Role of Parathyroid Hormone–Related Protein in Tubulointerstitial Apoptosis and Fibrosis after Folic Acid–Induced Nephrotoxicity

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Parathyroid hormone–related protein (PTHrP) is shortly upregulated in acute renal injury, but its pathophysiologic role is unclear. Investigated was whether PTHrP might act as a profibrogenic factor in mice that do or do not overexpress PTHrP in the proximal tubule after folic acid (FA) nephrotoxicity, a model of acute renal damage followed by partial regeneration and patchy tubulointerstitial fibrosis. It was found that constitutive PTHrP overexpression in these animals conveyed a significant increase in tubulointerstitial fibrosis, associated with both fibroblast activation (as α-smooth muscle actin staining) and macrophage influx, compared with control littermates at 2 to 3 wk after FA damage. Cell proliferation and survival was higher (P < 0.01) in the renal interstitium of PTHrP-overexpressing mice than in control littermates within this period after injury. Moreover, the former mice had a constitutive Bcl-XL protein overexpression.

In vitro studies in renal tubulointerstitial and fibroblastic cells strongly suggest that PTHrP (1-36) (100 nM) reduced FA-induced apoptosis through a dual mechanism involving Bcl-XL upregulation and Akt and Bad phosphorylation. PTHrP (1-36) also stimulated monocyte chemoattractant protein-1 expression in tubuloepithelial cells, as well as type-I procollagen gene expression and fibronectin (mRNA levels and protein secretion) in these cells and renal fibroblastic cells. Our findings indicate that this peptide, by interaction with the PTH1 receptor, can increase tubulointerstitial cell survival and seems to act as a proinflammatory and profibrogenic factor in the FA-damaged kidney.

Received July 7, 2005. Accepted March 6, 2006. Published online ahead of print. Publication date available at www.jasn.org. A.O. and D.R. contributed equally to this work.

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Both dysfunction and loss of tubular epithelial cells play central roles on the mechanisms of kidney damage after ischemic or toxic challenge (1,2). Shortly after renal damage, the tubular epithelium generates proinflammatory mediators that promote the influx of inflammatory cells into the tubulointerstitial space (3). Tubulointerstitial inflammation now is known to contribute to interstitial fibrosis and kidney damage progression (4,5). Apoptosis participates in the initial loss of intrinsic renal cells after acute kidney injury, but it also may provide a balance for the excessive mononuclear cell infiltration and the compensatory tubular hyperplasia in this setting (1,6,7).

Parathyroid hormone (PTH)-related protein PTHrP and the PTH1 receptor (PTH1R) are abundant throughout the renal parenchyma (8,9). In the tubular epithelium, PTHrP has mitogenic features, and its overexpression occurs rapidly in experimental models of several nephropathies (10–12). However, PTHrP overexpression in the renal proximal tubule has proved inefficient in protecting against ischemic or nephrotoxic renal injury in mice (13). On the other hand, recent data support the concept that PTHrP may act as a proinflammatory mediator in various pathophysiologic conditions (14,15). Moreover, angiotensin II (Ang II) infusion in rats was shown recently to induce the renal PTHrP/PTH1R system, associated with tubular damage and fibrosis (16).

Previous in vitro studies have shown that endogenous PTHrP seems to increase tubuloepithelial cell viability after injury (12,17). Moreover, whereas PTHrP can promote cell death in a rat intestinal cell line (18), it inhibits apoptosis in both pancreatic β cells and chondrocytes (19,20). At least in chondrocytes, this antia apoptotic effect seems to involve PTHrP interaction with the PTH1R and also its internalization into the nucleus (20). As stated above, a putative inhibitory effect of PTHrP on renal cell apoptosis might have a significant impact on the pathogenesis of kidney injury.

In this study, we examined the role of PTHrP in folic acid (FA) nephrotoxicity—in which acute renal damage occurs associated with an early upregulation of PTHrP followed by partial regeneration and patchy tubulointerstitial fibrosis.
within several weeks (17,21,22)—in mice that do or do not overexpress PTHrP in the renal proximal tubule. We also explored some of the mechanisms whereby PTHrP might contribute to the temporal changes in tubulointerstitial fibrosis in this model of acute tubular injury.

