Angiotensin II induces apoptosis in renal proximal tubular cells

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Angiotensin II induces apoptosis in renal proximal tubular cells. Am J Physiol Renal Physiol 284: F955–F965, 2003; 10.1152/ajprenal.00246.2002.—ANG II has been demonstrated to play a role in the progression of tubulointerstitial injury. We studied the direct effect of ANG II on apoptosis of cultured rat renal proximal tubular epithelial cells (RPTECs). ANG II promoted RPTEC apoptosis in a dose- and time-dependent manner. This effect of ANG II was attenuated by anti-transforming growth factor (TGF)-β antibody. Moreover, TGF-β triggered RPTEC apoptosis in a dose-dependent manner. ANG II also enhanced RPTEC expression of Fas and Fas ligand (FasL); furthermore, anti-FasL antibody attenuated ANG II-induced RPTEC apoptosis. In addition, ANG II increased RPTEC expression of Bax, a cell death protein. Both ANG II type 1 (AT1) and type 2 (AT2) receptor blockers inhibited RPTEC apoptosis. SB-202190, an inhibitor of p38 MAPK phosphorylation, and caspase-3 inhibitor also attenuated ANG II-induced RPTEC apoptosis. ANG II enhanced RPTEC heme oxygenase (HO)-1 expression. Interestingly, pretreatment with hemin as well as curcumin (inducers of HO-1) inhibited the ANG II-induced tubular cell apoptosis; conversely, pretreatment with zinc protoporphyrin, an inhibitor of HO-1 expression, promoted the effect of ANG II. These results suggest that ANG II-induced apoptosis is mediated via both AT1 and AT2 receptors through the generation of TGF-β, followed by the transcripion of cell death genes such as Fas, FasL, and Bax. Modulation of tubular cell expression of HO-1 has an inverse relationship with the ANG II-induced tubular cell apoptosis.

Bax; Bel-2; Fas; Fas ligand; proximal tubular epithelial cells; heme oxygenase-1

Angiotensin II has been demonstrated to contribute to the progression of renal injury through its hemodynamic effects (35, 36). These effects are confirmed by blocking its production and receptor sites (15, 16, 20). However, apart from its hemodynamic effects, the direct effects of ANG II on kidney cells are being increasingly recognized (2, 6, 9, 25, 27). It has been demonstrated that in addition to circulating ANG II, tissue (intrarenal) generation of ANG II is also important for its net effect (35).

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jury, induction of HO-1 has been demonstrated to provide protection from ongoing injury (8, 10, 17, 23, 31). In addition to the model of ANG II-induced tubular cell injury, enhanced tubular cell HO-1 expression has been shown to confer protection against cisplatin-induced tubular cell injury (16, 28).

In the present study, we evaluated the effect of ANG II on the apoptosis of cultured rat proximal tubular cells. We also studied the molecular mechanisms involved and the possible relationship between HO-1 expression and ANG II-induced tubular cell apoptosis.

MATERIALS AND METHODS

Renal Proximal Tubular Epithelial Cell Cultures

Rat proximal renal tubular epithelial cells (NRK-52E) were obtained from American Type Culture Collection, Rockville, MD. Cells were grown in DMEM (GIBCO, Grand Island, NY) containing 2% penicillin-streptomycin, 1% HEPES, 1.5 g NaHCO3, 2 mM l-glutamine, 1 mM sodium pyruvate, and 10% FCS. In the experimental protocols, cells were incubated in media containing 1% FCS.

Apoptotic Studies

Rat and human renal proximal tubular epithelial cells (RPTECs; HK-2) were treated under control and experimental conditions as indicated. At the end of incubation period, cells were stained with H-33342 and propidium iodide and evaluated for apoptosis as described previously (29, 30). In these studies, observers were blinded to experimental conditions.

Detection of tubular cell apoptosis by gel electrophoresis. This is a simple method that is specific for isolation and confirmation of DNA fragments from apoptotic cells (14). Because this method only picks up DNA fragments, one will not visualize any loading of samples that do not contain DNA fragments. RPTECs were treated under control and experimental conditions as indicated, and DNA was extracted and electrophoresed as described previously (29, 30).

