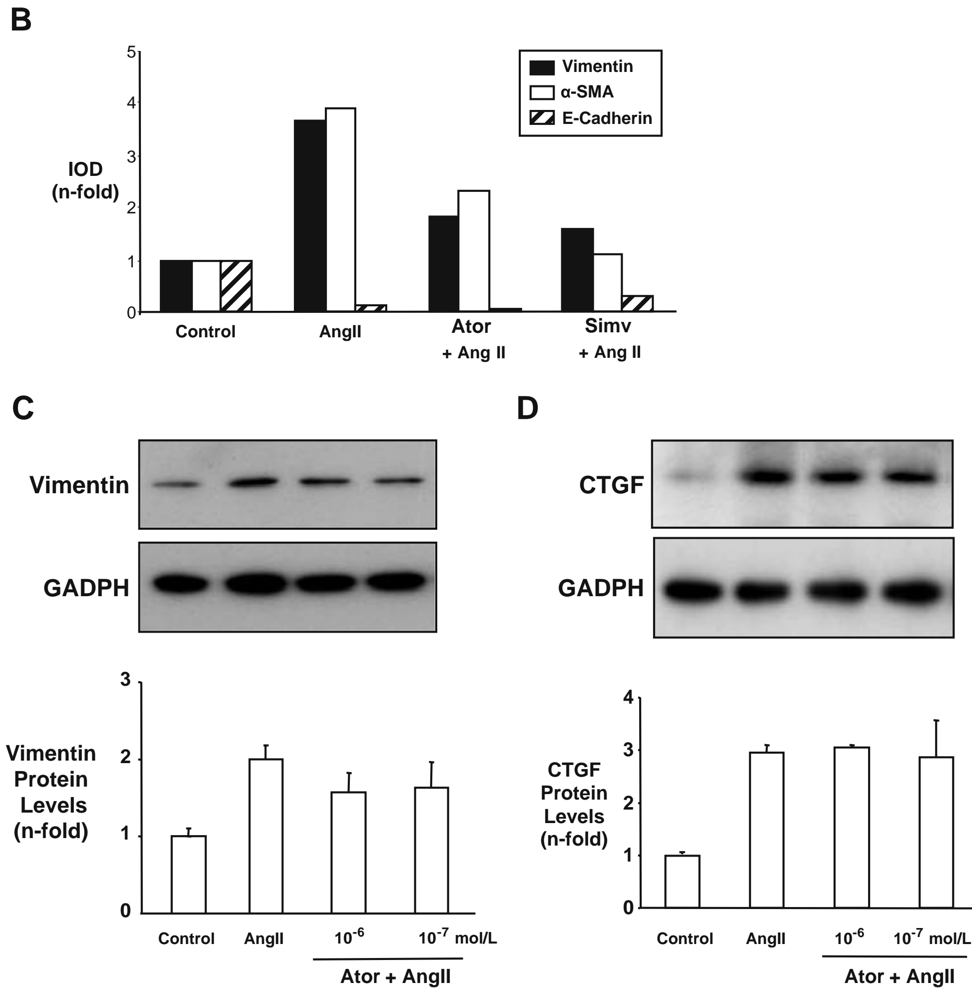


Fig. 6. Effect of HMG-CoA reductase inhibitors on AngII-induced EMT. Cells were pretreated for 1 h with two statins: atorvastatin or simvastatin (10^{-6}mol/l) and then stimulated with 10^{-7} mol/l AngII for 3 days. **A** Vimentin, α-SMA and E-cadherin were detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody, and evaluated by confocal microscopy. The figures of confocal microscopy show a representative experiment of three done. **B** Quantification of immunofluorescence data expressed as integrated optical density (IOD) as described in "Materials and Methods." **C** HK2 cells were pretreated for 1 h with atorvastatin (10^{-6}–10^{-7} mol/l) and then stimulated with 10^{-7} mol/l AngII for 24 h and vimentin expression was quantified by Western blot. Data are expressed as mean ± SEM of three experiments. *P<0.05 *vs* control, #P<0.05 *vs* AngII. **D** HMG–CoA reductase inhibitors did not modulate CTGF production caused by AngII. HK2 cells were pretreated for 1 h with atorvastatin (10^{-6} and 10^{-7}mol/l) and then stimulated with 10^{-7}mol/l AngII for 24 h. Results of total CTGF production were obtained from densitometric analysis and expressed as ratio CTGF/GAPDH as n-fold over control. Figures show in top panel a representative Western blot and bottom data total CTGF production as mean ± SEM of three independent experiments. *P<0.05 *vs* control.

overexpression of α-SMA, TGF-β, CTGF and matrix proteins (50,51). Recently, specific tubular inhibition of ROCK has shown renal protective effects in ischemia-reperfusion in rats (52). These investigations support the idea that treatments that inhibit RhoA/ROCK pathway in tubuloepithelial cells could be an appropriate choice as therapeutic strategies in chronic renal diseases.

