Angiotensin II Induces Renal Plasminogen Activator Inhibitor-1 and Cyclooxygenase-2 Expression Post-Transcriptionally via Activation of the mRNA-Stabilizing Factor Human-Antigen R

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Angiotensin (Ang) II-induced fibrosis of the kidney is characterized by the enhanced expression of profibrotic and proinflammatory genes, including the serine protease inhibitor plasminogen activator inhibitor-1 (PAI-1) and cyclooxygenase-2 (COX-2). In addition to transcriptional regulation, both genes are subject to post-transcriptional control by AU-rich destabilizing elements that reside within the 3' untranslated region of the mRNA. We demonstrated that the continuous infusion of AngII in rats induced fibrosis concomitant with a significant increase in glomerular PAI-1 and COX-2 expression levels. Using RNA pull-down assays and electromobility shift assays, we demonstrated the increased binding of the ubiquitous RNA-binding protein human-antigen R (HuR) to the mRNAs of both PAI-1 and COX-2 in the cytoplasmic fractions of renal homogenates from AngII-treated rats. Actinomycin D experiments in rat mesangial cells revealed that AngII stabilizes both mRNAs via HuR as proven by small interfering RNA. Mechanistically, AngII promotes an increase in nucleo-cytoplasmic HuR shuttling, which was blocked by the PKC inhibitor rottlerin and the type-I AngII (AT₁) receptor antagonist valsartan but was unaffected by both AT₂ receptor antagonists PD123319 and CGP42112. Coimmunoprecipitation revealed that AngII treatment caused an increase in nuclear PKC- δ concomitant with binding to nuclear HuR both *in vitro* and *in vivo*. The post-transcriptional regulation of PAI-1 and COX-2 by PKC- δ -dependent HuR shuttling may contribute to

the pathogenesis of hypertensive nephrosclerosis triggered by AngII. (Am J Pathol 2009, 174:1252–1263; DOI: 10.2353/ajpath.2009.080652)

Chronic hypertension in many cases is causally associated with the development of renal fibrosis. In this context, the multifunctional angiotensin (AngII) has emerged as a key player in the initiation and progression of fibrogenic processes in the kidney. Independent of its direct effect on smooth muscle contractility, AngII promotes fibrosis by exerting several prominent nonhemodynamic effects including proliferative, proinflammatory, and profibrotic activities.^{1,2} These may at least in part account for the compelling renoprotective effects of AngII-targeted therapies.² In addition to its inducible effect on collagen expression, AngII can exert a strong stimulatory effect on the transcription of plasminogen activator inhibitor (PAI)-1, a member of the serpin super family of serine protease inhibitors crucially involved in pathological extracellular matrix deposition.^{3–5} Considerable evidence has accumulated demonstrating that despite its cardinal function in regulating intravascular fibrinolysis, an up-regulation of PAI-1 is thought as a main cause for tissue fibrosis.^{4,6,7} Mechanistically, the AngII-induced extracellular matrix accumulation is predominantly mediated via the AT₁ receptor thereby leading to an increased expression of the profibrotic cytokine transforming growth factor (TGF)- β .^{8,9} Besides the transcriptional regulation, PAI-1 expression underlies a post-transcriptional control that is structurally related to a

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1.0-kb 3'-untranslated region (3'-UTR) targeting the exonucleolytic decay of PAI-1 mRNA.^{10,11} Only a limited number of physiological stimuli, including growth factors and hormones, have been shown to modulate PAI-1 synthesis by an involvement of post-transcriptional events.^{12,13} Although the 3'-UTR of PAI-1 bears various putative AU-rich destabilizing elements (AREs), the identity of mRNA binding proteins targeting these regulatory sequences is not known.

In addition to induction of profibrotic genes, AngII can critically modulate the glomerular response to injury through an increased synthesis of prostaglandins because of the enhanced expression of the inducible cyclooxygenase-2 (COX-2) enzyme. COX-2-derived prostanooids play a key role in pathophysiological processes such as tumorigenesis, inflammation, and hypertension.¹⁴ A constitutive expression of COX-2 has been demonstrated in the kidney and is specifically found in the late thick ascending limb of Henle and in the macula densa.¹⁴ In contrast, an up-regulation of glomerular COX-2 has been observed in response to proinflammatory cytokines¹⁵ and on stimulation with AngII.¹⁶ Similar to PAI-1, the induction of COX-2 in addition to transcriptional mechanisms is mediated by post-transcriptional events and structurally attributed to multiple AREs in the 3'-UTR of the COX-2 mRNA.^{17–20} Several studies demonstrated that the 3'-UTR of COX-2 mRNA is a specific target of the embryonic lethal abnormal vision protein HuR.^{19–23} Previously, we demonstrated a HuR-dependent stabilization of cytokine-induced COX-2 mRNA and a subsequent increase in prostaglandin E₂ (PGE₂) formation in human mesangial cells (MCs) by AngII.²⁰ As for PAI-1, post-transcriptional regulation of COX-2 within the kidney and the underlying signaling pathways are still poorly understood.

In this study, we attempted to prove a functional correlation between AngII-induced activation of the ubiquitous mRNA binding protein HuR and renal expression of COX-2 and PAI-1. We found that mRNAs of both genes are targets of HuR-dependent mRNA stabilization *in vitro* and *in vivo*. Moreover, post-transcriptional regulation of both genes by HuR highlights an additional nonhemodynamic effect by AngII that may be functionally important for inflammatory and fibrotic cell responses in the kidney.

Materials and Methods

Reagents

Human recombinant interleukin (IL)-1 β was from Cell Concept (Umkirch, Germany) and human recombinant tumor necrosis factor (TNF)- α from Knoll AG (Ludwigshafen, Germany). AngII and Ponceau red were purchased from Sigma-Aldrich (Deisenhofen, Germany). Actinomycin D (from *Streptomyces* species) was purchased from Alexis Biochemicals (Laeufelfingen, Switzerland). CGP42112, PD123319, rottlerin, staurosporin, and valsartan were obtained from Calbiochem (Schwalbach, Germany). Ribonucleotides and modifying enzymes were purchased from Life Technologies (Karlsruhe, Germany).

RNA oligonucleotides were derived from Whatman Biometra (Göttingen, Germany). Antibodies raised against β -actin, collagen-type IV, COX-2, HDAC1, HuR, PAI-1, anti-goat, anti-rabbit, and anti-mouse horseradish peroxidase-linked IgGs, were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The antibody raised against PKC- δ was obtained from New England Biolabs (Frankfurt am Main, Germany) and that raised against fibronectin was from Invitrogen (Karlsruhe, Germany).

