

Role of the Renin-Angiotensin System on the Parathyroid Hormone-Related Protein Overexpression Induced by Nephrotoxic Acute Renal Failure in the Rat

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Parathyroid hormone-related protein (PTHrP), a mitogenic factor for renal cells, is overexpressed in acute renal failure (ARF). Recent data support an association between PTHrP and the renin-angiotensin system in the damaged kidney. The effects of angiotensin II (Ang II) inhibitors (quinapril, enalapril, and/or losartan) on PTHrP and the PTH1 receptor (PTH1R) expression in rats with either folic acid (FA)- or gentamicin-induced ARF were analyzed. The decreased renal function and the PTHrP upregulation and PTH1R downregulation induced by the nephrotoxins were inhibited by the Ang II blockers. In tubuloepithelial cells NRK-52E, the rapid (10 min) increase in PTHrP mRNA by FA, associated with a perinuclear relocalization of Ang II/AT1 receptor, was inhibited by losartan but not candesartan, which traps Ang II receptors at the cell surface. Maximal PTHrP protein overexpression by FA (at 24 to 72 h)—or by exogenous Ang II—was abolished by both Ang II antagonists. PTHrP upregulation by FA was preceded by increased extracellular signal-regulated kinase (ERK) phosphorylation and inhibited by the ERK inhibitor PD098059. FA also activated cAMP response element-binding (CREB) protein, and this was prevented by losartan in these cells. Moreover, PTHrP mRNA overexpression by either FA or Ang II occurred in NRK 52E that were transfected with a CREB construct but not the dominant-negative CREB133 construct. These findings demonstrate that the decreased renal function and PTHrP overexpression in nephrotoxin-damaged kidney depends on renin-angiotensin system. In this setting, intracellular Ang II/AT1 receptor recycling seems to be related to PTHrP induction through ERK and CREB activation in tubuloepithelial cells.

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Ischemic and nephrotoxic acute renal failure (ARF) occurs frequently in hospitalized patients. Current hypothesis supports that renal dysfunction in ARF is caused by sublethally damaged—often structurally intact—tubuloepithelial cells that are able to recover after removal of the insult (1,2). Recovery from ARF occurs associated with an immediate early gene response that is similar to that triggered by growth factors. It is currently thought that autocrine/paracrine growth factors that are released at sites of nephron injury act as mediators of tubular regeneration (2,3).

Parathyroid hormone-related protein (PTHrP) is a widespread factor in fetal and adult tissues, where it exerts complex functions (4). In the kidney, PTHrP and the PTH1 receptor (PTH1R) are present along the nephron and in the intrarenal arterial tree (5,6). PTHrP expression is rapidly and transiently upregulated during the recovery period after renal ischemia or folic acid (FA)-induced ARF in rats (7,8). PTHrP has growth-regulatory properties in some

renal cell types (8–12). However, the putative role of PTHrP in the regenerative process of the damaged kidney is intriguing because the PTH1R gene is rapidly downregulated in tubular cells after acute renal injury (7,8).

The renin-angiotensin system (RAS) plays an important pathogenetic role in the progression of kidney damage, but its involvement in ARF is poorly understood (13–15). In rats with tubulointerstitial damage after protein overload or FA injection, both PTHrP and angiotensin-converting enzyme (ACE) genes are upregulated in the renal cortex (8,16). Moreover, angiotensin II (Ang II) stimulates PTHrP expression in renal and vascular smooth muscle cells both *in vivo* and *in vitro* (6,17). In fact, recent findings suggest that PTHrP might contribute to Ang II-induced tubular injury (6).

In this study, we examined the interaction between Ang II and PTHrP in nephrotoxic ARF, both in rats *in vivo* and in tubuloepithelial cells *in vitro*. Our findings provide further insights into the important role of Ang II on the pathogenesis of this condition.

Materials and Methods

Induction of Nephrotoxic ARF

Male Wistar rats (250 g body wt) were fed standard rat diet and given free access to water. Some rats received a single intraperitoneal injection of FA (Sigma, St. Louis, MO), 250 mg/kg in 300 mM NaHCO₃

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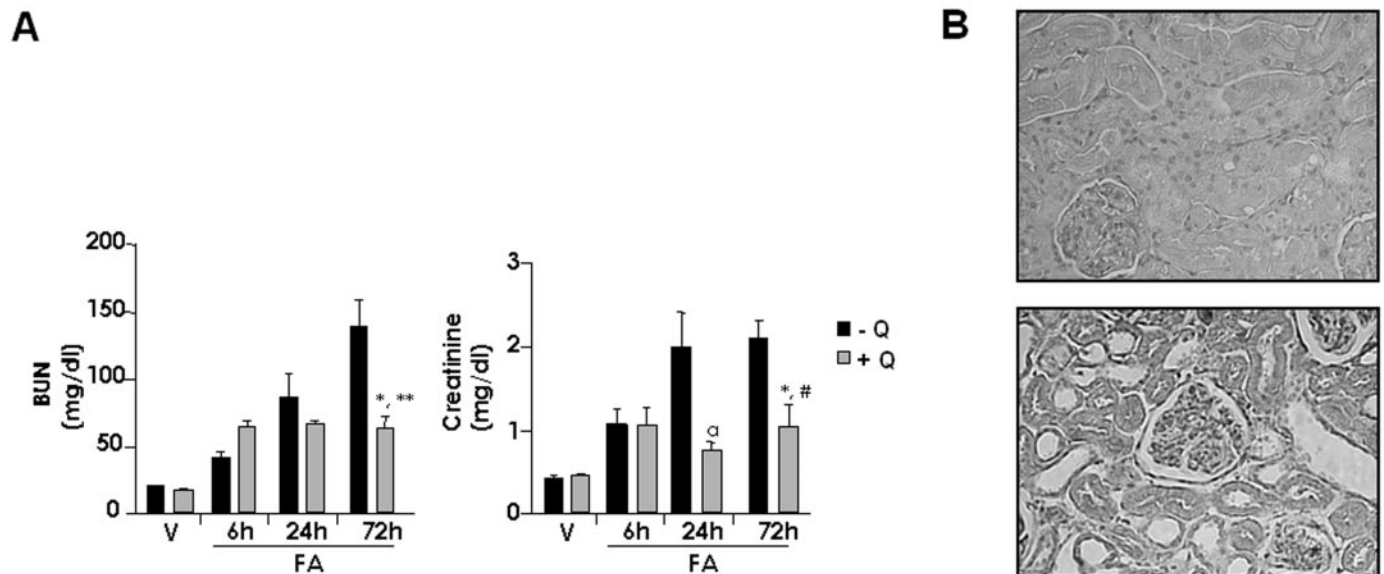


Figure 1. (A) Time course of changes in plasma creatinine and blood urea nitrogen (BUN) in rats with folic acid (FA)-induced injury, pretreated or not with quinapril (Q). Experimental values are mean \pm SEM of four to six animals per group at each time. V, vehicle-injected rats. * $P < 0.05$ versus $-Q$ at 72 h; ^a $P < 0.025$ versus $-Q$ at 24 h; ** $P < 0.01$; # $P < 0.05$ versus V. (B) Periodic acid-Schiff (PAS) staining in representative renal tissue samples of rats, pretreated (top) or not (bottom) with quinapril, at 6 h after FA injury. Histologic examination of kidney sections showed that tubular dilation and edema were prevented by pretreatment with this ACE inhibitor. Magnification, $\times 200$.