Materials and Methods

PTHRP-Overexpressing Transgenic Mice

PTHRP-overexpressing transgenic (PTHRP-TG) mice were a gift of Drs. A.F. Stewart and A. García-Ocaña (Department of Endocrinology and Metabolism, University of Pittsburgh School of Medicine, Pittsburgh, PA). These mice were generated by breeding hemizygote mice for two types of constructs: One that consists of a 2.2-kb γ-glutamyl transpeptidase promoter fragment upstream of a tetracycline transactivator fusion protein and another that contains a human PTHrP cDNA placed under the control of a tetracycline operator (13). Transgene-bearing founders were outbred continually to normal CD-1 mice to generate hemizygotes. Genotyping of these mice was carried out by tail DNA PCR. In all of the experiments described below, gender-unselected mice (4 to 8 mo of age), homogeneously distributed among groups, were used. The results that were obtained with PTHrP-TG mice were compared with those that were obtained with control littermates (those that bear either one of the previously mentioned constructs or normal CD-1 mice) (13). Studies were performed with the approval of and in accordance with guidelines established by Institutional Animal Care and Use Committee at Fundación Jiménez Díaz.

Induction of FA Nephrotoxicity

Renal failure was induced by a single intraperitoneal injection of FA (250 mg/kg body wt) in 300 mM sodium bicarbonate (vehicle) (21,23). At different periods (up to 4 wk) after injection of either FA or vehicle (controls), some mice were food deprived and placed in restrictive cages. After 24 h, urine was collected and blood was taken by cardiac puncture under anesthesia. Plasma and urine creatinine were determined by autoanalyzer (Dimension RXL; Dade Behring, Liederbach, Germany). All mice were killed, and the kidneys were harvested. One kidney from each animal in all groups was fixed in 4% p-formaldehyde for histologic studies. The remaining kidney of each animal was homogenized in lysis buffer (250 mM sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, 0.2 mM PMSF, and 1.4 μM aprotinin [pH 7.5]) and stored at −20°C for subsequent analysis.

Histology and Immunohistochemistry

Histologic evaluation by hematoxylin/eosin and staining analysis were performed routinely on serial paraffin-embedded renal tissue sections (2 μm) within the same mouse tissue. Apoptosis was identified by condensed nuclear chromatin and intact cell membranes (24). Alternatively, some tissue samples were incubated with 100 μg/ml RNase A (Sigma, St. Louis, MO) and 2 μg/ml propidium iodide (Sigma) in PBS for 10 min at 37°C in the dark and analyzed by fluorescence microscopy. Apoptotic cell death also was assessed by enzymatic in situ labeling of DNA strand breaks using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) method (In Situ Cell Death Detection Kit; Promega, Madison, WI) in mouse kidney samples.

Immunohistochemistry was performed using previously described protocols (15–17,21,25) and the following antibodies (dilution, -fold): Two rabbit polyclonal antibodies against proliferating cell nuclear antigen (PCNA; Dako, Glostrup, Denmark; 150) or laminin (Neo Markers, Fremont, CA; 100), respectively, and two mAb against the F4/80 antigen in murine monocytes/macrophages (Serotec, Oxford, UK; 50) or α-smooth muscle actin (α-SMA; Sigma; 150), respectively. The tissue sections were rehydrated, and endogenous peroxidase and nonspecific binding were blocked. Then, they were incubated for 30 min at room temperature (PCNA) or overnight at 4°C with the primary antibodies. Sections subsequently were incubated with the corresponding anti-IgG biotinylated-conjugated antibody followed by the avidin-biotin-peroxidase complex (Dako) or a polymer-peroxidase complex (Envision+ System; Dako; PCNA and α-SMA), and 3,3′-diaminobenzidine as chromogen. The sections were counterstained with hematoxylin. Some tissue samples were incubated without the primary antibody, as negative controls.

Staining was evaluated in five to 10 different high-power fields per section that contained at least one glomerulus in four sections from each experimental mouse in a total of three to five mice per group. Tubular and interstitial TUNEL staining was evaluated by counting the number of stained cell nuclei per field in the proximal and distal tubules (identified by morphologic criteria) and in the interstitium, respectively. In the latter compartment, the number of PCNA- and F4/80-positive cells also was counted per field. The percentage of stained area for Masson’s, laminin, and α-SMA was estimated by the following semiquantitative score: 0, no staining; 1, up to 25%; 2, between 25 and 50%; 3, between 50 and 75%; and 4, >75%. All evaluations were performed by two to three independent observers in a blinded manner, and the corresponding mean score value was obtained for each mouse.