Animals

All procedures performed on animals were done in accordance with National Institutes of Health guidelines and were approved by the local government authorities (Regierungspräsidium Darmstadt). Male Sprague-Dawley rats weighting 180 to 200 g (Harlan Winkelmann, Borcheln, Germany) were maintained under controlled conditions of light, temperature, and humidity. Osmotic minipumps (model 2001; Alzet, Cupertino, CA) that delivered 0.5 μ l/hour for the indicated time points were implanted subcutaneously under isoflurane anesthesia.

One group consists of control animals that received NaCl (0.9 g/L), the other group of rats were continuously infused with AngII at 400 ng/kg/minute up to 14 days as previously described. Systolic blood pressure was measured by the tail-cuff method. Rats were anesthetized by ketamine hydrochloride (5.8 mg/100 g) and xylazine hydrochloride (0.39 mg/100 g) and sacrificed either after 6 hours, or 7 or 14 days of treatment ($n = 3$ animals per group) by retrograde perfusion through the infrarenal abdominal aorta. Perfusion was conducted with phosphate-buffered saline (PBS), pH 7.4, for 3 minutes at a pressure level of 180 mmHg. One part of the renal tissue was snap-frozen in liquid nitrogen for biochemical analysis. A second portion of frozen tissue was grounded and homogenized in the Trizol reagent (Sigma). Another portion of the tissue destined for immunohistochemical analysis was embedded into paraffin.

Immunohistochemistry

Immunohistochemical analysis of paraffin-embedded tissue was performed as described.²⁴ Briefly, after removal of paraffin with xylene, and rehydration endogenous peroxidase was inactivated by a 5-minute incubation in 3% hydrogen peroxide. Antigen was retrieved by microwave treatment in 0.01 mol/L citrate buffer at pH 6.0 for 10 minutes at 300 W. Slides were rinsed with PBS before blocking for 30 minutes with 20% rat serum diluted in PBS. The following primary antibodies were first incubated for 1 hour at 37°C and overnight at 4°C: rabbit polyclonal anti-Coll-IV (1:50), rabbit anti-COX-2 (1:100), and rabbit anti-PAI-1 (1:100). After several washing steps in PBS, slides were incubated with the biotinylated goat anti-rabbit antibody (1:250, DAKO, Hamburg, Germany) for 30 minutes at 37°C. After several washing steps with PBS, slides were incubated for 30 minutes with ExtrAvidin-Peroxidase (1:100, Sigma) and peroxidase was de-

tested by 3-amino-9-ethylcarbazole chromagen (Sigma) diluted in 0.05 mol/L sodium acetate buffer, pH 5.0, 0.03% H₂O₂.

Immunofluorescence

After rehydration and antigen retrieval slides were blocked with 20% rat serum diluted in PBS. The slides were incubated with a rabbit anti-fibronectin antibody (1:2000) for 1 hour at 37°C and overnight at 4°C, washed with PBS, and incubated for 1 hour at 37°C with a Cy3-labeled goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA).

Cell Culture

Rat glomerular MCs were characterized as described²⁵ and grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 5 ng/ml insulin, 100 U/ml penicillin, and 100 µg/ml streptomycin. Serum-free pre-incubations were performed in Dulbecco's modified Eagle's medium supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin for 24 hours. All cell culture media and supplements were purchased from Life Technologies.

Cell Fractionation and Western Blot Analysis

Preparation of cytoplasmic and nuclear lysates from cells or whole kidney samples were performed according to a protocol from Dignam and colleagues²⁶ and subsequent Western blot analyses were performed using standard procedures. Fifteen to thirty µg of either nuclear or cytoplasmic fractions from MCs or tissue samples were used for assessment of intracellular HuR trafficking. For ensuring an equal sample loading of nuclear proteins, blots were reprobed with an anti-histone deacetylase 1 (HDAC-1)-specific antibody. For detection of total HuR, COX-2, and PAI-1 levels, whole cell lysates were prepared as described previously.²⁷ Total cell extracts containing 20 to 50 µg of protein were prepared in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was performed by standard procedures. Proteins were transferred to polyvinylidene difluoride membranes before the immunodetection. After 1 hour blocking in 2% bovine serum albumin or 5% milk powder in Tris-buffered saline containing 0.05% Tween, Western blots were probed with the primary antibody (1:1000) overnight at 4°C. After incubation with a horseradish peroxidase-conjugated secondary antibody (1:10,000) signals were detected with an enhanced chemiluminescence system, and densitometric data from Western blots were obtained and quantified using a 1D Digital Science Imaging System from Kodak (Eastman-Kodak, Rochester, NY).

Immunoprecipitation Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

HuR-bound mRNA isolated from cytoplasmic fractions from MCs or from tissue homogenates from whole kidneys was detected by using a pull-down RT-PCR assay as described previously.¹⁹ In brief, cells were treated with lysis buffer (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.1% Nonidet P-40, 50 mmol/L NaF, 10 mmol/L Na₃VO₄, 10 mmol/L sodium pyrophosphate, 50 mmol/L disodium glycerol phosphate, and 100 U/ml RNasin) and cell lysates immunoprecipitated with 2 µg of a anti-HuR antibody or, alternatively, the same amount of IgG from mouse origin in an overnight incubation at 4°C. Subsequently, protein G Sepharose CL-4B beads (Amersham-Biosciences, Freiburg, Germany) were added and incubated for a further 2 hours. After a short centrifugation (3000× *g*) the beads were successively washed with low- and high-salt buffer before total RNA was extracted by the Tri reagent (Sigma-Aldrich). The HuR-bound RNA was reversely transcribed using SuperScript reverse transcriptase (Invitrogen) and subjected to quantitative PCR (qRT-PCR). Normalization of input RNA was confirmed by RT reaction of total cellular RNA isolated from an equal amount of cell extract as was used for IP and subsequent assessment of GAPDH levels.