(vehicle) or the same volume of vehicle (8). Another group of rats received injections twice daily for 4 consecutive days with gentamicin (Calbiochem, San Diego, CA), 200 mg/kg in 0.9% NaCl (vehicle), or vehicle alone (18). Some rats were treated with 4 mg/L enalapril (MSD, Madrid, Spain) or quinapril (200 mg/L; Parke-Davis, Madrid, Spain) in the drinking water for 2 or 3 wk, respectively, before FA injection. Other groups of animals received losartan (20 or 100 mg/L; MSD) in the drinking water for 1 or 2 wk before either FA or gentamicin injection, respectively. These doses were selected because they have shown to elicit at least partial Ang II blockade (19–21). At different times, four to six animals from each group were killed under ether anesthesia, and kidneys were removed. One kidney from each animal in all groups was fixed in 4% buffered p-formaldehyde for light microscopy examination and immunohistochemistry. The remaining kidney of each animal was removed, weighed, and stored in RNAlater (Ambion, Austin, TX) at -20°C for up to 3 d for subsequent total RNA and protein extraction.

Blood was taken by cardiac puncture under ether anesthesia after overnight fast. Plasma creatinine and blood urea nitrogen (BUN) were determined by autoanalyzer (Hitachi Chemistry System, Boehringer Mannheim, Mannheim, Germany).

Histology and Immunohistochemistry

Fixed renal tissue sections were dehydrated by graded ethanols and xylene and embedded in paraffin. Paraffin-embedded tissue sections (4 μm) were mounted on 3-aminopropyltriethoxy-silane-treated slides for histologic evaluation (by periodic acid-Schiff reactive staining) or immunohistochemistry. The latter was performed using the following rabbit polyclonal antibodies (dilution, -fold): anti-PTHrP antiserum C6 (100); anti-Ang II antiserum (Peninsula Laboratories, San Carlos, CA; 400); and anti-proliferating cell nuclear antigen (PCNA) antiserum (Dako, Glostrup, Denmark; 150) (6,8). Immunostaining was performed as described in detail (6,8). After incubation with the corresponding primary antiserum for

either 1 h (PTHrP) or 30 min (Ang II and PCNA), the sections were incubated with a polymer-peroxidase complex (Envision+System; Dako) and 3,3'-diaminobenzidine and then counterstained with Carazzi's hematoxylin. Some tissue sections were incubated without the primary antibody as negative controls. PCNA staining was evaluated in at least four $\times 400$ microscopic fields by counting the number of stained tubular cell nuclei per field, and the corresponding mean from each rat was calculated.

Cell Culture Studies

Rat kidney tubuloepithelial cells NRK 52E (ATCC CRL 1571) were grown in DMEM with 5% FBS and antibiotics, in 5% CO_2 at 37°C , as described (8). NRK 52E cells were serum-depleted for 24 h before addition of FA, at 10 mM in 300 mM NaHCO_3 , a toxic dose for these cells (8). Some NRK 52E cells were transfected with pCMV-cAMP response element-binding (CREB; constitutively expressing the wild-type CREB) or pCMV-CREB133 (a dominant-negative mutant vector preventing Ser¹³³ phosphorylation of CREB; Clontech, Palo Alto, CA) using lipofectamine (Invitrogen, Groningen, the Netherlands), according to the manufacturer's instructions. The transfected cells were selected in 1 mg/ml gentamicin (Invitrogen) for 3 wk to establish stable cell lines. Subsequently, cells were treated with the agonists for different times before total RNA isolation.

In some experiments, NRK 52E cells that were grown on coverslips in multiwell plates were stimulated with 10 mM FA for different time periods in FBS-depleted medium. Then, they were fixed with 64% isopropanol/15% polyoxyethylene (Cell-fix; Shandon, Pittsburgh, PA) and either permeabilized or not with 0.1% Triton X-100 in PBS for 5 min at 4°C . After blocking with 1.5% normal goat serum in PBS for 30 min, the anti-Ang II antibody referred to above or a polyclonal AT1 receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were added, each at 1:200 dilution, for 1 h at room temperature. Then, FITC-conjugated anti-rabbit IgG antibody (Sigma), at 1:200 dilution, was added for 30 min. After extensive

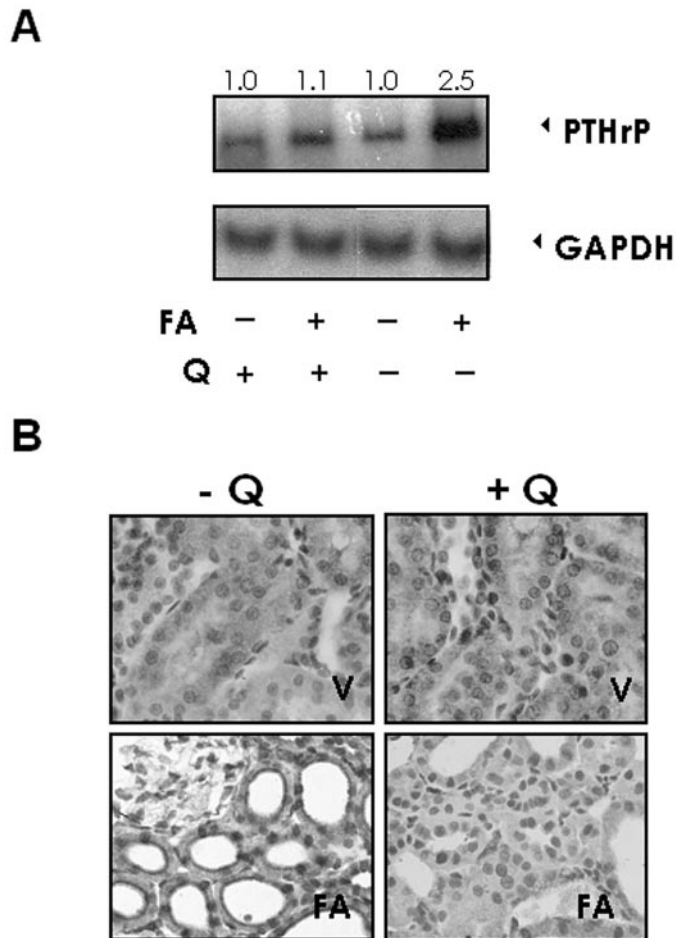


Figure 2. Parathyroid hormone-related protein (PTHrP) over-expression—both mRNA and protein—after FA injury. (A) PTHrP mRNA in the rat renal tissue at 6 h after FA injury, with or without quinapril (Q) pretreatment. PTHrP mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, a constitutive control, were evaluated by reverse transcription-PCR (RT-PCR). Relative intensities of PTHrP signal corresponding to four to six animals per group are indicated at the top. (B) PTHrP immunostaining with C6 antiserum in the rat renal cortex at 72 h after FA injury or vehicle (V) injection, with or without Q pretreatment. Magnification, $\times 1000$ (top) and $\times 500$ (bottom).

washing, cells were mounted in Mowiol (Calbiochem, San Diego, CA), and immunofluorescence analysis was performed with a Leica DM-IRB confocal microscope.

Total RNA and Protein Extraction

Total RNA and protein were isolated from either rat kidney homogenates obtained with a glass-Teflon homogenizer or FBS-depleted NRK 52E cells using TriReagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer’s instructions. Total RNA was quantified by A_{260}/A_{280} ratio. Protein was determined by the Bradford’s method (Pierce, Rockford, IL), using BSA as standard.

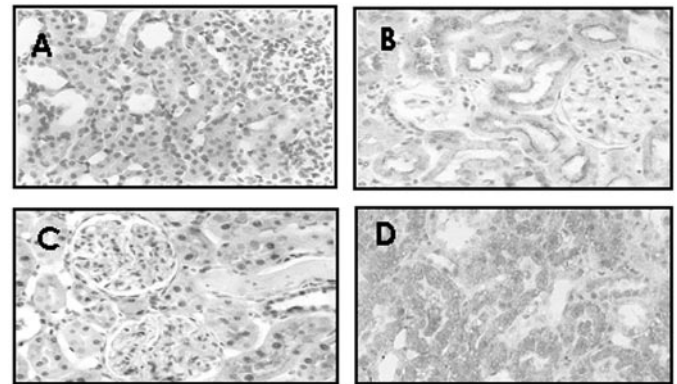


Figure 3. Effect of losartan (LST) pretreatment on angiotensin II (Ang II) immunostaining in the kidney of rats with FA injury. Ang II positivity in the rat renal cortex at 72 h after vehicle injection (B) or FA injury (C and D), with (D) or without (C) LST pretreatment. (A) Negative control (without primary antibody) is also shown. Magnification, $\times 100$.