Superoxide Assay

Equal numbers of RPTECs were plated in 100-mm petri dishes and grown to subconfluence. The cells were washed twice with normal saline and incubated in serum- and phenol red-free media containing either buffer, 10−8 M ANG II, or 1 μg/ml anti-TGF-β antibody or ANG II+anti-TGF-β antibody at 37°C for a 2-h period. Supernatants were collected into precooled microcentrifuge test tubes at 0, 30, 45, 60, and 120 min. A superoxide assay was subsequently carried out. In brief, 50 μl of each supernatant were pipetted into a 96-well plate, kept on ice, and mixed with 100 μl of phenol red washing solution containing 140 mM NaCl, 10 mM potassium phosphate buffer (pH 7.0), 5.5 mM dextrose, 0.1 g/l phenol red, 8.5 units/ml horseradish peroxidase, and 100 ng/ml phorbol myristate acetate. Incubation was carried out at 37°C for 45, 90, and 150 min and terminated by the addition of 10 μl of 1 M NaOH. Calorimetric reading was done in an ELISA microplate reader at 620 nm. Values are plotted against a standard curve generated from known concentrations of H2O2. Results are expressed in arbitrary units, and experiments were repeated four times, each in triplicate.

Protein Extraction and Western Blot Analysis

RPTECs were treated under control and experimental conditions as indicated. At the end of the incubation period, the cells were washed three times with PBS, scraped into a modified RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 0.1% SDS, 10 μl of protease inhibitor cocktail/ml of buffer, and 100 μg/ml of PMSF) and transferred, using a syringe fitted with a 21-gauge needle, into a microcentrifuge tube. The cell lysates were centrifuged at 15,000 g for 30 min at 4°C. The supernatant was analyzed for total protein content. Twenty micrograms of protein were heated at 100°C for 10 min, loaded, and separated on a 12% PAGE gel under nonreducing conditions. The proteins were electrotransferred to a nitrocellulose membrane in transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS, and 20% methanol at 4°C overnight. Nonspecific binding to the membrane was blocked for 1 h at room temperature with blocking buffer (0.5% BSA in PBS with 0.1% Tween 20). The membrane was then incubated for 16 h at 4°C with primary antibodies [mouse monoclonal anti-Bax antibody, Pharmingen, San Diego, CA; goat polyclonal anti-Bcl-2 antibody, rabbit polyclonal anti-Fas antibody, Santa Cruz Biotechnology, Santa Cruz, CA; mouse monoclonal anti-Fas ligand (FasL) antibody, Pharmingen; and mouse monoclonal anti-HO-1 antibody (Abcam, Cambridge, MA)] in blocking buffer, followed by incubation for 1 h at room temperature with the appropriate secondary antibody in blocking buffer. Signals were visualized by an enhanced chemiluminescence detection kit (Pierce) after exposure to X-ray film (Eastman Kodak, Rochester, NY) (29, 30). To determine loading, blots were stripped and reprobed for β-actin. Quantitative densitometry was performed on the identified bands by using a computer-based measurement system (IS-1000 Digital Imaging System, Alp Innotech, San Leandro, CA).

Statistical Analysis

Statistical analysis was performed using GraphPad Instat software. A Newman-Keuls multiple comparison test was used, and P values were calculated.

RESULTS

Studies Pertaining to Apoptosis

To study the dose-response effect of ANG II on RPTEC apoptosis, equal numbers of cells (10,000 cells/well, 24-well plates) were incubated in media containing either buffer (control) or 10−12 to 10−6 M ANG II for 16 h at 37°C. Subsequently, cells were evaluated for...
apoptosis and necrosis. Four series of experiments were carried out. ANG II induced apoptosis in RPTECs in a dose- and time-dependent manner (Fig. 1, A and B). ANG II induced a mild degree of necrosis in tubular cells (control, 1.2 ± 0.8%; 10^{-8} M ANG II, 3.5 ± 1.5%; 10^{-6} M ANG II, 4.5 ± 1.8% necrosed cells/field).

To confirm the occurrence of apoptosis, equal numbers of RPTECs were incubated in 100-mm petri dishes with media containing either buffer (control) or 10^{-8} and 10^{-6} M ANG II for 16 h. Subsequently, DNA was isolated and electrophoresed. In gel electrophoresis, DNA isolated from ANG II-treated RPTECs showed a classic ladder pattern, thus confirming the occurrence of apoptosis (Fig. 1C).