Cell Culture Studies

Rat tubulop epithelial NRK-52E (ATCC CRL 1571) and renal fibroblastic NRK-49F (ATCC CRL 1570) cells were grown in DMEM with 10% FBS, 1% nonessential amino acids, and antibiotics in 5% CO₂, at 37°C. Wild-type mouse cortical tubule (MCT) cells or those that constitutively overexpressed the human Bcl-X_L gene (MCT-Bcl-X_L) (23,26) were grown in RPMI 1640 with 10% FBS. For viability studies, subconfluent cells (50,000 cells/cm²) were incubated for 24 h with 10 mM FA—a toxic dose for renal epithelial cells (17,21)—or serum-depleted medium, with or without 100 nM PTHrP (1-36) and/or 1 μM [Asn¹⁰, Leu¹¹, D-Trp¹²] PTHrP (7-34) amide [PTHrP (7-34); Bachem, Bubendorf, Switzerland]. At specific points thereafter, nonadherent cells were collected and pooled with adherent cells (after gentle trypsinization). Total cell numbers and the percentage of cell viability were determined by trypan blue exclusion. Alternatively, cells were stained with the DNA fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (24). The number of nuclei with fragmented or condensed DNA/300 nuclei was taken as an index of apoptosis. For flow cytometry studies, cells were collected, as described above, and incubated in the dark for 1 h at 4°C in 60 μg/ml RNase A, 50 μg/ml propidium iodide, and 0.05% Nonidet P-40 in PBS. FACScan analysis then was performed using LYSIS II software. The percentage of hypodiploid cells, corresponding to apoptotic cells, was calculated on the basis of evaluation of 10,000 cells per experimental condition (23).

Western Blot Analysis

Kidney tissue or cell samples were homogenized in lysis buffer, and protein content was determined by the Bradford method (Pierce, Rockford, IL), using BSA as standard. To analyze Akt and Bad phosphorylation, a phosphatase-inhibitor cocktail (Set II; Calbiochem, San Diego, CA) was added to lysis buffer. Proteins (30 to 60 μg/lane) 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% defatted milk or 5% BSA (only for PTHrP) in PBS or 50 mM...
Tris-HCl (pH 7.5) and 150 mM NaCl (phospho-473Ser-Akt, Akt, phospho-136Ser-Bad, Bad) with 0.05% Tween-20, and incubated overnight at 4°C with the following rabbit polyclonal antibodies (dilution, -fold): Anti-PTHrP antisera C6 (2500) (15,16,21,25), anti-PTH1R Ab-IV (Covance, Berkeley, CA; 1000) (13,25), anti-monoctye chemoattractant protein-1 (MCP-1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 500), anti-Bcl-2 antibody (Santa Cruz Biotechnology; 2000), anti-Bcl-XL antibody (Santa Cruz Biotechnology; 500), anti-Bax antibody (Santa Cruz Biotechnology; 2000), anti-phospho-473Ser-Akt or anti-Akt antibodies (Cell Signaling Technology, Beverly, MA; 1000), anti-phospho-136Ser-Bad (Santa Cruz Biotechnology; 2000), or anti-Bad antibodies (Cell Signaling Technology, Beverly, MA; 1000), anti-phospho-473Ser-Akt or anti-Akt antibodies (Cell Signaling Technology, Beverly, MA; 1000), anti-phospho-136Ser-Bad (Santa Cruz Biotechnology; 2000), or anti-Bad antibodies (Cell Signaling Technology, Beverly, MA; 1000). The efficacy of protein transfer to the membranes was assessed by either α-tubuline or α-actin or by Ponceau S staining. The membranes subsequently were incubated with relevant peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, developed by ECL chemiluminescence (Amersham), and fluorogram bands were quantified by densitometry.

Real-Time PCR
Cell total RNA was isolated with Trizol (Invitrogen, Groningen, The Netherlands), and gene expression was analyzed by real-time PCR (27,28). Rat fibronectin-specific forward and reverse primers and probe were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA), whereas for rat type-1 procollagen, predeveloped TaqMan assay reagents, primers, and probe were used (Rn00584426_m1), as described in detail previously (27,28). 18S rRNA served as housekeeping gene and was amplified in parallel with genes of interest.

Statistical Analyses
All results are expressed as mean ± SEM. Variance analysis followed by Dunnett test was performed to assess the effect of FA on the various parameters that were evaluated throughout the course of study in vivo. Mann-Whitney test was performed to analyze the differences between PTHrP-TG mice and their control littersmates. Correlation between two numerical variables was assessed by coefficient. The effects of the different treatments in vitro were assessed by parametric (Dunnett test) or nonparametric (Kruskal-Wallis test) ANOVA or Mann-Whitney test as appropriate. P < 0.05 was considered significant.