Semiquantitative Reverse Transcription-PCR

Total RNA from rat kidney or NRK 52E cells (10 to 100 ng) was reverse-transcribed, and resulting cDNA was amplified using the Titanium One-step RT-PCR kit (Clontech) with specific primers for rat PTHrP (6,8,16). Either the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified using specific primers (6,8), or modified 18S primers (QuantumRNA 18S Internal Standards; Ambion) were used for 18S coamplification, as constitutive controls.

The reaction mixture (10 μ l) was incubated for 45 min at 48°C and 2 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 60 to 62°C, and 2 min at 68°C, with a final extension of 7 min at 68°C. Preliminary experiments established that these conditions provided a linear cDNA amplification in each case. PCR products were separated on 2% agarose gels, and bands were visualized by ethidium bromide staining. Values that were obtained after densitometric scanning of PCR products were normalized against those of the corresponding glyceraldehyde-3-phosphate dehydrogenase and 18S PCR products.

Western Blot Analysis

Total protein extracts (60 μ g of protein) from rat kidney or NRK 52E cells were separated on 12.5% polyacrylamide-SDS gels under reducing conditions. After electrophoresis, samples were transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK), blocked with either 5% BSA (PTHrP) or 5% defatted milk (PTH1R, p42/p44 extracellular signal-regulated kinases [ERK1/2] and phospho(p)ERK 1/2) in PBS with 0.05% Tween-20, and then incubated overnight with an anti-pERK1/2 (Thr²⁰²/Tyr²⁰⁴) or an anti-ERK1/2 antibody (Cell Signaling Technology, Beverly, MA), each at 1:1000 dilution; the anti-PTHrP antiserum C6, at 2500-fold dilution; or affinity-purified anti-PTH1R antibody Ab-VII (Covance, Berkeley, CA), at 5 μ g/ml (6). β -Actin and α -tubulin were detected with a specific rabbit polyclonal antibody (Santa Cruz Biotechnology) or mouse monoclonal antibody (Sigma), respectively, as constitutive controls. After extensive washing, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG and developed by enhanced chemiluminescence (Amersham). The corresponding fluorogram bands were quantified by densitometric scanning (Image-Quant; Molecular Dynamics, Sunnyvale, CA).

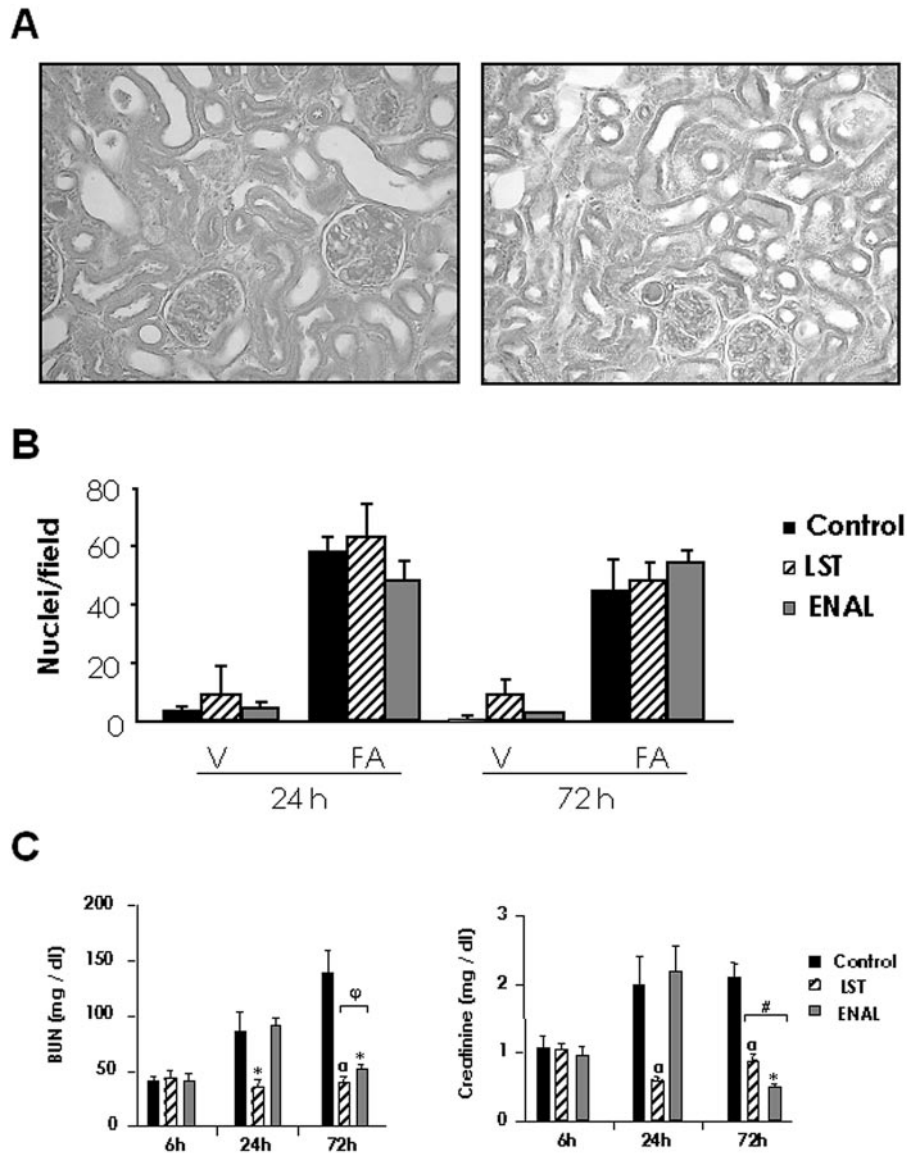


Figure 4. (A) PAS staining in representative renal tissue samples of rats that were pretreated with enalapril (ENAL; left) or losartan (LST; right), at 72 h after FA injury. Histologic examination of kidney sections showed that tubular dilation and edema were not prevented by pretreatment with these Ang II inhibitors. (B) Renal tubular cell proliferation at 24 to 72 h after FA injury in rats that were pretreated or not with LST or ENAL. $P < 0.01$ at 24 h or 72 h versus the corresponding proliferating cell nuclear antigen score values of vehicle (V)-injected rats. (C) Time course of changes in plasma creatinine and BUN in rats with FA-induced renal injury, pretreated or not with LST or ENAL. Experimental values are mean \pm SEM of four to six animals per group at each time period. * $P < 0.025$, ^a $P < 0.05$ versus the corresponding untreated values; # $P < 0.05$, ^e $P < 0.01$ versus values of V-injected rats: 18 ± 3 mg/dl (BUN) and 0.5 ± 0.1 mg/dl (plasma creatinine). Magnification, $\times 200$ (left) and $\times 150$ (right) in A.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to a commercially available procedure (NE-PER, Pierce), as described previously (22). The synthetic double-stranded CRE oligonucleotide 5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3' was 5'-end-labeled with 10 μ Ci (γ -³²P)ATP and T4 polymerase. Nuclear extracts (4 μ g of protein) were incubated with 200,000 dpm of ³²P-labeled oligonucleotide probe in 20 μ l of a reaction mixture that contained 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, and 1 μ g of poly (dI-dC) for 20 min at 4°C. Protein-DNA complexes were resolved on native 5% polyacrylamide/0.25 \times

TBE gels. Gels then were dried and exposed to radiosensitive film. As specificity controls, nuclear extracts were preincubated with a 100-fold excess of either unlabeled oligonucleotide or another oligonucleotide that had an NF- κ B or core-binding factor α 1 (cbfa1) binding site, for 20 min at 4°C, before addition of the labeled probe.