To evaluate whether this effect of ANG II was species specific, we evaluated the effect of ANG II on human proximal tubular cells (HK-2). Equal numbers of cells were incubated in media containing either buffer or 10^{-8} and 10^{-6} M ANG II for 16 h. Subsequently, cells were stained for apoptosis. ANG II promoted HK-2 cell apoptosis (control, 1.5 ± 0.5%; 10^{-8} M ANG II, 15.5 ± 1.2%; 10^{-6} M ANG II, 25.5 ± 2.0%; P < 0.001). These findings suggest that the apoptotic effect of ANG II is not species specific.

Studies Pertaining to AT_{1} and AT_{2} Receptors

To evaluate the role of AT_{1} and AT_{2} receptors in the induction of apoptosis, equal numbers of RPTECs were incubated in media containing vehicle (control), the AT_{1} inhibitor losartan (10^{-8} M, Sigma, St. Louis, MO), or the AT_{2} inhibitor PD-123319 (10^{-6} M, Sigma) with or without 10^{-8} or 10^{-7} M ANG II for 16 h. Subsequently, cells were stained for apoptosis. Four series of experiments were carried out. As shown in Fig. 2A, both losartan and PD-123319 inhibited ANG II-induced RPTEC apoptosis. This effect of ANG II receptor blockers was also confirmed by DNA gel electrophoresis (Fig. 2B).

Studies Pertaining to the Role of TGF-β

To determine whether the effect of ANG II is mediated through TGF-β, equal numbers of cells were incubated in media containing either vehicle (control) or 1 μg/ml anti-TGF-β antibody (Santa Cruz Biotechnology) with or without 10^{-8} M ANG II for 16 h. Subsequently, cells were prepared for DNA fragmentation assay. In parallel experiments, equal numbers of cells were treated with either vehicle (control) or 1 μg/ml anti-TGF-β antibody with or without 10^{-8} or 10^{-6} M ANG II for 16 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live, apoptosed, and necrosed cells was recorded. Values are means ± SE of 4 sets of experiments, each carried out in triplicate. *P < 0.001 compared with control and 10^{-8} M ANG II. **P < 0.001 compared with 10^{-10} M ANG II. ***P < 0.001 compared with 10^{-6} M ANG II. *P < 0.01 compared with 10^{-8} M ANG II. 