Real-Time RT-PCR

Two-step real-time-PCR was performed using a TaqMan (ABI 7000) from Perkin Elmer (Emeryville, CA). Total RNA from MCs or from whole kidney homogenates was isolated for measurement of COX-2, PAI-1, and GAPDH contents by standard procedures. Levels of specific mRNAs were determined by using a protocol according to the hot start real-time PCR procedure with Quanti-Tec SYBR green (Qiagen, Hilden, Germany). Equal amounts of RNA were reverse-transcribed with the reverse transcriptase (Invitrogen) by using random hexamer primers. The following primers from the complete sequence of the rat mRNAs were used for PCR: COX-2 (GenBank accession no. S67722); (forward): 5'-CTCTGCGATGCTCTTC-CGAG-3', (reverse): 5'-AAGGATTGCTGCATGGCTG-3'; PAI-1 (GenBank accession no. M 24067); (forward): 5'-GAGCCAGATTCATCATCAACG-3', (reverse): 5'-CT-GCAATGAACATGCTGAGG-3' and GAPDH (GenBank accession no. AB017801); (forward): 5'-CCTTCATTGAC-CTCAACTAC-3', (reverse): 5'-GGAAGGCCATGCCAGT-GAGC-3'. The C(T) values of COX-2 and PAI-1 mRNA level were normalized to the C(T) values of GAPDH mRNA within the same sample.

RNA Electromobility Shift Assay (RNA-EMSA) and Supershift Analysis from Whole Tissue

RNA-EMSA for assessment of *in vivo* RNA binding of HuR were accomplished as described previously.¹⁹ Briefly, a

single-stranded RNA oligonucleotide, radioactively labeled by T4 polynucleotide kinase (30 kcpm/reaction) was incubated with 5 μ g of cytoplasmic extract from whole kidney homogenates and incubated at room temperature for 20 minutes in a buffer containing 10 mmol/L HEPES, pH 7.6, 3 mmol/L $MgCl_2$, 40 mmol/L KCl, 2 mmol/L dithiothreitol, 5% glycerol, and 0.5% Nonidet P-40. To reduce nonspecific binding yeast total RNA (200 ng/ml final concentration) was added. The total volume of each reaction was 10 μ l. RNA-protein complexes were separated in 6% nondenaturing polyacrylamide gels and run in Tris-borate EDTA. After electrophoresis, the gels were fixed and analyzed by a phosphorimager using an automated detector system BAS 1500 from Fuji film (Raytest, Staubenhardt, Germany).

The sequence of RNA oligonucleotides used for EMSA were in accordance to the pentameric ARE sequences (italics) were as followed: PAI-1-ARE-1: 5'-GUGAUUGAAUUAUUUAUCUUGU-3'; PAI-1-ARE-2: 5'-AGCCUUUUUAUUUAAACCAUGG-3'; PAI-1-ARE-3: 5'-CA GAAACCAUUUUACUGAAA-3'; COX-2-ARE-1: 5'-CC AUAUUUAUUUAUUUAU-3'; COX-2-ARE-2: 5'-A AUUUAAUUUAUUUAUUA-3'. Supershift analysis was done by addition of 200 ng of antibody 20 minutes after the addition of the radioactive labeled RNA oligo and incubated for a further 15 minutes at room temperature. All antibodies used for supershift analysis were purchased from Santa Cruz Biotechnology.

RNA Interference

Gene silencing in renal MCs was performed using either a predesigned siRNA corresponding to the rat HuR cDNA sequence (GenBank accession no. NM_001108848) (5'-GAUGCCAACUUGUACAUCA-3') from Eurogentec (Searing, Belgium) or a nongene specific control siRNA that was derived from Dharmacon (Lafayette, Chicago). Subconfluent (30 to 50%) MCs were transfected for 48 hours with 25 nmol/L of siRNA by use of the Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation of HuR

Immunoprecipitation of HuR was performed as described previously.¹⁹ Briefly, nuclear extracts (400 μ g) either from MCs or from whole kidneys were incubated overnight at 4°C with either 2 μ g of a monoclonal anti-HuR antibody or with the same amount of mouse IgG (both diluted in lysis buffer containing 5% fetal calf serum). Subsequently protein G Sepharose CL-4B beads were added and incubated for another 2 hours. After a short centrifugation step at 3000 $\times$ g, the precipitated complexes were successively washed three times with low-salt buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.2% Triton X-100, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.1% SDS) and once with high-salt buffer (50 mmol/L Tris-HCl, pH 7.5, 500 mmol/L NaCl, 0.2% Triton X-100, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.1% SDS). After the washing steps beads were subjected to SDS-PAGE

and co-immunoprecipitated proteins were detected by Western blot analysis.

Determination of PGE₂ and PAI-1 Levels in Conditioned Media and in Whole Tissue Homogenates

Levels of PGE₂ in cell supernatants or in tissue homogenates from whole kidneys were determined by the Correlate-EIA prostaglandin E₂ enzyme-linked immunosorbent assay kit (Assay Designs, Ann Arbor, MI). Isolation of prostaglandins was done by resuspending the thawed tissue homogenates in a 10-fold volume of bidist water followed by a short centrifugation (3000 $\times$ g). For immunodetection, either 100 μ l of conditioned media, or 100 μ l of supernatants from tissue homogenates were directly transferred into the microtest strip wells of the enzyme-linked immunosorbent assay plate and further procedures performed according to the manufacturer's instructions. The absorbance at 405 nm was measured in a microtest plate spectrophotometer and PGE₂ contents determined by a calibration curve using PGE₂ as a standard.

Levels of rat PAI-1 antigen were determined by the Imclone rat PAI-1 enzyme-linked immunosorbent assay from American Diagnostica (Stanford, CT). One hundred μ l of 1:10 diluted conditioned media were directly transferred to the microtest strip wells of the enzyme-linked immunosorbent assay plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 450 nm was measured by a spectrophotometer and PAI-1 levels were determined with a calibration curve using rat PAI-1 as a standard.

Statistical Analysis

Results are expressed as means $\pm$ SD. The data are presented as x-fold induction compared with untreated control or compared with stimulated values. Statistical analysis was performed using the Kruskal-Wallis test. *P* values <0.05, <0.01, and <0.005 were considered significant.