Results
Changes in Renal Function in Mice with FA Nephrotoxicity
We recently reported that both PTHrP-TG mice and their control littersmates exhibited a normal and similar renal function in basal condition and the same levels of renal impairment up to 1 wk after FA injury (13). Three weeks after FA injection, the latter mice showed a complete recovery of the renal function according to the creatinine clearance: 0.14 ± 0.01 versus 0.12 ± 0.01 ml/min in vehicle-treated mice. However, PTHrP-TG mice still showed a dramatic decrease in this parameter (0.04 ± 0.02 ml/min; P < 0.01 versus corresponding value in control littersmates).

Increased Extracellular Matrix Deposition and Interstitial α-SMA Immunostaining in the Kidney of PTHrP-TG Mice with FA Nephrotoxicity
In previous studies (13,17), we found that the kidneys from uninjured PTHrP-TG mice showed normal structure upon light microscopy examination. We also had found that early in FA injury, an increase in PTHrP associated with PTH1R downregulation occurs (13,17,21). We found herein that between 2 and 3 wk after FA, the PTHrP levels remained elevated in the kidney of PTHrP-TG mice, whereas the renal PTHrP/PTH1R system normalizes in control littersmates (Figure 1A). At this time, interstitial fibrotic areas (assessed by Masson’s and laminin staining) that surrounded atrophic tubules were observed in FA-injected animals, which were higher in PTHrP-TG mice. At 4 wk, patchy fibrosis remained elevated in both types of mice, with a tendency to higher values—which did not reach statistical significance—in PTHrP-TG mice (Figure 1, B and C).

Consistent with the aforementioned results on extracellular matrix deposition, we found a significant increase in interstitial staining for α-SMA—a marker of fibroblast activation (5)—in the renal cortex of PTHrP-TG mice by 2 to 3 wk after FA damage. At 4 wk, this staining decreased but was still detectable in both types of mice (Figure 2A).
Increased Interstitial Proliferation and Survival and Macrophage Influx in PTHrP-TG Mice after FA Damage

An increased number of PCNA-positive cells was observed in the renal interstitium of control and PTHrP-TG mice after FA injury or V injection. PCNA positivity in the kidney of representative control and PTHrP-TG mice after V or FA injection at 2 wk is shown. PCNA-positivity score values are mean ± SEM of four mice per group at each period. Positivity for proliferating cell nuclear antigen (PCNA) in the renal cortical interstitium (left) and for terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (right) in the latter (B) and in the proximal and distal tubules (C) of control and PTHrP-TG mice at different periods after FA injury. Staining score values are mean ± SEM of four to six mice per group at each period. *P < 0.01, **P < 0.05 versus corresponding V-treated value; aP < 0.01, bP < 0.05 versus corresponding control value. Magnification, ×100.

Figure 2. (A) Time course of changes in α-smooth muscle actin (α-SMA) immunostaining in the renal interstitium of control (□) and PTHrP-TG (■) mice after FA injury or V injection. α-SMA positivity in the kidney of representative control and PTHrP-TG mice after V or FA injection at 2 wk is shown. α-SMA-positivity score values are mean ± SEM of four mice per group at each period. Positivity for proliferating cell nuclear antigen (PCNA) in the renal cortical interstitium (left) and for terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (right) in the latter (B) and in the proximal and distal tubules (C) of control and PTHrP-TG mice at different periods after FA injury. Staining score values are mean ± SEM of four to six mice per group at each period. *P < 0.01, **P < 0.05 versus corresponding V-treated value; aP < 0.01, bP < 0.05 versus corresponding control value. Magnification, ×100.

Increased Interstitial Proliferation and Survival and Macrophage Influx in PTHrP-TG Mice after FA Damage

An increased number of PCNA-positive cells was observed in the renal interstitium of PTHrP-TG mice, compared with that in control littermates, within 1 to 2 wk after nephrotoxic injury (Figure 2B, left). In the former mice, a decrease in TUNEL-positive interstitial cells also was found at this stage after FA (Figure 2B, right). This decrease might reflect, at least in part, an increased clearance of these cells as a result of an enhanced macrophage infiltration (29). In fact, in the renal interstitium, the number of F4/80-positive cells was significantly higher in PTHrP-TG mice than in their control littermates at 1 to 3 wk after FA (Figure 3A). Moreover, there was a significant correlation between this number and that of TUNEL-positive cells (r = −0.788, P < 0.01) but also between the latter and the score values for α-SMA (r = −0.810, P < 0.05) in these animals. Therefore, a prolonged survival of interstitial fibroblasts seems to occur associated with renal PTHrP overexpression and fibrosis in mice. In addition, we found that PTHrP (1-36) can stimulate MCP-1 protein in tubuloepithelial cells NRK-52E after stimulation with 100 nM PTHrP (1-36). Representative autoradiograms corresponding to Western blot analysis of MCP-1 and α-actin, a constitutive control, in these cells’ protein extracts are shown. Values are mean ± SEM from at least two independent experiments in duplicate. *P < 0.01 versus basal value (100%). Magnification, ×200.