Many efforts have been done to find a biomarker for the progression of chronic renal diseases, but until now there is not a good candidate. CTGF is upregulated in many human renal diseases and mediates TGF-β-induced fibrosis and EMT (21,53). We have previously shown that CTGF is a downstream mediator of AngII-induced renal fibrosis (24). In this paper, we have observed that CTGF blockade by a CTGF antisense oligonucleotide or an inhibitor of CTGF-receptor, diminished vimentin induction caused by AngII both at 24 and 72 h, showing that CTGF also contributes to AngII-induced EMT. Similar findings were previously described (18), supporting the importance of CTGF as a mediator of EMT. Moreover, recently it have been demonstrated that the treatment with a CTGF antisense oligonucleotide ameliorates renal damage in experimental diabetes (54).

Fig. 6. (continued)



In these animals a correlation between renal and urine levels of CTGF has been found, indicating the potential importance of this growth factor as a biomarker in renal diseases. In different cell types MAPKs and ROCK are involved in CTGF regulation. In fibroblasts, ERK1/2 and JNK, but not p38 inhibition, decreased AngII-induced CTGF upregulation (28), while in mesangial cells only the p38 inhibitor SB203580 diminished CTGF (27). In cultured renal fibroblasts activation of Rho is involved in CTGF overexpression caused by TGF-β and AngII (55,56). The involvement of Rho in CTGF regulation has been described in many cell types, including VSMC and lung fibroblasts (13,57). In this paper we have observed that in human tubuloepithelial cells AngII regulates CTGF *via* activation of three MAPKs (p38, ERK and JNK) and ROCK, showing a similar response to EMT regulation (Fig. 8). Although more studies are needed to define whether CTGF can be used as a biomarker in renal patients, the effect of these kinase inhibitors support the idea that CTGF could be a molecular target for the regulation of EMT.

The HMG-CoA reductase inhibitors are effective in controlling hypercholesterolemia, even in advanced stages of renal failure and in patients who are on chronic dialysis, and present cardiovascular protective effects (58), however their renoprotective effects in human renal diseases are not proven. Although several experimental models of kidney injury have shown beneficial effects, there are some contradictory data. A meta-analysis of several smaller studies of patients with various forms of renal diseases concluded that lipid-lowering drugs can reduce the decline of the glomerular filtration rate, but large clinical trials are warranted (59). Chronic treatment with the hydrophilic rosuvastatin, but not the lipophilic simvastatin had renoprotective effects in spontaneously hypertensive stroke-prone rats, a model characterized by proteinuria, inflammatory cell infiltration, α-SMA-positive cells, degenerative changes in podocytes, and severe fibrosis (15). In murine adriamycin nephropathy statins failed to ameliorate renal damage (60). In the model of unilateral ureteral obstruction in rats simvastatin diminished renal interstitial inflammation and fibrosis. Simvastatin also prevented tubular activation and transdifferentiation, as shown by decreased vimentin and α-SMA expression (61). In cultured human tubuloepithelial cells we have found that two statins, atorvastatin and simvastatin only partially diminished AngII-induced EMT changes. Similar findings were found with pravastatin in response to TGF-β mediated EMT and extracellular matrix deposition, and only the combination with PPAR-gamma agonists markedly inhibited these processes (17). The pleiotropic effect of statins are due to their inhibition of cellular responses, as a result of the inhibition of the mevalonate pathway induced by these agents, which