Results

AngII Induces a Cytoplasmic HuR Accumulation in Renal Tissue

We have previously reported an AngII-dependent stabilization of COX-2 mRNA that is mainly because of enhanced HuR activity and accompanied by an increased HuR shuttling from the nucleus to the cytoplasm.²⁰ To test for a possible *in vivo* relevance, we measured cytoplasmic HuR content in whole kidney samples from rats treated with AngII for 6 hours, 7 or 14 days. Western blot analysis revealed that kidneys from AngII-treated rats contained high cytoplasmic HuR lev-

Figure 1. AngII induces a nucleo-cytoplasmic HuR shuttling and a subsequent increase in HuR binding to COX-2 and PAI-1 mRNAs *in vivo*. **A:** Representative Western blot analysis showing AngII-induced changes in the cytoplasmic HuR content in whole kidney homogenates. Rats were infused for the indicated time periods with vehicle (–) or with AngII (+) (400 ng/kg/minute). Whole kidney homogenates from three animals of each group were pooled and prepared for cell fractionation. Thirty μ g of cytoplasmic fractions or 10 μ g of nuclear extracts were subsequently subjected to SDS-PAGE and immunoblotted with an anti-HuR-specific antibody. To correct for variations in the protein loading, blots were stripped and successively incubated with anti- β -actin and anti-histone deacetylase 1 (HDAC1) antibodies, respectively. **B:** Pull-down assay from cytoplasmic fractions (300 μ g) of tissue homogenates from rats treated for 14 days with either vehicle or with AngII (400 ng/kg b.w.), which were immunoprecipitated with 2 μ g of anti-HuR (a.-HuR) or with the same amount of mouse IgG (IgG). The RNA-bound by HuR was harvested and subjected to qRT-PCR by using either COX-2- or PAI-1-specific primers as indicated from the coding regions of the corresponding genes. Amounts of input RNA added to the IP reaction mixture were normalized by assessment of GAPDH levels from input RNA by RT-PCR (not shown). Data show the results of a pull-down experiment from cytoplasmic extracts originating from a pool of three animals (per group) and are depicted as -fold induction compared with vehicle-treated animals.

els with a maximal cytoplasmic HuR accumulation already observed at day 7 whereas cytoplasmic HuR was undetectable in the extracts from vehicle-treated rats (Figure 1A, top). By contrast, the nuclear content of HuR remained unchanged in each group of animals tested (Figure 1A, bottom).

AngII Infusion Increases Cytoplasmic HuR-Binding to COX-2 and PAI-1 mRNAs

To further elucidate whether the increase in cytoplasmic HuR by AngII is functionally relevant and results in an increase in HuR-bound target mRNAs we performed a RNA-pull-down assay. To this end, crude cytoplasmic extracts from whole kidney homogenates were subjected to immunoprecipitation with an anti-HuR antibody. Subsequently, HuR-bound mRNAs were amplified by qRT-PCR. Because of the high amount of cytoplasmic protein required for the IP, we pooled samples from equally treated animals. First we monitored an increased binding to COX-2 mRNA because COX-2 constitutes a prominent target of HuR-triggered mRNA stabilization. Interestingly, cytoplasmic fractions from rats treated for 14 days with AngII contained an overall eightfold higher HuR-COX-2 mRNA content when compared with fractions from vehicle-treated animals (Figure 1B, black bars), although an equal input level of mRNA and protein was used for the IP (data not shown). Additionally, we tested for the pull-down of PAI-1 mRNA, which because of several pentameric AUUUA-motifs in its 3'-UTR might be a further HuR target mRNA. In fact, we measured a robust increase in PAI-1 cDNA levels in the extracts from AngII-perfused rats when compared with the homogenates from control animals (Figure 1B, gray bars). By contrast, no cDNA was amplified when instead of PAI-1- or COX-2-specific primers, GAPDH-specific primers were applied to the PCR (data not shown). Also, no cDNA was amplified if serum IgG was used for pull-down (Figure 1B) thus indicating that the results were specific for the HuR-interaction.

AngII Augments the Cytoplasmic HuR Binding to PAI-1- and COX-2-Specific AREs *In Vivo*

Next, we performed RNA-EMSA to determine whether the increase in HuR-binding revealed by the pull-down experiments, is attributable to the increased HuR-binding to AREs of corresponding UTRs. First, we tested different RNA oligonucleotides each encompassing one of the three putative pentameric AREs from the 3'-UTR of rat PAI-1 mRNA denominated as ARE-1, ARE-2, and ARE-3, respectively (Figure 2A). To this end, the cytoplasmic extracts from whole kidney homogenates that were used for the pull-down assays were assessed for *in vitro* RNA-binding. We observed a low constitutive binding of two complexes irrespective of which RNA oligo was applied for the binding reaction (Figure 2A). In contrast, the binding of a faster migrating complex (complex II) was strongly increased in extracts from AngII-treated animals when compared with controls (Figure 2A). Next, we performed supershift analysis to prove the presence of HuR in the AngII-induced complex II. The addition of the anti-HuR antibody resulted in a complete disappearance of the AngII-inducible complex II, which indicates that the antibody used for supershift analysis masks the RNA-binding domain (Figure 2A). By contrast, the addition of a supershift antibody raised against AUF-1 (adenosine, uri-

Figure 2. AngII induces RNA binding of a HuR-containing complex to PAI-1 (**A**)- and COX-2 (**B**)-specific AREs *in vivo*. Schematic representations of the 3'-UTRs of rat PAI-1 (**A**) or COX-2 (**B**) mRNAs. Gray boxes depict the position of single pentameric AUUUA motifs (bold, underlined) within a specific ARE and the sequences below show the composition of corresponding RNA oligonucleotides used for RNA-EMSA. Cytoplasmic extracts (5 μ g) pooled from three animals infused for 14 days with either vehicle (–) or with AngII (+) were added to the EMSA reaction together with the 32 P-labeled RNA oligonucleotide and RNA-protein complexes were resolved from unbound RNA by nondenaturing gel electrophoresis (complex I and complex II). Supershift analysis identifies HuR in the case of PAI-1 (**A**), and additionally AUF-1 as the predominant constituents of the AngII-induced complex binding to AREs from COX-2 (**B**). Supershift analysis was performed as described in the Materials and Methods.

dine-rich element factor-1), another prominent RNA-stability regulatory protein had no inhibitory effect on the RNA binding to the complexes (Figure 2A). These data identify HuR as a prominent constituent of the AngII-inducible complex binding to the 3'-UTR of rat PAI-1 mRNA.