Fig. 1. A: dose-response effect of ANG II on rat proximal tubular epithelial cell (RPTEC) apoptosis. Equal nos. of cells were incubated in media containing either buffer (control) or 10^{-12} and 10^{-8} M ANG II for 16 h. At the end of the incubation period, cells were stained for apoptosis and necrosis. Values are means ± SE of 4 sets of experiments, each carried out in triplicate. *P < 0.001 compared with control and 10^{-12} M ANG II. **P < 0.001 compared with 10^{-10} M ANG II. ***P < 0.001 compared with 10^{-6} M ANG II. B: time course effect of ANG II on RPTEC apoptosis. Equal nos. of cells were incubated in media containing either buffer (control) or 10^{-8} M ANG II for variable time periods (6, 12, 18, and 24 h). At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live, apoptosed, and necrosed cells was recorded. Values are means ± SE of 4 series of experiments, each carried out in triplicate. *P < 0.001 compared with respective controls. **P < 0.001 compared with ANG II (6 h). ***P < 0.001 compared with ANG II (6 and 12 h). ****P < 0.001 compared with ANG II (6–16 h). C: representative gel showing the effect of ANG II on tubular cell apoptosis. Equal nos. of RPTECs were incubated in media containing either buffer (control) or 10^{-8} and 10^{-6} M ANG II for 16 h. At the end of the incubation period, cells were lysed, and DNA was extracted and run on gel electrophoresis. Lane 1, molecular marker; lane 2, control; lanes 3 and 4, 10^{-8} and 10^{-6} M ANG II-treated cells, respectively.
Fig. 2. A: effect of ANG II type 1 (AT₁) and type 2 (AT₂) receptor antagonists on ANG II-induced tubular cell apoptosis. Equal nos. of RPTECs were incubated in media containing vehicle (control), 10⁻⁶ M losartan (LOS), or 10⁻⁶ M PD-123319 (PD) with or without 10⁻⁶ or 10⁻⁷ M ANG II for 16 h. At the end of the incubation period, cells were stained for apoptosis. Values are means ± SE of 4 sets of experiments, each carried out in triplicate. *P < 0.001 compared with control, ANG II+LOS, ANG II+PD, and ANG II+LOS+PD. **P < 0.05 compared with ANG II+LOS+PD and control. B: representative gel showing the effect of ANG II on tubular cell DNA fragmentation. Equal nos. of RPTECs were incubated in media containing either buffer (control) or 10⁻⁶ or 10⁻⁷ M ANG II with or without losartan or PD-123319 (cells were pretreated with either losartan or PD-123319 for 60 min before addition of ANG II) for 16 h. At the end of the incubation period, cells were lysed, and DNA was extracted and run on gel electrophoresis. Lanes 1 and 7, molecular markers; lane 2, control; lanes 3 and 4, 10⁻⁶ and 10⁻⁷ M ANG II-treated cells, respectively; lanes 5 and 6, 10⁻⁶ M ANG II+losartan and 10⁻⁷ M ANG II+PD-123319, respectively. C: representative gel showing the effect of anti-Transforming growth factor (TGF)-β antibody on ANG II-induced tubular cell DNA fragmentation. Equal nos. of RPTECs were incubated in media containing either buffer (control), 10⁻⁶ M ANG II, or anti-TGF-β antibody+ANG II for 16 h. Subsequently, DNA was extracted and run on gel electrophoresis. Lane 1, molecular marker; lane 2, control; lanes 3 and 4, 10⁻⁶ M ANG II- and anti-TGF-β antibody+ANG II-treated cells, respectively. D: effect of anti-TGF-β antibody (Ab) on ANG II-induced apoptosis. Equal nos. of cells were incubated in media containing vehicle (control); CONT) or 1 μg/ml anti-TGF-β antibody (Pharmingen) with or without 10⁻⁶ or 10⁻⁷ M ANG II for 16 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live, apoptosed, and necrosed cells was recorded. Values are means ± SE of 4 sets of experiments, each carried out in triplicate. *P < 0.001 compared with respective controls. ***P < 0.001 compared with 10⁻⁶ M ANG II without anti-TGF-β antibody. +++P < 0.01 compared with 10⁻⁶ M ANG II without anti-TGF-β antibody.

ANG II for 16 h. Subsequently, cells were stained for apoptosis. Four sets of experiments were carried out. As shown in Fig. 2, C and D, anti-TGF-β antibody inhibited ANG II-induced RPTEC apoptosis.

To evaluate the effect of TGF-β1 on RPTEC apoptosis, equal numbers of RPTECs were incubated in media containing either buffer (control) or variable concentrations of TGF-β1 (50–2,000 pg/ml; Collaborative Biomedical Products, Bedford, MA) for 16 h. Subsequently, cells were stained for apoptosis. Eight sets of experiments were carried out. TGF-β1 triggered RPTEC apoptosis in a dose-dependent manner (Fig. 3A).

To establish a causal relationship between TGF-β and the occurrence of RPTEC apoptosis, we studied the effect of anti-TGF-β antibody on TGF-β-induced tubular cell apoptosis. Equal numbers of RPTECs were incubated in media containing either buffer, 10 ng/ml TGF-β1, 1 μg/ml anti-TGF-β1 antibody, or anti-TGF-β antibody+TGF-β1 for 16 h. Subsequently, cells were assayed for apoptosis. Anti-TGF-β antibody attenuated the proapoptotic effect of TGF-β (Fig. 3B).

Studies Pertaining to the Role of FasL

To evaluate the contribution of the FasL pathway in ANG II-induced tubular cell apoptosis, equal numbers of RPTECs were incubated in media containing buffer (control) or 10⁻⁸ or 10⁻⁷ M ANG II with or without anti-FasL antibody (1 μg/ml, mouse monoclonal antibody, Pharmingen) for 16 h. Subsequently, cells were stained for apoptosis. As shown in Fig. 3C, anti-FasL
antioxidants such as diphenyleneiodonium chloride (DPI, Sigma), ascorbic acid (AA), and N-acetyl cysteine (NAC). Equal numbers of RPTECs were incubated in media containing buffer (control), 10 μM DPI, 100 μM AA, or 50 μM NAC with or without 10⁻⁸ M ANG II for 24 h. At the end of the incubation period, cells were assayed for apoptosis. DPI, NAC, and AA inhibited (P < 0.01) the effect of ANG II (Fig. 4A). These findings indicate that oxidative stress plays a causal role in ANG II-induced RPTEC apoptosis.