Figure 3. (A) Changes in interstitial F4/80 immunostaining in the renal cortex of control and PTHrP-TG mice after FA injury or V injection. F4/80 staining in representative control (□) and PTHrP-TG (■) mice at 2 wk is shown (top). F4/80 positivity score values are mean ± SEM of four to eight mice per group at each period. *P < 0.01 versus corresponding V-treated value; aP < 0.01, bP < 0.05 versus corresponding control value. (B) Monocyte chemoattractant protein-1 (MCP-1) protein expression in tubuloepithelial cells NRK-52E after stimulation with 100 nM PTHrP (1-36). Representative autoradiograms corresponding to Western blot analysis of MCP-1 and α-actin, a constitutive control, in these cells’ protein extracts are shown. Values are mean ± SEM from at least two independent experiments in duplicate. *P < 0.01 versus basal value (100%). Magnification, ×200.
Bcl-X<sub>L</sub> Is Overexpressed in the Kidney of PTHrP-TG Mice

We next determined the expression of various cell death–related proteins in the mouse kidney at different times after FA injury by Western blot. It is interesting that we found that PTHrP-TG mice showed basal overexpression of the antiapoptotic protein Bcl-X<sub>L</sub>, which remained unchanged after FA injury (Figure 4A). However, in these mice, the basal protein levels of either Bax or Bcl-2 (a pro- and antiapoptotic protein, respectively [30]) were similar to those in control littermates (Figure 4A, top). In the latter mice, FA induced an increase in both Bcl-X<sub>L</sub> and Bax and a decrease in Bcl-2 protein levels at 72 h, which normalized at 2 wk (Figure 4A, bottom). In PTHrP-TG mice, however, changes in both Bax and Bcl-2 proteins occurred accordingly to those in their control littermates.

To confirm that renal PTHrP overexpression was responsible for the observed Bcl-X<sub>L</sub> upregulation and tubulointerstitial changes in PTHrP-TG mice, doxycycline (20 mg/L) was administered (in the drinking water) to these mice to reverse PTHrP overexpression (13). At 1 wk after this administration, renal Bcl-X<sub>L</sub> protein overexpression in PTHrP-TG mice decreased to normal levels (Figure 4B, top). In these animals, doxycycline treatment for an extended period of 1 wk after FA changed the number of PCNA-positive cells or cells that underwent apoptosis in the renal interstitium to levels that were indistinguishable from those in normal mice (Figure 4B, bottom).

**Figure 4.** (A) Evolution of apoptosis-related proteins in the kidney of control and PTHrP-TG mice after FA damage. Representative autoradiograms corresponding to Western blot analysis of these proteins in basal conditions are shown (top). Relative densitometric values are mean ± SEM of four to six mice per group at each period versus those of V-treated mice. *P < 0.01, **P < 0.05 versus V value (100%); *P < 0.01 versus corresponding values in control mice at the same point. (B) Effect of doxycycline treatment on Bcl-X<sub>L</sub> protein expression and interstitial proliferation and apoptosis in the kidney of PTHrP-TG mice. Basal Bcl-X<sub>L</sub> protein expression (by Western blot) and renal interstitial cell proliferation (PCNA) and apoptosis (by propidium iodide) at 1 wk of FA injury were evaluated in the kidney of control mice (■) and PTHrP-TG mice that were treated (▲) or not treated (□) with doxycycline (20 mg/L). Relative intensities of Bcl-X<sub>L</sub> signals are indicated at the top (top). Experimental intensities of Bcl-X<sub>L</sub> signals are mean ± SEM of four to six mice per group. *P < 0.01, **P < 0.05 versus corresponding V-treated value; †P < 0.01, ‡P < 0.05 versus corresponding PTHrP-TG value.