Statistical Analyses

Results are expressed as mean \pm SEM throughout the text. Statistical analysis was performed by either Kruskal-Wallis test or Mann-Whitney test, when appropriate. $P < 0.05$ was considered significant.

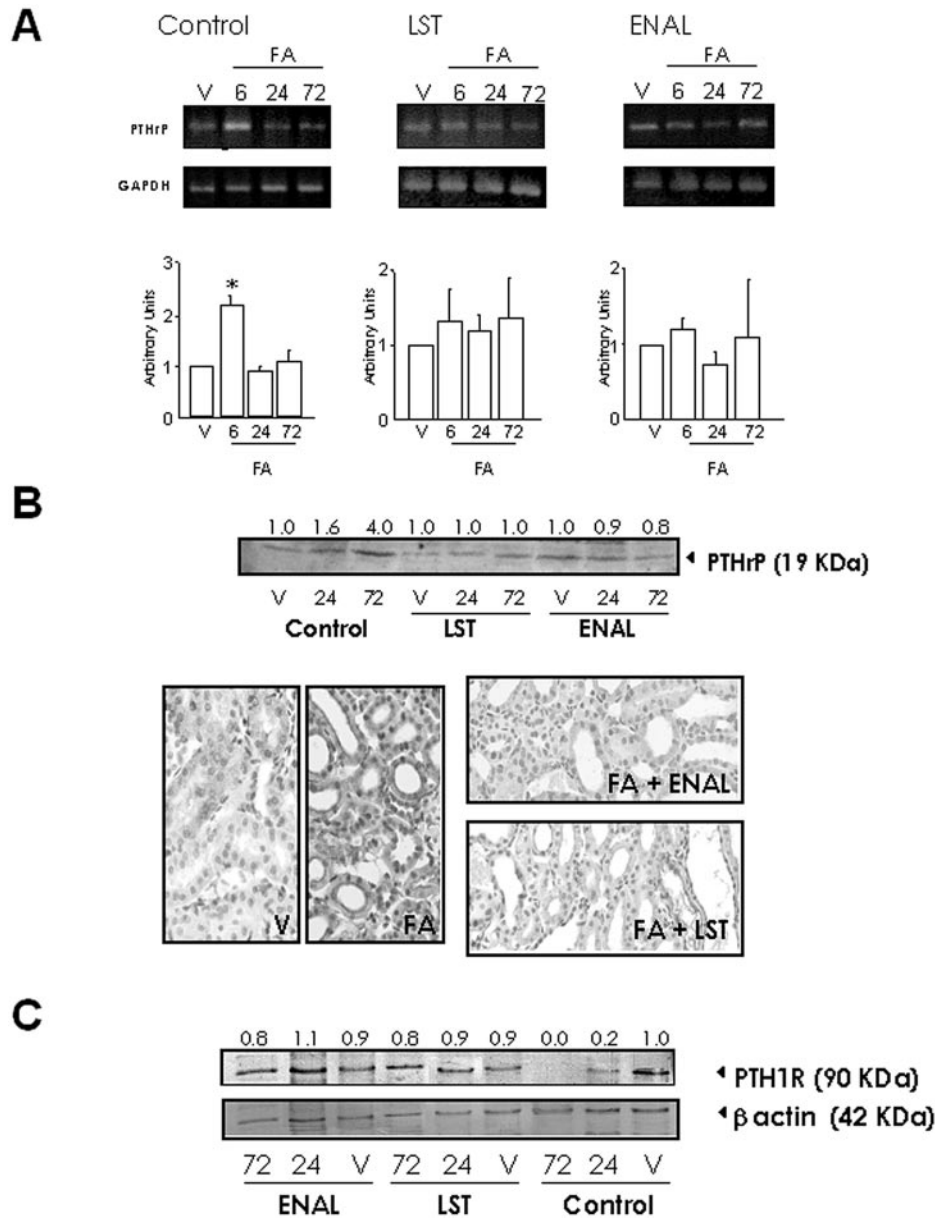


Figure 5. PTHrP and PTH1 receptor (PTH1R) in the FA-injured kidney of rats after enalapril (ENAL) or losartan (LST) pretreatments. (A) Representative autoradiograms corresponding to PTHrP mRNA changes (evaluated by RT-PCR) in the kidney at 6 to 72 h after FA injury, with or without (control) LST or ENAL pretreatment, or vehicle (V) injection. GAPDH mRNA was included as a constitutive control. Relative densitometric values, as mean \pm SEM of four to six animals per group at each time period, *versus* those of V-injected rats are also shown. * $P < 0.01$ *versus* V value. (B) PTHrP protein was evaluated in the rat kidney by Western blot, at 24 h and 72 h, or immunohistochemistry, at 72 h after FA or V. (C) Western blot analysis of PTH1R protein levels at 24 h and 72 h after FA, pretreated or not with the Ang II inhibitors, or V injection. Protein loading was similar in each well, as assessed by using Ponceau S staining (data not shown; B), or β -actin as an internal control (C). Relative intensities of PTHrP and PTH1R signals, indicated at the top (B and C), are shown.

Results

Effect of Ang II Blockade on Changes in Renal Function and PTHrP Expression Induced by Nephrotoxic ARF

We found that the decreased renal function was significantly improved by quinapril pretreatment within 24 to 72 h after FA (Figure 1A). Moreover, histologic findings showed that tubular dilation and edema, which were observed already at 6 h after FA injection, were prevented by pretreatment with this ACE

inhibitor (Figure 1B). In addition, PTHrP upregulation—both mRNA (by reverse transcription-PCR) and protein (by immunohistochemistry)—induced by FA in the rat renal cortex was not observed following Ang II blockade by quinapril (Figure 2).

We further evaluated whether inhibition of PTHrP overexpression after FA might be a consequence of the dramatic decrease in histologic alterations in the rat renal cortex associated with quinapril administration. Thus, in the next series of