Furthermore, we tested for a modulation of RNA-binding to two sequences from the 3'-UTR of the rat COX-2 mRNA, each of them encompassing three pentameric ARE motifs (ARE-1 and ARE-2). Both sites revealed a strong sequence homology to a functional HuR-binding site of the human correlate mRNA.²² Similar to the binding observed with the PAI-1-specific RNA probes, the RNA binding of a lower migrating complex to the COX-2-specific ARE-1 (Figure 2B, gray boxes) was markedly increased in the cytoplasmic extracts originating from AngII-perfused rats when compared with RNA binding measured in vehicle-treated animals (Figure 2B, left). Similarly, the RNA-binding of a lower migrating complex to ARE-2 (Figure 2B, white boxes) was enhanced in the same extracts although with an overall lower intensity (Figure 2B, left). Supershift analysis revealed that the AngII-triggered RNA-binding to both AREs was strongly reduced by addition of either a HuR-specific, or by an antibody raised against AUF-1 (Figure 2B, right). These data suggest that both RNA-binding proteins are involved in the AngII-mediated COX-2 mRNA binding.

AngII Infusion Leads to an Increase in Glomerular COX-2 and PAI-1 Expression

In accordance to the data from the pull-down assay, steady-state levels of COX-2 (16-fold induction, $n = 3$) and PAI-1 (eightfold induction, $n = 3$) mRNA levels were significantly higher in kidneys of rats receiving a 14-day infusion of AngII when compared with vehicle-treated control rats (Figure 3A). These data implicate that the AngII-induced increase in HuR binding results in increased mRNA level of PAI-1 and COX-2. Moreover, these data identify the PAI-1 message as a novel target of HuR-triggered mRNA stability. Consistent with the increase in mRNA levels, Western blot analysis revealed an accumulation of cytoplasmic HuR in AngII-treated rats (Figure 3B, top) whereas the level of nuclear (Figure 3B, middle) and total HuR (Figure 3B, bottom) remained unchanged independent of which group was tested. Concomitant with cytoplasmic HuR accumulation, we observed a significant rise in the total PAI-1 and COX-2 protein levels (Figure 3B, bottom). Corresponding to these biochemical data, immunohistochemical analysis of 14-day treated rats revealed a profound increase in glomerular PAI-1 staining in the sections from AngII-perfused animals (Figure 4). Similar to PAI-1 expression, staining of cortical COX-2 was markedly intensified by AngII (Figure 4) and mainly detected in mesangial cells and podocytes (Figure 4, COX-2 cort.). By contrast, a clear cortical COX-2 staining in control animals was only detectable in cells of the macula densa region. Immuno-

Figure 3. The increase in HuR shuttling by AngII is accompanied by an up-regulation of COX-2 and PAI-1 *in vivo*. **A:** Corresponding to the increase in HuR-bound mRNAs, AngII infusion causes an increase in the total steady-state levels of COX-2 and PAI-1 mRNA as determined by qRT-PCR and normalized COX-2 and PAI-1 mRNA contents to GAPDH mRNA level. Data represent means $\pm$ SD ($n = 3$) and are presented as fold induction compared with vehicle ($*P \leq 0.05$, $**P \leq 0.01$). **B:** Whole kidney homogenates from rats that had been infused for 14 days with either vehicle or with AngII were fractionated for assessment of cytoplasmic HuR (cytoplasmic), total HuR, or PAI-1 and COX-2 contents (total), respectively. Thirty μ g of each fraction were subjected to Western blot analysis and cytoplasmic fractions were successively probed with anti-HuR- and with anti- β -actin-specific antibodies. Total protein extracts were additionally probed with an anti-PAI-1- and anti-COX-2-specific antibody. The Western blots shown indicate an up-regulation of cytoplasmic HuR (**top**) in three individual animals corresponding with an increase in the total COX-2 and PAI-1 levels. **Bottom:** Summary of total COX-2, PAI-1, cytoplasmic, as well as nuclear HuR protein levels in rats treated for 14 days with either vehicle or with AngII (400 ng/kg b.w.). To correct for variations in the protein loading, blots were stripped and successively incubated with anti- β -actin and anti-HDAC1 antibodies, respectively. Results are means $\pm$ SD ($n = 3$) and are presented as induction versus vehicle-treated control animals ($*P \leq 0.05$, $***P \leq 0.005$).

Figure 4. AngII-induced modulation of protein expression by immunohistochemical analysis. Paraffin-embedded sequential sections were stained with anti-PAI-1-specific antiserum and with a polyclonal anti-COX-2 antibody and finally counterstained with hematoxylin as described in the Materials and Methods. COX-2 immunoreactivity was additionally assessed in a medullary (med.) region of the kidney. Concomitant with increased cortical (cort.) COX-2 and PAI-1 levels, an increased staining of the profibrotic marker proteins type-IV collagen (Coll-IV) and fibronectin (FN) is mainly found in cortical areas of kidneys from AngII-treated animals as revealed by either immunohistochemical (Coll-IV) or immunofluorescent (FN) detection. Original magnifications, $\times 400$.

histochemical analysis furthermore revealed an increased expression of medullary COX-2 in AngII-infused animals (Figure 4, COX-2 med.) thus indicating an elevation of COX-2 protein by AngII in the cortex and in the medulla. In addition, the increased staining of type-IV collagen (Coll-IV) and fibronectin (FN) in the cortical regions of kidneys from AngII-treated animals (14 days) indicates the presence of renal fibrosis (Figure 4). Concomitant with the histological changes, systolic blood pressure in AngII-infused animals was significantly higher when compared with vehicle-treated animals (Table 1).

Table 1. Physiological Parameters from Vehicle and AngII-Treated Sprague-Dawley Rats

Day	Mean systolic BP (mmHg)		BW (g) at sacrifice	
	Vehicle	AngII*	Vehicle	AngII
1	79 ± 11	126 ± 31 [†]	217 ± 30	219 ± 71
7	96 ± 29	162 ± 30 [‡]	249 ± 20	207 ± 34
14	118 ± 10	159 ± 11 [†]	262 ± 21	208 ± 32

Data are means ± SEM ($n = 3$ in each group). BP, blood pressure; BW, body weight.

*Infusion rate: 400 ng/kg/minute, [†] $P \leq 0.05$, [‡] $P \leq 0.01$.