**PTHRP (1-36) Is a Survival Factor for Renal Tubuloepithelial and Fibroblastic Cells**

To assess further whether PTHrP may act as a survival factor in renal tubulointerstitial cells in vitro, we used NRK-52E cells, a rat tubuleepithelial cell line of proximal tubule origin (17,31), which shows a growth response to PTHrP (17), and NRK-49F, a rat renal fibroblast cell line [32]. Treatment of serum-deprived NRK-52E cells with 100 nM PTHrP (1-36) for 48 h significantly decreased the number of apoptotic cells—assessed by DAPI staining—from 53 ± 7 to 16 ± 4% (P < 0.05, n = 4); similar to 13 ± 8% in cells that were grown in 10% FBS-containing medium. Moreover, flow cytometry indicated that the increase in apoptotic hypodiploid cells' number by 10 mM FA was decreased by PTHrP (1-36) in a dose-dependent manner (Figure 5A). However, the percentage of NRK-52E cells that underwent apoptosis upon addition of FA was unaffected by this peptide in the presence of 1 μM PTHrP (7-34), a PTH1R antagonist (33): 22% (FA alone) or 26% (FA + both peptides). This was confirmed by using trypan blue staining to assess cell viability (Figure 5B, left). In addition, we found that PTHrP (1-36) increased the survival of FA-treated NRK-49F cells, an effect that also was inhibited by PTHrP (7-34) (Figure 6A, left).

Activation of caspases has been shown to have a role in cisplatin-induced nephrotoxicity and other renal injuries (6,7,34). We found that the pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (zVAD-fmk; Calbiochem, San Diego, CA), at 50 μM, significantly inhibited the effect of 10 mM FA on cell death in both NRK-52E and -49F cells, in the presence or absence of 100 nM PTHrP (1-36) (Figures 5B and 6A, right).

**PTHRP (1-36) Induces Bcl-X<sub>L</sub> Protein Expression in Both NRK-52E and -49F Cells**

To confirm that Bcl-X<sub>L</sub> protein is in fact a target for PTHrP antiapoptotic action in the kidney, as suggested by our in vitro findings, we performed further experiments in renal cells in vivo. In both NRK-52E and -49F cells, FA significantly reduced the Bcl-X<sub>L</sub>/Bax protein ratio (by decreasing the former and increasing the latter) within 24 h. However, PTHrP (1-36), at 100 nM, markedly increased Bcl-X<sub>L</sub> protein expression and the Bcl-X<sub>L</sub>/Bax protein ratio in the presence of FA in both cell lines; an effect abrogated by 1 μM PTHrP (7-34) (Figures 5C and 6B).
Figure 5. PTHrP (1-36) decreases apoptosis that was induced by FA toxicity in tubuloepithelial cells NRK-52E. (A) Apoptosis was assessed by flow cytometry after cell incubation with 10 mM FA and various PTHrP (1-36) doses (or saline V) for 24 h. (B) The stimulatory effect of PTHrP (1-36) at 100 nM on cell viability (estimated by trypan blue) was abrogated by either 1 mM PTHrP (7-34) (left) or the caspase pan-inhibitor Z-VAD-fmk (at 50 μM) (right). The inhibitors were added 1 h before FA and PTHrP (1-36). (C) PTHrP (1-36) at 100 nM stimulates the Bcl-XL/Bax protein ratio that was decreased by FA in NRK-52E cells; this effect was abrogated by 1 mM PTHrP (7-34). Values are mean ± SEM from at least three independent experiments in triplicate. *P < 0.01 versus nonstimulated basal value and/or Z-VAD-fmk alone; †P < 0.01 versus FA- and/or PTHrP (7-34)-treated values.
A Dual Mechanism Is Responsible for the Antiapoptotic Effect of PTHrP (1-36) in Renal Tubuloepithelial Cells

We next determined whether changes in Bcl-XL protein indeed was critical for the effect on cell survival that was induced by PTHrP (1-36) in renal tubuloepithelial cells. We used MCT cells, which show a proliferative response to this peptide (35) and in which protection from apoptosis can be conferred by constitutive expression of Bcl-XL (26). MCT cells that did or did not overexpress Bcl-XL protein were exposed to 10 mM FA for 24 h, in the presence or absence of PTHrP (1-36). Bcl-XL overexpression resulted in decreased cell death that was induced by FA (Figure 7A). However, the presence of PTHrP (1-36) improved cell viability similarly in MCT cells that did or did not overexpress Bcl-XL (Figure 7A), suggesting that its effect on cell survival, at least in these tubuloepithelial cells, is not fully accounted for by an increase in Bcl-XL.