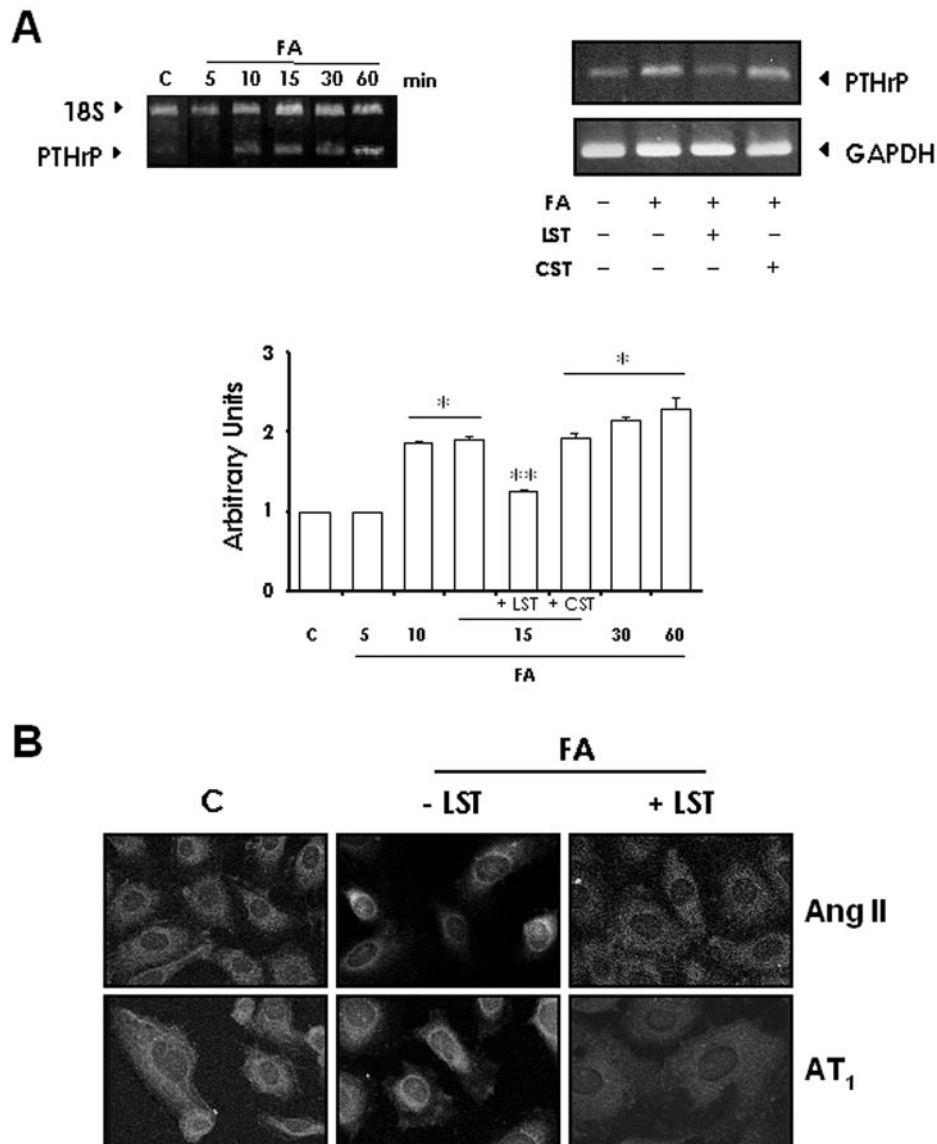


Figure 6. (A) PTHrP mRNA induction by FA, in the presence or absence of either losartan (LST) or candesartan (CST), in NRK 52E. Representative autoradiograms corresponding to PTHrP mRNA changes (analyzed by RT-PCR) after addition of FA (10 mM) for different time periods (left) or 15 min (right). Either 18S or GAPDH mRNA were used as constitutive controls. C, saline control. Relative densitometric values, as mean \pm SEM from three different experiments, are also shown. * $P < 0.05$ versus C value; ** $P < 0.05$ versus FA alone at 15 min. (B) FA induced, within 10 min, a rapid relocalization of the Ang II/AT₁ receptor complex to the nuclear envelope of NRK 52E cells, which was inhibited by LST. Both Ang II antagonists (at 10 μ M) were added 1 h before FA (A and B).

experiments we used less efficient doses of another ACE inhibitor, enalapril, and of an AT₁ antagonist, losartan, for a shorter period than that used for quinapril to neutralize partially Ang II action. This pretreatment with enalapril increased ACE mRNA expression in vehicle-injected rats, as well as its FA-induced overexpression (8), which is likely due to ACE activity inhibition (15) (data not shown). Ang II staining was observed mainly in the brush border of cortical tubules in vehicle-injected rats but localized to the nuclei of tubule cells after FA injection, as previously reported (8). However, this relocalization was prevented by losartan pretreatment (Figure 3).

With the use of this maneuver, there were no significant

changes in the renal histologic alterations (Figure 4A) or the increased PCNA immunostaining in renal tubule cells (Figure 4B) acutely observed after FA in rats that were pretreated with these Ang II antagonists. However, both enalapril and losartan pretreatments significantly decreased the acute deterioration of the renal function after FA injection (Figure 4C). This improvement in the renal function induced by the Ang II inhibitors was associated with a lack of renal PTHrP (mRNA and protein) upregulation after FA (Figure 5, A and B). Moreover, the PTH1R levels in the FA-injured kidney were not downregulated but remained similar to those of vehicle-injected controls after pretreatment with both Ang II antagonists (Figure 5C).

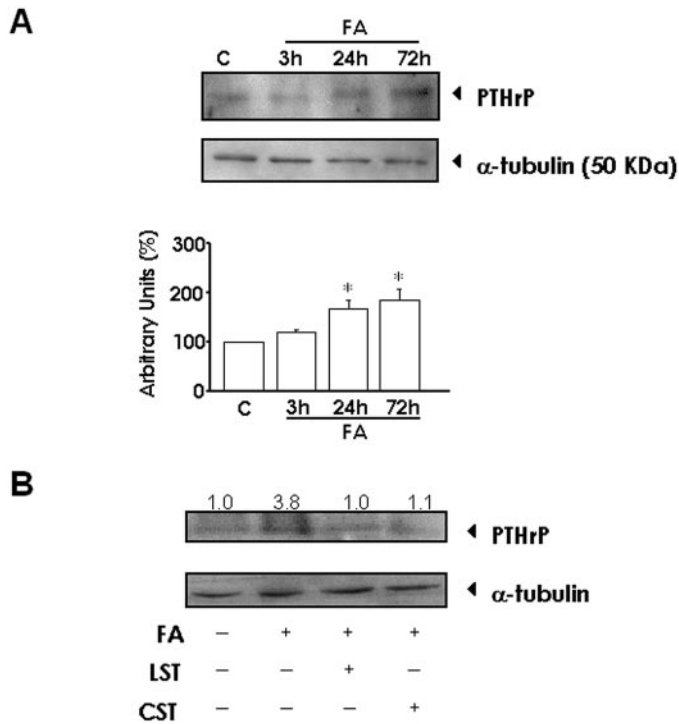


Figure 7. Western analysis of PTHrP protein levels (using anti-serum C6) in NRK 52E cells after incubation with FA (10 mM), at different time periods (A) or at 24 h, in the presence or absence of Ang II antagonists (B). Losartan (LST) or candesartan (CST) was added (at 10 μ M) 1 h before FA. Protein loading was similar in each well, as assessed by α -tubulin as an internal control. (A) Relative densitometric values, as mean \pm SEM from at least three different experiments, are shown. * $P < 0.05$ versus control (C) value. (B) Relative intensities of PTHrP signal are indicated at the top.

To confirm independently the apparent association between RAS activation and PTHrP overexpression in nephrotoxic ARF, we assessed the effect of losartan on the PTHrP response to a distinct model of induction of this condition by injecting high doses of gentamicin into rats. Renal function in gentamicin-injected animals, which was dramatically deteriorated at day 4 after gentamicin injection, improved significantly after losartan pretreatment. Thus, plasma creatinine and BUN values (mg/dl) were, respectively, 0.25 ± 0.05 and 10.5 ± 0.5 (control animals; $n = 4$) versus 6.0 ± 0.6 or 3.1 ± 0.7 , and 162 ± 16 or 103 ± 18 , in gentamicin-injured animals, either untreated or treated with losartan ($P < 0.05$; $n = 6$). Four days after gentamicin injection, we also found significant changes in the renal PTHrP/PTH1R system: PTHrP protein levels increased to $191 \pm 8\%$, but those of the PTH1R protein decreased to $69 \pm 8\%$, compared with the corresponding values in control rats (100%; $P < 0.05$ or less; $n = 6$). After losartan pretreatment, the PTHrP protein values ($107 \pm 17\%$) normalized to those in control animals, whereas the downregulated PTH1R protein levels remained low ($82 \pm 7\%$ versus $69 \pm 8\%$; $P > 0.05$) but showed a tendency to reach those of vehicle-injected (control) rats.