HuR Is Indispensable for AngII-Induced Stabilization of PAI-1 and COX-2 mRNAs in Rat MCs

Next, we performed experiments with the general transcription inhibitor actinomycin D in rat renal MCs. To ensure an initial and profound increase in expression of both genes, MCs were pretreated for 16 hours with a cytokine mix consisting of TNF- α and IL-1 β (both at 2 nmol/L) before actinomycin D was added. Subsequently, MCs were additionally treated with vehicle or with AngII (100 nmol/L) and time-dependent mRNA decay was measured by real-time PCR. The reduction in cytokine-induced PAI-1 and COX-2 mRNA levels occurred with half-lives of ~2 hours (Figure 5A) and ~5 hours (Figure

5B), respectively. The addition of AngII (100 nmol/L) markedly increased the half-lives of both mRNA species to ~8 hours for PAI-1 and more than 8 hours for COX-2 (Figure 5, A and B). To test for the involvement of HuR in the AngII-induced stabilization of both mRNAs, we applied a siRNA approach by transfecting either a HuR-specific siRNA or, alternatively, a nonspecific control siRNA. Simultaneous stimulation of MCs with cytokine mix and AngII displayed a significant increase in the cytokine-triggered steady-state levels of PAI-1 and COX-2 mRNAs (Figure 6A). Interestingly, silencing of HuR exclusively prevented the AngII-dependent rise in both mRNAs without affecting the cytokine-induced gene expression (Figure 6A). Consistently, attenuation of HuR specifically interfered with the AngII-dependent increase of the cytokine-evoked release of PAI-1 (Figure 6B) and PGE₂ (Figure 6C). In summary, these data indicate that the increase in PAI-1 and COX-2 by AngII is mainly attributed to HuR-dependent stabilization of the corresponding mRNAs.

AngII Induces Nucleo-Cytoplasmic HuR Shuttling by an AT₁- and PKC-Dependent Mechanism

Because HuR is predominantly localized in the nucleus under basal conditions, nucleo-cytoplasmic shuttling is an important prerequisite for its stabilizing effects on cognate target mRNAs.²⁸ We could previously show that PKC- α and PKC- δ play important roles in the stimulus-dependent HuR shuttling in human MCs.^{19,20} Stimulation of rat MCs with AngII caused a substantial increase in cytosolic HuR content, whereas the level of nuclear and total HuR remained unchanged independent of which stimulus was applied (Figure 7A). Similar to human MCs, the increase in the nuclear HuR export by AngII was strongly diminished in cells that were co-treated with either valsartan (100 nmol/L), a specific inhibitor of the AT₁ receptor subtype or, alternatively, with rottlerin (10 μ mol/L) an inhibitor of the Ca²⁺-independent PKC- δ isoenzyme (Figure 7A). By contrast, the AngII-induced increase in the cytoplasmic HuR level was unaffected by CGP42112 an AT₂ receptor-specific inhibitor (100 nmol/L) and only weakly impaired by the broad-spectrum PKC inhibitor staurosporine (100 nmol/L) (Figure 7A). To further confirm a specific AT₁-dependence of AngII-induced HuR-shuttling *in vivo*, we measured cytoplasmic HuR contents in whole kidney samples from animals that were treated for 6 hours with AngII (400 ng/kg/min) in the presence or absence of either valsartan (20 mg/kg/day), or the AT₂-receptor antagonist PD123319 (30 mg/kg/day). Similar to the modulation observed in MCs, the AngII-induced increase in cytoplasmic HuR accumulation was totally abrogated in the presence of valsartan but, in contrast, was not affected by PD123319 (Figure 7B). No cytoplasmic HuR was detectable when animals were treated with either AT receptor antagonists alone (Figure 7B). Measurement of tissue PGE₂ levels, the most abundant prostanoid in the kidney, revealed a significant increase in the basal PGE₂ production in response to

Figure 5. AngII stabilizes cytokine-induced PAI-1 (A) and COX-2 (B) mRNAs in rat MCs. Quiescent MCs were treated for 16 hours with a cytokine mix containing IL-1 β and TNF- α (both at 2 nmol/L) and were then washed twice before actinomycin D (5 μ g/ml) was added. After a short preincubation of 30 minutes, MCs were additionally treated for the indicated times with vehicle (control, filled rhombi) or with 100 nmol/L AngII (AngII, filled squares) before cells were harvested and extracted for total cellular RNA. PAI-1 (A) or COX-2 (B) mRNA levels were quantified by qRT-PCR using GAPDH as a normalization control. Graphs show means ± SD ($n = 3$) and depict the percentage of remaining PAI-1 (A) or COX-2 (B) mRNA levels compared with the levels of both mRNA species measured immediately before the addition of Act D (0 hours).

Figure 6. Silencing of HuR prevents the AngII-triggered increase in PAI-1 and COX-2 mRNA levels and correspondingly inhibits the increase in PAI-1 and PGE₂ release. Rat MCs were either left untransfected (–) or were transfected (+) with either siRNA duplexes of HuR (siRNA-HuR) or control siRNA duplexes (siRNA-control) as described in the Materials and Methods. After transfection, cells were serum-starved for 16 hours and then treated for a further 24 hours with vehicle (–), or with a cytokine mix (cyt.-mix.) containing IL-1β and TNF-α (both at 2 nmol/L) in the presence (+) or absence (–) of AngII (100 nmol/L) or with AngII alone as indicated. **A:** The efficiency of silencing RNA on HuR protein levels was proven by the assessment of the total HuR level by Western blot analysis using an anti-HuR-specific antibody. An overall reduction in the cellular protein content was excluded by the successive β-actin staining. Changes in the PAI-1 (black bars) and COX-2 (hatched bars) mRNA levels were determined by qRT-PCR and by normalizing both mRNAs to GAPDH mRNA levels. The results are means ± SD (*n* = 3) and are presented as fold induction (**P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.005) compared with nonstimulated controls or (°*P* ≤ 0.05) to cytokine-stimulated values, (n.s.) not significant. **B** and **C:** PAI-1 protein (**B**) and PGE₂ (**C**) levels in cell supernatants derived from MCs treated as described in **A**. Data represent means ± SD (*n* = 3). ****P* ≤ 0.005 compared with control or (°*P* ≤ 0.01, °°*P* ≤ 0.005) to cytokine-induced conditions, (n.s.) not significant.