Studies Pertaining to the Role of Oxidative Stress

To determine the role of oxidative stress in ANG II-induced RPTEC apoptosis, we studied the effect of antioxidants such as diphenyleneiodonium chloride (DPI, Sigma), ascorbic acid (AA), and N-acetyl cysteine (NAC). Equal numbers of RPTECs were incubated in media containing buffer (control), 10 μM DPI, 100 μM AA, or 50 μM NAC with or without 10⁻⁸ M ANG II for 24 h. At the end of the incubation period, cells were assayed for apoptosis. DPI, NAC, and AA inhibited (P < 0.01) the effect of ANG II (Fig. 4A). These findings indicate that oxidative stress plays a causal role in ANG II-induced RPTEC apoptosis.

Fig. 3. A: effect of TGF-β in ANG II-induced RPTEC apoptosis. Equal nos. of RPTECs were incubated in media containing either buffer (control) or variable concentrations of TGF-β1 (50, 500, 1,000 pg/ml) for 16 h. Subsequently, cells were stained with H-33342 and propidium iodide. The percentage of live, apoptosed, and necrosed cells was recorded. Values are means ± SE of 8 series of experiments, each carried out in triplicate. *P < 0.01 compared with control and 50 pg/ml TGF-β1. ***P < 0.001 compared with control and 50–500 pg/ml TGF-β1. B: effect of anti-TGF-β antibody on TGF-β1-induced RPTEC apoptosis. Equal nos. of RPTECs were incubated in media containing either buffer, TGF-β1 (TGF-β1, 10 ng/ml), anti-TGF-β antibody (A-TGF-ab; 1 μg/ml), or anti-TGF-β antibody + TGF-β for 16 h. Subsequently, cells were assayed for apoptosis. *P < 0.01 compared with control, A-TGF-ab alone, and TGF-β1 + A-TGF-ab. C: effect of anti-Fas ligand (FasL) antibody in ANG II-induced tubular cell apoptosis. Equal nos. of RPTECs were incubated in media containing buffer (control) or 10⁻⁶ or 10⁻⁸ M ANG II with or without anti FasL antibody (anti-FasL-ab; 1 μg/ml) for 16 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live, apoptosed, and necrosed cells was recorded. Values are means ± SE of 4 sets of experiments, each carried out in triplicate. *P < 0.001 compared with control and ANG II-stimulated states in the presence or absence of anti-TGF-β antibody. D: effect of anti-FasL antibody in TGF-β1-induced tubular cell apoptosis. Equal nos. of RPTECs were incubated in media containing buffer (control) or TGF-β1 (TGF-b; 10 ng/ml) with or without 1 μg/ml A-FasL-Lab for 16 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live, apoptosed, and necrosed cells was recorded. Values are means ± SE of 3 series of experiments, each carried out in triplicate. *P < 0.001 compared with control and TGF-b + A-FasL-ab.

To evaluate the contribution of the FasL pathway in TGF-β-induced tubular cell apoptosis, equal numbers of RPTECs were incubated in media containing buffer (control) or 10 ng/ml TGF-β with or without 1 μg/ml anti-FasL antibody (anti-FasL-ab; 1 μg/ml) for 16 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live, apoptosed, and necrosed cells was recorded. Values are means ± SE of 8 series of experiments, each carried out in triplicate. *P < 0.01 compared with control and 50 pg/ml TGF-β1. **P < 0.001 compared with control and 50–1,000 pg/ml TGF-β1.

To determine the role of HO-1 preinduction on ANG II-induced tubular cell apoptosis, equal numbers of cells were incubated in media containing either buffer or ANG II for 120 min. Supernatants were collected at 0, 30, 45, 60, 90, and 120 min and assayed for superoxide and hydrogen peroxide. As shown in Fig. 4, B and C, ANG II promoted RPTEC generation of both superoxide and hydrogen peroxide at 45 min. The generation of ROS plateaued at 120 min (data on 60, 90, and 120 min are not shown). However, anti-TGF-β antibody inhibited the effect of ANG II.