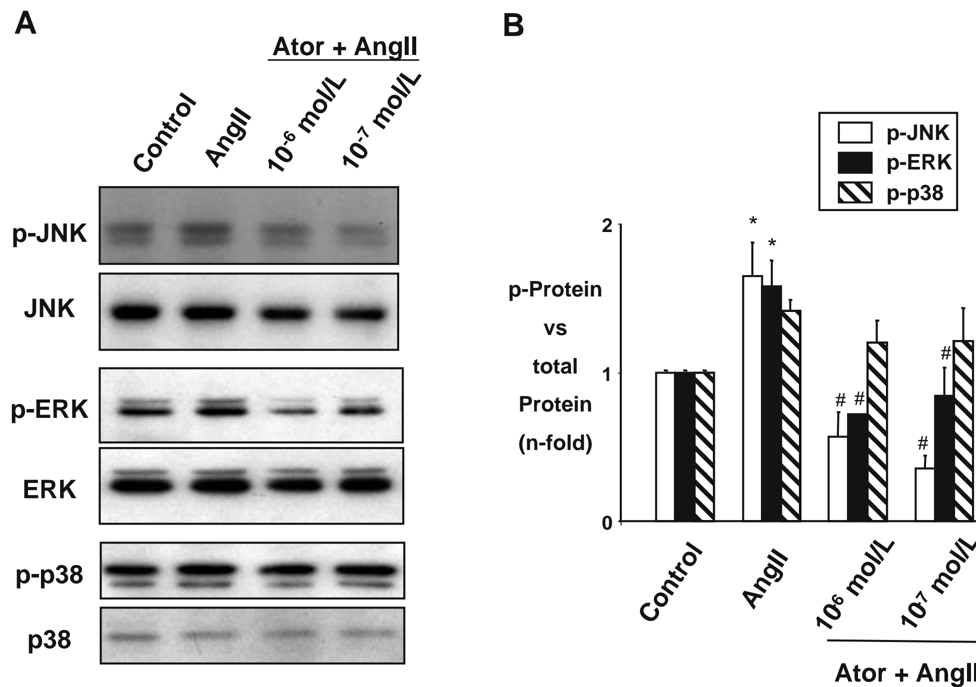


Fig. 7. Atorvastatin diminishes MAPK activation in AngII-treated human tubuloepithelial cells. Cells were pretreated for 1 h with atorvastatin (ator: 10^{-6}–10^{-7} mol/l), and then stimulated with 10^{-7} mol/l AngII for 20 (for JNK) and 30 min (ERK and p38). Figure shows a representative Western blot of phospho-JNK (p-JNK1/2), phospho-ERK (p-ERK1/2) and phospho-p38 (p-p38), JNK, ERK and p38 (used as controls). Figure show data as mean ± SEM of three to four experiments. *$P<0.05$ *vs* control. #$P<0.05$ *vs* AngII.

includes the activation of the small G protein Rho and MAPKs (11–13). In tubuloepithelial cells we have observed that atorvastatin inhibited AngII-induced MAPKs activation. Similar findings were observed with lovastatin, simvastatin, and pravastatin in the reduction of RhoA and Rac1 activation and in the inhibition of EMT caused by activated peripheral blood mononuclear cells conditioned-medium (16). However, our studies in cultured tubuloepithelial cells showed that the inhibitory effect of statins was lower than those of kinase inhibitors on AngII-induced EMT. Interestingly, we have observed that atorvastatin did not diminish CTGF production caused by AngII, showing a different response to MAPKs and ROCK inhibitors, which abolished AngII-induced CTGF upregulation. The regulation of CTGF seems to be dependent on the cell type and stimuli, as described above for MAPK pathway. In this sense, in Wistar rat VSMC and several fibroblasts cell lines statins diminished CTGF induced by AngII and TGF-β (13,62–64), while this effect was not observed in tubuloepithelial cells. Future studies are needed to further investigate the mechanisms involved in statins action in the kidney and in renal cells.

CONCLUSION

Our data show that MAPKs and ROCK inhibitors abolished CTGF upregulation and EMT, suggesting that the blockade of these pathways could be an important therapeutic choice for renal diseases. Future *in vivo* investigation of the effect of kinase inhibitors in chronic renal diseases could improve the current clinical treatments of renal patients.

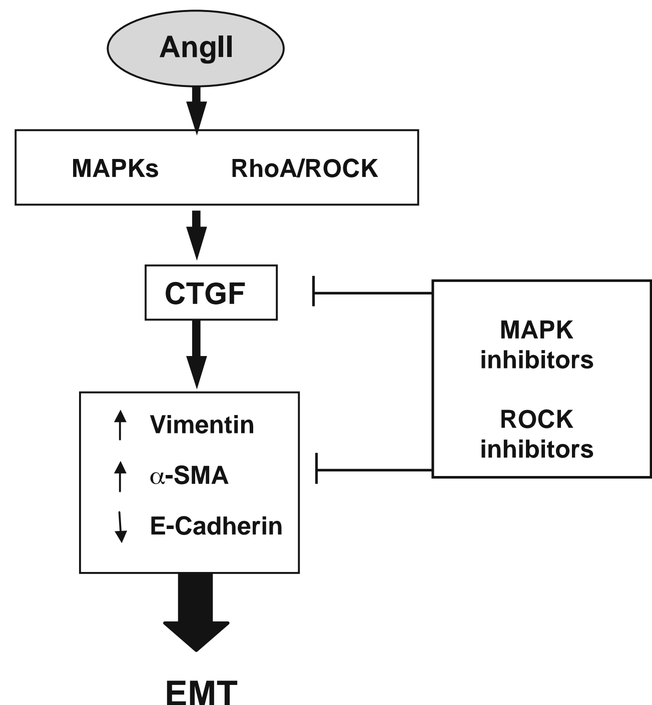


Fig. 8. AngII through the activation of the MAPKs cascade and the RhoA/ROCK pathway regulates CTGF and EMT markers.

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