Figure 7. AngII induces cytoplasmic accumulation of HuR and nuclear import of PKC-δ. **A:** Representative Western blot showing cytoplasmic (**top**), nuclear (**middle**), and total (**bottom**) HuR levels after treatment with different pharmacological inhibitors. MCs were serum-starved for 16 hours and subsequently remained untreated (–) or were stimulated for an additional 2 hours with AngII (+) (100 nmol/L) in the absence (vehicle) or presence of either valsartan (100 nmol/L), CGP42112 (100 nmol/L), staurosporine (100 nmol/L), or rottlerin (10 μmol/L) as indicated. All inhibitors were preincubated for 30 minutes before the addition of AngII. Protein lysates from cytoplasmic (20 μg), nuclear (10 μg), or whole cell extracts (30 μg) were subjected to SDS-PAGE and immunoblotted with an anti-HuR-specific antibody. To correct for variations in the protein loading, blots were successively incubated with anti-β-actin and anti-HDAC1 antibodies, respectively. **B: Top:** AngII increases cytoplasmic HuR levels *in vivo* by an AT₁-dependent mechanism. Rats were infused for 6 hours with vehicle (–) or with AngII (+) (400 ng/kg/minute) in the absence (+ vehicle) or presence of either valsartan (20 mg/kg/day), or PD123319 (30 mg/kg/day) or, alternatively, with each inhibitor alone. Whole kidney homogenates from three animals of each group were pooled and prepared for cell fractionation. Thirty μg of cytoplasmic fractions were subsequently subjected to SDS-PAGE and immunoblotted with an anti-HuR-specific antibody. To correct for variations in the protein loading, blots were stripped and successively incubated with an anti-β-actin antibody. PGE₂ levels in the corresponding tissue homogenates are depicted in the **bottom** panel with the data representing means ± SD (*n* = 3). **P* ≤ 0.05 compared with animals treated for 6 hours with vehicle, or °*P* ≤ 0.05 versus AngII-treated animals. **C:** AngII promotes nuclear translocation of PKC-δ.

AngII, which was completely blocked in the presence of valsartan (Figure 7B, bottom). By contrast, systemic administration of PD123319 caused a further increase in both the AngII-induced and basal PGE₂ production, which may indicate that the synthesis of PGE₂ is under an additional control of the AT₂ receptor.

Based on the present and our previous findings in human MCs, we checked whether PKC-dependent HuR shuttling is preceded by a nuclear translocation of PKC- δ , which is one of the abundant Ca²⁺-independent novel PKC isoforms in MCs.²⁹ Time-course experiments revealed that AngII promotes a rapid import of PKC- δ into the nucleus. Corresponding to the increase in nuclear PKC- δ , we observed a reduction in the cytoplasmic PKC- δ content with a maximal effect observed at 30 minutes of AngII stimulation (Figure 7C). Collectively, these data indicate that AngII-induced HuR shuttling in the rat kidney is regulated by an AT₁ receptor-dependent mechanism that is triggered by PKC- δ .

AngII Induces a Physical Interaction of PKC- δ with HuR in the Nucleus

Next, we tested for a direct physical interaction of both proteins, which is an important prerequisite for the post-translational modification of HuR by PKC- δ .²⁰ Using IP with a HuR-specific antibody revealed appearance of co-immunoprecipitated PKC- δ in the nucleus only in the fractions from AngII-treated MCs, whereas no PKC- δ was precipitated by the HuR antibody when an equal protein amount of the corresponding cytoplasmic fractions was applied to the IP (Figure 8A). In addition, no PKC- δ was detectable when instead of the anti-HuR antibody a similar amount of mouse IgG was used for IP (Figure 8A). In accordance with these findings from cell culture, physical interactions between HuR and PKC- δ were exclusively found in the nuclear homogenates from AngII-treated rats whereas no PKC- δ was pulled down in kidney samples from vehicle-treated animals (Figure 8B). Collectively, these data demonstrate that AngII does initiate a rapid translocation of PKC- δ to the nucleus that is followed by an increased PKC- δ binding to nuclear HuR *in vivo* and in cultured MCs.

Discussion

During the last decade much evidence has been accumulated demonstrating that AngII can directly trigger renal fibrosis independently from its hemodynamic effects. Thereby AngII can propagate extracellular matrix accumulation either by directly promoting collagen ex-

Figure 8. AngII promotes a physical interaction between PKC- δ and nuclear HuR in rat MCs (A) and *in vivo* (B). **A:** Immunoprecipitation (IP) of cytoplasmic and nuclear HuR with PKC- δ in rat MCs stimulated either without (–) or with 100 nmol/L of AngII (+) for 30 minutes. For IP, a total protein amount of 500 μ g of either nuclear or cytoplasmic extracts was incubated overnight with 2 μ g of either anti-HuR antibody (a.-HuR) or a nonspecific IgG isotype antibody (IgG). Co-immunoprecipitated PKC- δ was identified by Western blot analysis (W.b.) by use of a PKC- δ -specific antibody. Equal amounts of immunoprecipitated HuR were furthermore ascertained by stripping the blot and reincubating with the anti-HuR antibody (W.b.) used for IP. The data shown are representative of three independent experiments giving similar results. **B:** IP of nuclear PKC- δ by an anti-HuR antibody in kidney homogenates derived from rats that were either treated with vehicle (–), or from animals continuously infused with AngII (+) for 14 days. For IP, a total protein amount of 500 μ g of nuclear extract from corresponding tissue homogenates pooled from three different animals was incubated with 2 μ g of an anti-HuR-specific antibody (a.-HuR) or with control IgG (IgG) as described above. The position of a precipitated band at 78 kDa, the size of PKC- δ , is indicated by an **arrow** and was determined by use of standard molecular weight markers. Equal amount of immunoprecipitated HuR was ascertained by reincubation with the anti-HuR antibody (HuR) used for IP.

pression or via an induction of tissue inhibitors of metalloproteinases (TIMPs) and/or plasminogen-activator inhibitors (PAIs).^{3–5} In this study we show that concomitant with the occurrence of tubular and glomerular fibrosis in rats, a continuous AngII infusion leads to a robust increase in PAI-1 and COX-2 at the protein and mRNA levels. Our findings underline several reports that demonstrated that AngII despite the propagation of profibrotic cell responses, exerts proinflammatory functions, indicated by the strong up-regulation of COX-2 *in vitro* and *in vivo*.^{16,30} Our data extend our previous studies demonstrating that AngII induces COX-2 expression in human MCs via an increase in HuR shuttling,²⁰ and give evidence for the *in vivo* relevance of AngII-triggered activation of HuR in the post-transcriptional regulation of COX-2 and PAI-1 expression. The supplementary *in vitro* studies with siRNAs confirmed the functional relevance of increased HuR shuttling by demonstrating that HuR is indispensably involved in the increase in PAI-1 and COX-2 mRNA levels and subsequent release of corresponding enzyme products by AngII (Figure 6).