To evaluate the role of ANG II and TGF-β on the generation of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, we measured the production of superoxide and H₂O₂ by RPTECs under control and ANG II-stimulated states in the presence or absence of anti-TGF-β antibody. Equal numbers of cells were incubated in media containing either buffer or ANG II for 120 min. Supernatants were collected at 0, 30, 45, 60, 90, and 120 min and assayed for superoxide and hydrogen peroxide. As shown in Fig. 4, B and C, ANG II promoted RPTEC generation of both superoxide and hydrogen peroxide at 45 min. The generation of ROS plateaued at 120 min (data on 60, 90, and 120 min are not shown). However, anti-TGF-β antibody inhibited the effect of ANG II.

AJP-Renal Physiol • VOL 284 • MAY 2003 • www.ajprenal.org
compared with control, NAC, DPI, AA, NAC/H11001, ANG II, and AA

buffer or 5 RPTECs were preincubated in media containing either induction) or treated simultaneously. Equal numbers of cells that were either pretreated with hemin (preinduc-
tion, we evaluated the effect of ANG II on tubular
effect of ANG II on RPTECs.

B, ZnP accentuated the proapoptotic As shown in Fig. 5 for 16 h. Subsequently, cells were stained for apoptosis. Values are means ± SE of 3 sets of experiments, each carried out in triplicate. *P < 0.001 compared with control, NAC, DPI, AA, NAC + ANG II, DPI + ANG II, and AA + ANG II. B: effect of ANG II and anti-TGF-β antibody on RPTEC superoxide generation. Equal nos. of cells were incubated in media containing either buffer or ANG II for 120 min. Supernatants were collected at 0, 30, 45, 60, 90, and 120 min and assayed for superoxide. The generation of superoxide is shown at 45 min. Values are means ± SE of 3 sets of experiments, each carried out in triplicate. *P < 0.001 compared with control and anti-TGF-β antibody + ANG II.

To determine the effect of HO-1 inhibition, equal numbers of RPTECs were pretreated in media containing either buffer (control) or zinc protoporphyrin (ZnP; 100 μM, Sigma) for 4 h, followed by incubation in media containing vehicle (control) or 10⁻⁸ M ANG II for 16 h. Subsequently, cells were stained for apoptosis. As shown in Fig. 5B, ZnP accentuated the proapoptotic effect of ANG II on RPTECs.

Studies Pertaining to Cell Death Pathways

To determine the role of caspase-3 in ANG II-induced tubular cell apoptosis, equal numbers of cells were curcumin-induced modulation of the ANG II effect. Equal numbers of RPTECs were incubated in media containing either buffer, 10⁻⁶ M ANG II, 15 μM curcumin, 50 μM ZnP, curcumin + ANG II, curcumin + ZnP, or curcumin + ZnP + ANG II for 16 h. Subsequently, cells were assayed for apoptosis. ZnP attenuated the antiapoptotic effect of curcumin on ANG II-treated cells (Fig. 5D). These findings suggest that the curcumin-mediated effect on ANG II-treated cells may have been induced through HO-1 expression.

Studies Pertaining to p38 MAPK Activation

To evaluate the role of p38 MAPK in ANG II-induced RPTEC apoptosis, we studied the effect of a selective p38 MAPK inhibitor, SB-202190 (Calbiochem, La Jolla, CA), on ANG II-induced RPTEC apoptosis. Equal numbers of cells were incubated in media containing either buffer (control) or 5 μM SB-202190 for 1 h. At the end of the incubation period, cells were reincubated in media containing vehicle (control) or 10⁻⁸ or 10⁻⁶ M ANG II for 16 h. Subsequently, cells were stained for apoptosis. As shown in Fig. 6A, SB-202190 partially inhibited the effect of ANG II on RPTEC apoptosis.

Studies Pertaining to p38 MAPK Activation

To evaluate the role of p38 MAPK in ANG II-induced RPTEC apoptosis, we studied the effect of a selective p38 MAPK inhibitor, SB-202190 (Calbiochem, La Jolla, CA), on ANG II-induced RPTEC apoptosis. Equal numbers of cells were incubated in media containing either buffer (control) or 5 μM SB-202190 for 1 h. At the end of the incubation period, cells were reincubated in media containing vehicle (control) or 10⁻⁸ or 10⁻⁶ M ANG II for 16 h. Subsequently, cells were stained for apoptosis. As shown in Fig. 6A, SB-202190 partially inhibited the effect of ANG II on RPTEC apoptosis.