Role of Ang II on PTHrP Overexpression Associated with Nephrotoxic-Induced Injury in Renal Tubule Epithelial Cells

To explore further the relationship between Ang II and PTHrP overexpression after nephrotoxic-induced ARF, we used rat renal epithelial cells NRK 52E. These cells express RAS and can synthesize Ang II, and they have AT1 receptors (23). In addition, we previously demonstrated that these cells produce PTHrP, whose gene expression increased within 6 h after treatment with 10 mM FA (8). In this study, this concentration of FA rapidly (within 10 min) induced PTHrP mRNA in NRK 52E cells (Figure 6A). This early onset of PTHrP mRNA overexpression was associated with a rapid relocalization of the Ang II/AT1 receptor complex (visualized by immunostaining with specific Ang II or AT1 antibodies) mainly to the perinuclear area of these cells (Figure 6B). It is interesting that both events were inhibited by 10 μ M losartan (Figure 6). However, 10 μ M candesartan, another Ang II antagonist that—in contrast to losartan—prevents AT1 receptor internalization and recycling (24), was inefficient in this regard (Figure 6A). In contrast, the maximal increase in PTHrP protein, which was observed within 24 to 72 h after FA (Figure 7A), was abolished by either losartan or candesartan in NRK 52E cells (Figure 7B). Both Ang II antagonists were equally effective in inhibiting the stimulatory effect of exogenously added Ang II, at 100 nM, on PTHrP in these cells. Thus, PTHrP mRNA levels increased to $181 \pm 15\%$ ($P < 0.05$) at 1 h by Ang II alone and were $96 \pm 6\%$ or $94 \pm 3\%$ with losartan or candesartan, respectively, compared with control (100%; $n = 3$). Meanwhile, PTHrP protein levels were $316 \pm 17\%$ ($P < 0.01$) at 24 h after Ang II and either $100 \pm 5\%$ or $99 \pm 5\%$ in the simultaneous presence of losartan or candesartan, respectively, with regards to control (100%; $n = 3$).

Mechanism by which Ang II Induces PTHrP Overexpression after FA-Induced Injury in Renal Tubule Epithelial Cells

Ang II interaction with AT1 receptors activates various transcription factors, including CREB protein, which elicits changes in PTHrP gene transcription in various cell types (25–27). In this study, Ang II was found to stimulate rapidly (within 10 min) CRE-binding activity in NRK 52E cell nuclear extracts, and this was inhibited by losartan (Figure 8A, left). ERK1/2 are rapidly activated (phosphorylated) by Ang II via AT1 receptors, leading to CREB activation in various cell types (25,27,28). We found that Ang II, within a time course similar to that of CREB activation, increases ERK1/2 phosphorylation (without affecting total ERK levels) in NRK 52E cell extracts (Figure 8A, right).

We next assessed whether CREB and ERK1/2 activation might be involved in the mechanism of FA-induced PTHrP overexpression through Ang II in NRK 52E cells. We found that FA rapidly induced CREB activation in NRK 52E cell extracts, and this was inhibited by losartan (Figure 8B, left). FA also stimulated ERK1/2 phosphorylation within a similar time course in these cell extracts (Figure 8B, right). Furthermore, PTHrP mRNA overexpression triggered by either FA or Ang II did occur in NRK 52E that were transfected with a wild-type CREB construct but not in those with the mutant CREB133 construct (Figure 9A). In addition, PTHrP

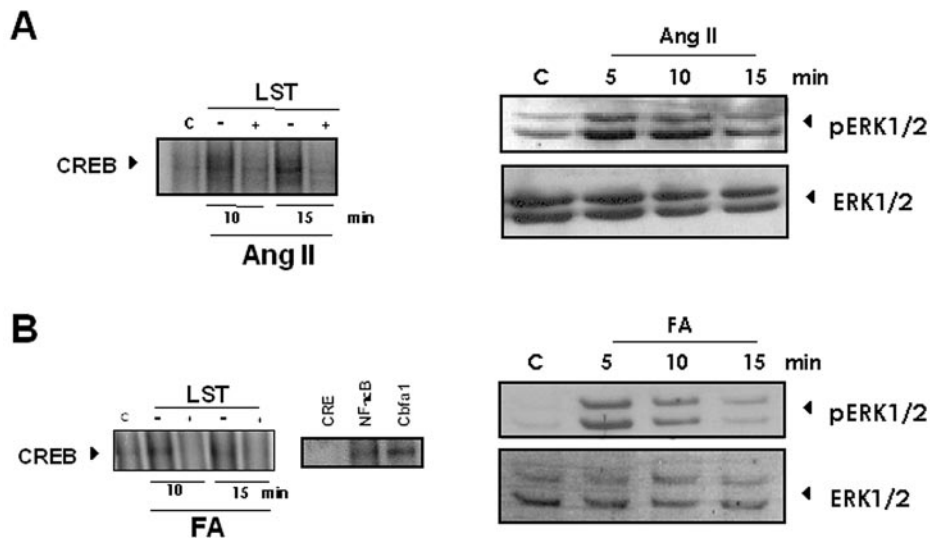


Figure 8. cAMP response element-binding (CREB) activation and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation induced by exogenously added Ang II or FA in NRK 52E cells. By using electrophoretic mobility shift assay with a synthetic double-stranded CRE oligonucleotide, it was found that Ang II (100 nM) as well as FA (10 mM) stimulated CREB activity within 10 min in NRK 52E cell nuclear extracts, and this activation was inhibited by previously (1 h) adding 10 μM losartan (LST; A and B, left). The retarded band in these extracts from FA-stimulated cells, at 10 min, disappeared with an excess of unlabeled CRE oligonucleotide but not with two noncompetitive oligonucleotides, confirming the specificity of the binding (B, middle). ERK1/2 phosphorylation by Ang II and FA in NRK 52E cell extracts (A and B, right). Western blot analysis using antibodies against phosphorylated forms of ERK (pERK1/2) or ERK1/2 was performed. C, nonstimulated control (at 5 min).

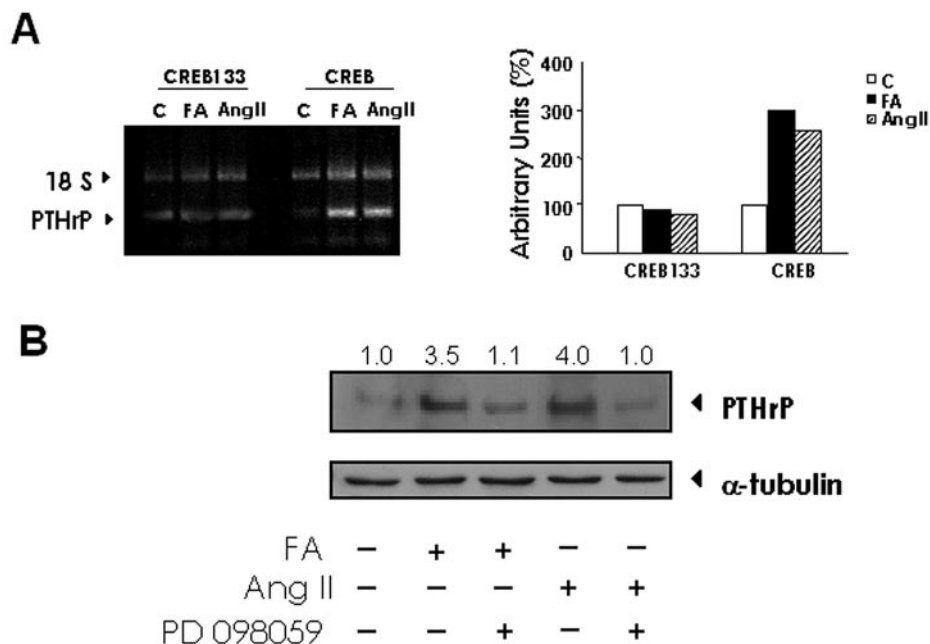


Figure 9. (A) Inhibition of PTHrP mRNA overexpression induced by either FA or Ang II in NRK 52E that were transiently transfected with a mutant of CREB (CREB133) but not in those that were transfected with a wild-type CREB construct. Representative autoradiogram showing PTHrP mRNA, and 18 S mRNA as a constitutive control, in transfected cells with each plasmid after 1-h stimulation with the agonists. C, saline control (left). Densitometric analysis of PTHrP mRNA levels corresponding to mean \pm SEM (<5% of the mean in all cases) from three different experiments (right). (B) Western blot analysis of PTHrP protein levels (using antiserum C6) in NRK 52E cells after incubation with either FA or Ang II for 24 h, with or without 10 μM PD098059. The inhibitor was added 1 h before the nephrotoxin or Ang II. Protein loading was similar in each well, as assessed by α-tubulin as an internal control. FA and Ang II were used at 10 mM and 100 nM, respectively. Relative intensities of PTHrP signal, indicated at the top, are shown.

protein upregulation by FA or Ang II was inhibited by 10 μ M PD098059, an ERK kinase inhibitor (29), in these cells (Figure 9B).