Another proapoptotic Bcl-2 family member, Bad, has the ability to bind directly to Bcl-XL, blocking its survival function (36). The pro-death activity of Bad depends on its phosphorylated status, controlled by phosphoinositol 3-kinase (PI3-K)-mediated Akt phosphorylation (36,37). We found herein that LY294002 (Sigma; Figure 7A) or wortmannin (data not shown), two PI3-K inhibitors (37,38), significantly inhibited the stimulatory effect of PTHrP (1-36) on cell survival in both wild-type and Bcl-XL–overexpressing MCT cells after FA incubation. Furthermore, PTHrP (1-36), at 100 nM, rapidly and transiently stimulated Akt and Bad phosphorylation at Ser (473) and Ser (136), respectively (without significantly affecting the respective total protein levels) in MCT cells (Figure 7B).

Figure 7. The effect of PTHrP (1-36) on cell survival that was decreased by FA in tubuloepithelial cells is related to phosphoinositol 3-kinase (PI3-K) pathway activation. (A) Subconfluent mouse cortical tubule (MCT) cells that did (■) or did not (□) overexpress Bcl-XL were treated with 10 mM FA for 24 h, in the presence or absence of PTHrP (1-36) (at 100 nM) and/or the PI3-K inhibitor LY294002 (at 10 μM). The inhibitor was added 1 h before FA and PTHrP (1-36). Cell viability was determined by trypan blue. Values are mean ± SEM from three independent experiments in triplicate. *P < 0.01 versus corresponding nonstimulated basal value; **P < 0.01 versus corresponding FA value in wild-type MCT cells; *P < 0.01 versus corresponding FA value; *P < 0.01 versus corresponding FA + PTHrP (1-36) values. (B) PTHrP (1-36) stimulates 473Ser-Akt and 136Ser-Bad phosphorylation in MCT cells. Wild-type MCT cells were serum-depleted for 24 h, and then fresh medium that contained this peptide (at 100 nM; or not; basal) was added. At different time points, cell extracts were obtained for Western blot analysis of phospho-473Ser-Akt and phospho-136Ser-Bad and total Akt and Bad (as internal controls).

Figure 6. PTHrP (1-36) increases cell viability, which was decreased by FA in renal fibroblastic cells NRK-49F. Subconfluent cells were stimulated with the agonists for 24 h, and then cell viability was assessed by trypan blue. PTHrP (1-36) and FA were used at 100 nM and 10 mM, respectively. (A) The stimulatory effect of PTHrP (1-36) on cell viability was abrogated by either 1 μM PTHrP (7-34) (left) or the caspase pan-inhibitor Z-VAD-fmk (at 50 μM; right). The inhibitors were added 1 h before FA and PTHrP (1-36). (B) PTHrP (1-36) stimulates the Bcl-XL/Bax protein ratio that was decreased by FA in NRK-49F cells; this effect was abrogated by 1 μM PTHrP (7-34). Values are mean ± SEM from at least three independent experiments in triplicate. *P < 0.01 versus nonstimulated basal value and/or Z-VAD-fmk alone; aP < 0.01 versus corresponding FA value; bP < 0.01 versus corresponding FA- and PTHrP (1-36)-treated values.

PTHRP (1-36) Induces a Fibrogenic Phenotype in Both NRK-52E and -49F cells

We further explored in vitro the hypothesis that PTHrP may contribute to the process of fibrogenesis. PTHrP (1-36), at 100
nM, was found to increase α-SMA protein expression in NRK-49F at 48 h, an effect that was inhibited by 1 μM PTHrP (7-34) (Figure 8A). In addition, PTHrP (1-36) at the same concentration increased the gene expression of the extracellular matrix proteins type-1 procollagen and fibronectin (27) and also the release of the latter protein into the cell-conditioned medium in these cells and NRK-52E cells (Figure 8, B and C).

Discussion

PTHrP has growth-regulatory features in tubulointerstitial cells (11,12,17,35), but current evidence does not support that it has a significant role in the renal regenerative process after acute kidney injury: (1) The PTH1R gene is downregulated rapidly, and targeted delivery of PTHrP to the proximal tubule does not provide protection in this setting (13,17,39); and (2) renal PTHrP upregulation but not tubular hyperplasia in the FA-injured kidney can be prevented by Ang II blockers (21). However, PTHrP seems to be involved in the mechanisms that are associated with Ang II–induced renal injury (16,21). Furthermore, PTHrP overexpression correlates with the development of proteinuria in rats with tubulointerstitial damage that was induced by protein overload and in an experimental mouse model of diabetic nephropathy (25,40).