Although the PAI-1 mRNA from different species bearing long 3'-UTRs with various AREs,³¹ the mechanisms involved in the post-transcriptional regulation of PAI-1 are poorly understood. To the best of our knowledge this is the first study demonstrating that

Figure 7 continued. Time course of PKC- δ entry into the nucleus by AngII. Quiescent MCs were treated with vehicle (5 minutes, 120 minutes) or with AngII (100 nmol/L) for the indicated time periods before cells were lysed for nuclear and cytoplasmic extracts. Nuclear (50 μ g) or cytoplasmic (20 μ g) extracts were subjected to SDS-PAGE and immunoblotted with an anti-PKC- δ -specific antibody. To ascertain equal protein contents within the extracts, the blots were stripped and reprobed with an anti- β -actin and with a HDAC1-specific antibody. The Western blots shown are representative of two independent experiments with similar results.

PAI-1 mRNA is a target of the mRNA-stabilizing protein HuR *in vitro* as well as *in vivo*. By RNA-EMSA we document a profound and persistent increase in HuR binding to three different pentameric AUUUA motifs in rats receiving AngII, which is in well correlation to the short-term effects observed in cultured MCs. It is interesting to note, that PAI-2, the main inhibitor of urokinase, is an additional target of HuR although in that specific case, HuR binding promotes the decay rather than stability of PAI-2 mRNA.³² Because it is widely assumed that HuR is associated with other ARE-binding proteins this somehow unexpected observation may be explained by the binding of other, possibly mRNA-destabilizing factors competing for the binding to the same ARE.³³

Similar to PAI-1 mRNA, the AngII-induced RNA-binding to COX-2 mRNA remained almost unchanged during the whole infusion period of 14 days, a finding that may substantiate the notion that HuR activation by AngII is a sustained process. Interestingly, a previous study demonstrated that AngII-dependent degradation of AT₁ receptor mRNA is mediated via binding of opposing mRNA-binding proteins including AUF-1, HuR, and the heterogeneous ribonucleoprotein A1.³⁴ From these data it becomes obvious that the destabilization of AT₁ receptor via HuR displacement by competing destabilizing factors may reflect an important autoregulatory feedback by which AngII limits the AngII-induced mRNA stabilization. Because HuR is predominantly localized within the nucleus, the stimulus-dependent mRNA binding and export of HuR from the nucleus is a major prerequisite for its stabilizing effects on mRNA.²⁸

Mechanistically, the regulation of HuR shuttling is a target of different signaling pathways, including the mitogen-activated protein kinases (MAPKs), the AMPK, and different members of the PKC family.³⁵ Previously, we have delineated the AngII-induced HuR trafficking in human MCs by demonstrating that the Ca²⁺-independent PKC- δ is essentially involved in the nucleo-cytoplasmic HuR shuttling and concomitantly critical for stabilization of COX-2 mRNA. Using co-IP and co-localization we had also shown that a physical interaction of nuclear HuR with PKC- δ is accompanied by a PKC-dependent phosphorylation and subsequent HuR export to the cytoplasm.²⁰ In the present study, we prove that AngII caused a physical interaction of PKC- δ with nuclear HuR *in vivo*. Coincidentally, with these *in vivo* analyses, we found that AngII induces a rapid import of PKC- δ to the nucleus suggesting that HuR is a direct binding partner of PKC- δ in the nucleus (Figure 7).

Besides the increased fibrotic responses attributed to an increase in PAI-1 synthesis, the HuR-mediated rise in COX-2 mRNA expression by AngII is accompanied by an increased prostaglandin E₂ synthesis in rat MCs (Figure 6C) and in tissue homogenates from whole kidneys (Figure 7B). PGE₂ is known as the dominant prostanoid in the kidney, which in most cases elicit a vasodilatory response but, in some circumstances, may also exert vasoconstrictive effects, depending on the specific subtype of PGE₂ receptor involved.³⁶ In the current study we found, that the AngII-mediated increase in COX-2 expression is specifically mediated by the AT₁ receptor

without an involvement of the AT₂ receptor. However, it should be noted that different effects of AngII on cortical COX-2 expression have been documented in the literature. Consistent with our data, previous studies by Jaimes and colleagues³⁰ demonstrated a strong up-regulation of glomerular COX-2 by AngII via the AT₁ receptor and increased reactive oxygen species generation.^{16,30} By contrast, another study reported an inhibitory effect of AngII on cortical COX-2, which was under a differential control by both types of AT receptors.³⁷ There could be several reasons for these discrepant findings. AngII additionally activates mesangial phospholipases C and A₂,³⁸ the rate-limiting enzymes of prostaglandin synthesis. Thus, temporal and/or spatial activation patterns or more down-stream PG synthases may also influence the balance between vasoconstrictory (eg, thromboxane) and vasodilatory (eg, prostacyclin) prostanoids and thereby alter renal blood flow and pressure, which by itself can modulate COX-2 expression.¹⁴ A previous study by Faour and colleagues³⁹ demonstrated that PGE₂ modulates the stability of COX-2 mRNA. This finding highlights the important role of a negative feedback regulation of COX-2 by prostanoids. It is tempting to speculate that, in addition to differences in the prostanoid levels, changes in the AT₁ and AT₂ as well as in prostanoid receptor expression may account for the reported opposite effects of AngII on cortical COX-2 expression. Further experimental evidence is needed to firmly define the impact of different prostanoids and their receptors on the AngII-triggered COX-2 expression in distinct areas of the kidney.

Because COX-2-derived prostaglandins in addition to maintaining renal and vascular homeostasis participate in the pathogenesis of inflammatory processes,¹⁴ the AngII-induced stabilization of PAI-1 and COX-2 may critically contribute to the AngII-triggered fibrosis and inflammation. The proinflammatory effects of AngII are furthermore highlighted by the immunosuppressive and anti-inflammatory effects of the AT₁ receptor antagonist candesartan in chronic kidney diseases.⁴⁰ Collectively, we suggest that post-transcriptional gene regulation by HuR may provide a novel target for intervention in the pharmacological treatment of common renal diseases. In addition, our study indicates that HuR shuttling and subsequent stabilization of target mRNAs by AngII is a long-lasting process that critically depends on PKC- δ . This emphasizes the viability of current pharmacological strategies aiming on a specific inhibition of distinct PKC isoenzymes because they could provide powerful tools to specifically interfere with deregulated HuR functions relevant for inflammation and tissue fibrosis.

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