Discussion

Current evidence indicates the important role of RAS in the pathophysiologic mechanisms of renal injury (6,13–16). In fact, Ang II, the main RAS agonist, is now considered as a growth factor that plays an important role in the progression of kidney damage (14). This is related to the well-documented findings that Ang II can induce the synthesis of several mediators, such as TNF- α , IL-6, monocyte chemoattractant protein-1, and the activity of the transcription factor NF- κ B, associated with the presence of glomerular and interstitial inflammatory cells in the kidney (19,30). Furthermore, previous studies support that this system is also involved in the mechanisms associated with ARF. Thus, activation of local components of RAS, including Ang II, seems to occur early after either FA injection or ischemia/reperfusion, and administration of Ang II antagonists exerts beneficial effects on renal function in the latter setting (8,15,20,31). Moreover, Ang II inhibition attenuates glycerol-induced ARF (32). This study demonstrates that Ang II blockade causes a significant improvement in the renal function after FA- or gentamicin-induced acute renal injury in rats.

Renal functional recovery after nephrotoxic or ischemic ARF requires the repair of the injured tubular epithelium, a process that seems to be modulated by a variety of renal growth factors and cytokines (2,3). In the kidney, Ang II can also affect renal cell growth besides its effects on extracellular matrix accumulation; *e.g.*, it promotes EGF-stimulated proximal tubule cell proliferation (33,34). In this study, we found that the dramatic quinapril-induced amelioration of renal function after FA was associated with low (normal) tubular cell proliferation (by PCNA immunostaining) and normal renal histology. However, pretreatment with lower doses of two different Ang II antagonists (enalapril and losartan) led to a significant improvement of rat renal function without significant changes in either tubular PCNA staining or histologic morphology in FA-injured kidney. Moreover, previous studies using the renal ischemic model have shown that AT1 receptor mRNA was downregulated in the renal cortex early after reperfusion, in the presence of an intense tubular proliferation (31). Collectively, these findings and the results herein support the notion that Ang II does not interact significantly with the mechanisms involved in tubular hyperplasia in both models of ARF.

In a previous study, we showed that PTHrP (1-36), exogenously administered as a single dose shortly after FA injection in rats, moderately but significantly increased the number of PCNA-stained tubular cells, without affecting the deteriorated renal function, at 24 h (8). In this study, we found that the renal PTHrP overexpression in rats with FA-induced ARF was abolished by two ACE inhibitors or the AT1 receptor antagonist losartan, even by using low doses of these antagonists, which did not affect tubular cell hyperplasia. These findings indicate that the initial proliferative response of the renal tubule to FA can occur independent of PTHrP, and Ang II antagonists are rather to exert beneficial effects in FA-injured kidney by probably affecting intrarenal hemodynamics.

It is interesting that pretreatment with the Ang II antagonists, in addition to preventing PTHrP overexpression, impaired the PTH1R protein downregulation observed in the acutely damaged rat kidney. Collectively, our findings support the notion that RAS activation is responsible for PTHrP induction and that Ang II blockade leads to normalization of the renal PTHrP/PTH1R system in nephrotoxic ARF.

We found that a nephrotoxic concentration of FA rapidly stimulates PTHrP mRNA in NRK 52E cells by a mechanism inhibited by losartan but not by candesartan, two agents that differ in their cell permeability (24). However, both antagonists inhibited PTHrP induction by exogenous Ang II, which suggests a similar effectiveness in their interaction with AT1 receptors on the cell surface in these cells. It is interesting that losartan also blocked the perinuclear relocalization of Ang II and the AT1 receptor that occurs shortly after FA addition to NRK 52E cells. The kidney accumulates Ang II from the circulation, but it can clearly be synthesized locally within the renal cells and act in an autocrine, paracrine, or even intracrine manner (15,24,35). Regarding the last, intracellular Ang II may accumulate as a result of Ang II/AT1 receptor complex formation in cell endosomes or as a result of its interaction with Ang II nuclear receptors, associated with disease severity in various pathologic conditions (35,36). The aforementioned difference between the efficacy of losartan and candesartan suggests that intracellular Ang II retention might be responsible for the early PTHrP gene induction by FA in renal tubule cells. This is consistent with previous findings in other cell types indicating that intracellular Ang II can increase gene transcription (24). However, both Ang II antagonists were shown to be equally effective in inhibiting PTHrP protein induction triggered by FA, suggesting that an autocrine mechanism may also contribute to this response at a later stage.

Ang II interaction with the AT1 receptor activates the MAPK pathway, leading to CREB activation (25,27,28). In this study, FA rapidly induced an AT1 receptor-mediated increase in CREB activation in NRK 52E cells. We also found herein that FA, in a similar manner to Ang II, induced ERK1/2 phosphorylation in NRK 52E cells. Moreover, inhibition of either ERK or CREB activation abolished FA-induced upregulation of PTHrP in these cells. Collectively, these findings support the hypothesis that in renal tubuloepithelial cells, PTHrP overexpression by FA is coupled to CREB activation through a pathway that requires ERK1/2 phosphorylation.

We recently demonstrated a correlation between the improvement of tubular damage and fibrosis and PTHrP inhibition after AT1 receptor blockade in rats with Ang II-induced renal injury (6). These findings further support the notion that PTHrP upregulation might be related to the mechanisms associated with Ang II-induced kidney injury. It was suggested previously that, in the arterial wall, PTHrP might act locally to antagonize some of the effects of Ang II on vascular smooth muscle cells (4,17,37). Thus, although further work is needed to define the true pathogenetic role of both proteins in ARF, PTHrP might either exert a reciprocal control on or recapitulate at least some Ang II effects in the acutely damaged kidney.

In conclusion, the findings presented here demonstrate that

PTHrP overexpression in nephrotoxic ARF depends on RAS activation. In addition, we have identified a mechanism by which a nephrotoxic concentration of FA, through activation of a local Ang II/AT1 receptor system, can induce PTHrP overexpression in the renal tubule.