To investigate further the putative role of PTHrP in the mechanism(s) of renal damage, we used herein previously characterized PTHrP-TG mice (13). Although their early response to either ischemic or FA-induced acute renal injury was similar to that of their control littermates (13), we have extended our initial period of study to several weeks after FA damage in both types of mice. Three weeks after FA injection, PTHrP-TG mice showed a significant impairment of renal function; meanwhile, it was back to normal values in control littermates. As expected (22), focal areas of tubulointerstitial fibrosis (as assessed by changes in Masson’s, laminin, and α-SMA staining) were evident in control mice within this period after FA, but they were significantly higher in PTHrP-TG mice. These in vivo findings were supported further by our in vitro data demonstrating that PTHrP (1-36) can induce α-SMA in a renal fibroblastic cell line and stimulates type-1 procollagen and fibronectin expression in this cell line and in tubulointerstitial cells. Collectively, these results suggest that PTHrP seems to act as a fibrogenic mediator in FA nephrotoxicity.

Apoptosis was shown recently to be an important component of the acute response of the tubular epithelium to FA injury in mice (23). Whereas apoptosis of tubular cells after an acute renal insult is considered to be deleterious by facilitating tubular atrophy, apoptosis of interstitial cells may be a mechanism to prevent fibrogenesis (1,6). We found that PTHrP overexpression and fibrosis were associated with an imbalance of apoptosis and cell proliferation in favor of the latter, which will result in a net increase of interstitial fibroblasts in the mouse kidney after FA injury. Our in vivo and in vitro findings also suggest that, in this setting, PTHrP can promote macrophage influx by directly increasing the synthesis of MCP-1 in tubulointerstitial cells. This is consistent with previous studies showing that PTHrP also is able to induce MCP-1 synthesis in smooth muscle cells, whereby it might promote monocye recruitment in human atherosclerotic plaques (15,41,42). Because macrophage infiltration is closely related to tubulointerstitial fibrosis (5), our findings also suggest that PTHrP may act directly as a fibrogenic mediator in FA nephrotoxicity.

These results also demonstrate that PTHrP may act directly as a survival factor for tubulointerstitial cells. Therefore, constitutive upregulation of Bcl-XL was found to occur in the kidney of PTHrP-TG mice, and this upregulation as well as the proliferative and antiapoptotic response of renal interstitial cells to FA were prevented by doxycycline-induced reversal of
PTHRp overexpression in these mice. Moreover, our in vitro data indicate that PTHrP (1–36) increases cell survival that is associated with an increase in Bcl-XL/Bax ratio in tubulointerstitial and renal fibroblastic cells that are treated with the nephrotoxin. Both effects were abolished by a PTH1R antagonist, suggesting that they might be mediated through this receptor in these cells. In addition, a pan-caspase inhibitor dramatically reduced the FA-induced decrease in cell survival, in the presence or absence of PTHrP (1–36), in both renal cell types, further supporting the antiapoptotic effect of this peptide. However, PTHrP (1–36) was equally efficient in protecting MCT cells that did or did not overexpress Bcl-XL from FA injury, suggesting that PTHrP might affect a mechanism downstream of Bcl-XL protein to increase tubulointerstitial cell survival. Our results suggest that such a mechanism is likely to involve activation of the PI3-K/Akt/Bad pathway (36–38).

Conclusion

This study demonstrates that the overall effects of PTHrP seem to be proinflammatory and profibrogenic in the FA-damaged kidney. Collectively, our findings support the hypothesis that PTHrP might contribute to the progression of renal damage in nephrotoxic renal injury by complex mechanisms: Increasing tubulointerstitial cell survival and inducing macrophage infiltration and fibroblastic activation.

Acknowledgments

This work was supported by grants from the Spanish Ministerio de Ciencia y Tecnología (SAF 2002-04356-C02-02), Comunidad Autónoma de Madrid (CAM) (CAM 08.6/0038.1/2000-2 and GR/SAL/0415/2004), Instituto de Salud Carlos III (C03/08), FIS (PI020513), and Spanish Society of Nephrology. A.O. and J.A.A. were supported by Conchita Rábago Foundation and D.R. and V.E. by “Ikerketa eta Prestakuntza Programa” from the Basque Government and Fondo de Investigación Sanitaria (FIS), respectively. A.O. is currently a recipient of a research contract from “Juan de la Cierva” Program from the Spanish Ministry of Education and Science.

Portions of this study were presented at the Acute Renal Failure Satellite Symposium, June 13 to 15, 2003, Ghent, Belgium; and the XLI Congress of the European Renal Association, May 15 to 18, 2004, Lisbon, Portugal.

We thank A.F. Stewart and A. García-Ocaña for critical reading of the manuscript and L.M. Blanco-Colio (Vascular and Renal Research Laboratory, Fundación Jiménez Díaz) for kindly supplying MCT-Bcl-X(L) cells.

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