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References

- Lieberthal W, Levine JS: Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. *Am J Physiol* 271: F477–F488, 1996
- Humes HD, Lake EW, Liu S: Renal tubule cell repair following acute renal injury. *Miner Electrolyte Metab* 21: 353–365, 1995
- Hammerman MR, Safirstein R, Harris RC, Toback FG, Humes HD: The role of growth factors in the process of renal regeneration and repair. *Am J Physiol* 279: F3–F11, 2000
- Clemens TL, Cormier S, Eichinger A, Endlich K, Fiaschi-Taesch N, Fisher E, Friedman PA, Karaplis AC, Massfelder T, Rossert J, Schüler K-D, Silve C, Stewart AF, Takene K, Helwig JJ: Parathyroid hormone-related protein and its receptors: Nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. *Br J Pharmacol* 134: 1113–1136, 2001
- Massfelder T, Stewart AF, Endlich K, Soifer NE, Judes C, Helwig JJ: Parathyroid hormone-related protein detection and interaction with NO and cyclic AMP in the renovascular system. *Kidney Int* 50: 1591–1603, 1996
- Lorenzo O, Ruiz-Ortega M, Esbrit P, Rupérez M, Ortega A, Santos S, Blanco J, Ortega L, Egido J: Angiotensin II increases parathyroid hormone-related protein (PTHrP) and the type 1 PTH/PTHrP receptor in the kidney. *J Am Soc Nephrol* 13: 1595–1607, 2002
- Soifer NE, Van Why SK, Ganz MB, Kashgarian M, Siegel NJ, Stewart AF: Expression of parathyroid hormone-related protein in the rat glomerulus and tubule during recovery from renal ischemia. *J Clin Invest* 92: 2850–2857, 1993
- Santos S, Bosch RJ, Ortega A, Largo R, Fernández-Agulló T, Gazapo R, Egido J, Esbrit P: Up-regulation of renal parathyroid hormone-related protein in FA-induced ARF: Relationship to the renal regenerative process. *Kidney Int* 60: 982–995, 2001
- Bosch RJ, Rojo-Linares P, Torrecillas-Casamayor G, Iglesias-Cruz MC, Rodríguez-Puyol D, Rodríguez-Puyol M: Effects of parathyroid hormone-related protein on human mesangial cells in culture. *Am J Physiol* 277: E990–E995, 1999
- García-Ocaña A, Galbraith SC, Van Why SK, Yang K, Golovyan L, Dann P, Zager RA, Stewart AF, Siegel NJ, Orloff JJ: Expression and role of parathyroid hormone-related protein in human renal proximal tubule cells during recovery from ATP depletion. *J Am Soc Nephrol* 10: 238–244, 1999
- De Miguel F, García-Cañero R, Esbrit P: Copurification of calcium transport-stimulating and DNA synthesis-stimulating agents with parathormone-like activity isolated from the hypercalcemic strain of the Walker 256 tumor. *Eur J Cancer* 27: 1022–1026, 1991
- García-Ocaña A, De Miguel F, Peñaranda C, Albar JP, Sarasa JL, Esbrit P: Parathyroid hormone-related protein is an autocrine modulator of rabbit proximal tubule cell growth. *J Bone Miner Res* 10: 1875–1884, 1995
- Harris RC, Martínez-Maldonado M: Angiotensin II-mediated renal injury. *Miner Electrolyte Metab* 21: 328–335, 1995
- Mezzano SA, Ruiz-Ortega M, Egido J: Angiotensin II and renal fibrosis. *Hypertension* 38: 635–638, 2001
- Allred AJ, Chappell MC, Ferrario CM, Diz DI: Differential actions of renal ischemic injury on the intrarenal angiotensin system. *Am J Physiol* 279: F636–F645, 2000
- Largo R, Gómez-Garre D, Santos S, Peñaranda C, Esbrit P, Egido J: Renal expression of parathyroid hormone-related protein (PTHrP) and PTH/PTHrP receptor in a rat model of tubulointerstitial damage. *Kidney Int* 55: 82–90, 1999
- Pirola CJ, Wang HM, Kaymar A, Wu S, Enomoto H, Sharifi B, Forrester JS, Clemens TL, Fagin JA: Angiotensin II regulates parathyroid hormone-related protein expression in cultured rat aortic smooth muscle cells through transcriptional and post-transcriptional mechanisms. *J Biol Chem* 268: 1987–1994, 1993
- Piron A, Leonard I, Nonclercq D, Toubeau G, Falmagne P, Heuson-Stiennon JA, Laurent G: In vitro demonstration of a mitogenic activity in renal tissue extracts during regenerative hyperplasia. *Am J Physiol* 274: F348–F357, 1998
- Gómez-Garre D, Largo R, Tejera N, Fortes J, Manzarbeitia F, Egido J: Activation of NF-kappa B in tubular epithelial cells of rats with intense proteinuria: Role of angiotensin II and endothelin-1. *Hypertension* 37: 1171–1178, 2001
- Long GW, Misra DC, Juleff R, Blossom G, Czako PF, Glover JL: Protective effects of enalaprilat against postischemic renal failure. *J Surg Res* 54: 254–257, 1993
- Mimran A, Ribstein J: Angiotensin receptor blockers: Pharmacology and clinical significance. *J Am Soc Nephrol* 10[Suppl]: S273–S277, 1999
- Guillén C, Martínez P, De Gortázar A, Martínez MA, Esbrit P: Both N- and C-terminal domains of parathyroid hormone-related protein increase interleukin-6 by nuclear factor-kappa B activation in osteoblastic cells. *J Biol Chem* 277: 28109–28117, 2002
- Miyajimi A, Chen J, Kirman I: Interaction of nitric oxide and transforming growth factor-beta 1 induced by angiotensin II and mechanical stretch in rat renal tubular epithelial cells. *J Urol* 164: 1729–1734, 2000
- Cook JL, Zhang Z, Re RN: In vitro evidence for an intracellular site of angiotensin action. *Circ Res* 89: 1138–1146, 2001

25. Cammarota M, Bevilacqua LRM, Dunkley PR, Rostas JAP: Angiotensin II promotes the phosphorylation of cyclic AMP-responsive element binding protein (CREB) at Ser133 through an ERK1/2-dependent mechanism. *J Neurochem* 79: 1122–1128, 2001
26. Chilco PJ, Leopold V, Zajac JD: Differential regulation of the parathyroid hormone-related protein gene P1 and P3 promoters by cAMP. *Mol Cell Endocrinol* 138: 173–184, 1998
27. Funakoshi Y, Ichiki T, Takeda K, Tokuno T, Iino N, Takeshita A: Critical role of cAMP-response element-binding protein for angiotensin II-induced hypertrophy of vascular smooth muscle cells. *J Biol Chem* 277: 18710–18717, 2002
28. Bernhard M, Montminy M: Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev* 2: 599–609, 2001
29. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: PD098059 is a specific inhibitor of the activation of mitogen-activated kinase kinase in vitro and in vivo. *J Biol Chem* 270: 27489–27494, 1995
30. Ruiz-Ortega M, Ruperez M, Lorenzo O, Esteban V, Blanco J, Mezzano S, Egido J: Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney. *Kidney Int* 62: S12–S22, 2002
31. Kontogiannis J, Burns KD: Role of AT1 angiotensin II receptors in renal ischemic injury. *Am J Physiol* 43: F79–F90, 1998
32. Abdulkader RC, Yuki MM, Paiva AC, Marcondes M: Prolonged inhibition of angiotensin II attenuates glycerol-induced acute renal failure. *Braz Med Biol Res* 21: 233–239, 1988
33. Sadoshima J: Cytokine actions of angiotensin II. *Circ Res* 86: 1187–1189, 2000
34. Yang CW, Ahn HJ, Kim WY, Shin MJ, Kim SK, Park JH, Kim YO, Kim YS, Kim J, Bang BK: Influence of the renin-angiotensin system on epidermal growth factor expression in normal and cyclosporine-treated rat kidney. *Kidney Int* 60: 847–857, 2001
35. Navar LG, Harrison-Bernard LM, Nishiyora A, Kobari H: Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39: 316–322, 2002
36. Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Jinard B, Anversa P: Myocardial cell death in human diabetes. *Circ Res* 87: 1123–1132, 2000
37. Wu S, Pirola CJ, Green J, Yamaguchi DT, Okano K, Jueppner H, Forrester JS, Fagin JA, Clemens TL: Effects of N-terminal, midregion, and C-terminal parathyroid hormone-related peptides on adenosine 3',5'-monophosphate and cytoplasmic free calcium in rat aortic smooth muscle cells and UMR-106 osteoblast-like cells. *Endocrinology* 133: 2437–2444, 1993