Studies Pertaining to Cell Death Pathways

To determine the role of caspase-3 in ANG II-induced tubular cell apoptosis, equal numbers of cells were...
incubated in media containing either buffer (control), or 10^{-8} \text{ M ANG II with or without caspase-3 inhibitor (5 \text{ M, no. 218750, Calbiochem}) for 16 h. Subsequently, cells were stained for apoptosis. Four series of experiments were carried out. Caspase-3 inhibitor attenuated the proapoptotic effect of ANG II (Fig. 6B).}

To determine the role of cell death proteins (Bax, Fas, FasL) and cell survival protein (Bcl-2) in ANG II-induced RPTEC apoptosis, equal numbers of tubular cells were incubated in media containing either buffer (control), or 10^{-8} \text{ and } 10^{-6} \text{ M ANG II for 16 h. Subsequently, protein was extracted, and Western blots were prepared and probed for Bax, Bcl-2, Fas, and FasL. Tubular cells showed an increased (P < 0.001) expression of Fas in response to ANG II treatment compared with control (Fig. 7B, depicted in the form of Fas/actin ratios). A representative gel is shown in Fig. 7A. Similarly, ANG II-treated cells showed increased expression of FasL compared with control (Fig. 7D, depicted in the form of FasL/actin ratios). A representative gel showing the effect of ANG II on FasL expression is shown in Fig. 7C. ANG II enhanced RPTEC expression of Bax (Fig. 8B, depicted in the form of Bax/actin ratios). A representative gel showing the effect of ANG II on Bax expression is shown in Fig. 8A. On the other hand, ANG II decreased RPTEC expression of Bcl-2 (Fig. 8D, shown in the form of Bcl-2/actin ratios). A representative gel showing the effect of ANG II on Bcl-2 expression is shown in Fig. 8C.

**Studies Pertaining to Tubular Cell HO-1 Expression**

To study the effect of ANG II on RPTEC HO-1 induction, equal numbers of cells were incubated in
To determine the role of TGF-β in ANG II-induced HO-1 expression, equal numbers of cells were treated with either buffer (control) or $10^{-8}$ and $10^{-6}$ M ANG II with or without anti-TGF-β antibody for 16 h. Subsequently, protein was extracted, and Western blots were generated and probed for HO-1. As shown in Fig. 9D, the effect of ANG II on RPTEC was inhibited by anti-TGF-β antibody. A representative gel showing the effect of anti-TGF-β antibody on ANG II-induced HO-1 expression is shown in Fig. 9C. These studies suggest that ANG II-induced tubular cell-HO-1 induction may be mediated through TGF-β.

To determine the effect of curcumin and hemin on HO-1 expression, equal numbers of RPTECs were incubated in media containing either buffer (control), 5 μM hemin, 15 μM curcumin, or hemin+curcumin for 16 h. Subsequently, cells were prepared for Western blot analysis and probed for HO-1. Both curcumin and hemin promoted HO-1 expression. However, curcumin did not enhance the effect of hemin on tubular cell HO-1 expression (Fig. 9, E and F).

**DISCUSSION**

The present study demonstrates that ANG II promotes tubular cell apoptosis. Because anti-TGF-β antibody attenuated this effect and TGF-β promoted tubular cell apoptosis, it appears that ANG II-induced tubular cell apoptosis may be mediated through TGF-β. Both AT₁ and AT₂ receptor antagonists partially blocked ANG II-induced tubular cell apoptosis. ANG II also promoted the expression of HO-1 and tubular cell expression of FasL, whereas anti-FasL antibody inhibited the effect of ANG II as well as TGF-β on tubular cell apoptosis. ANG II not only promoted tubular cell expression of Bax but also inhibited the expression of Bcl-2. Pretreatment of tubular cells with inducers of HO-1 (hemin and curcumin) attenuated the response of ANG II, thus suggesting that preinduction of HO-1 expression perhaps provides protection against ANG II-induced tubular cell injury. On the other hand, pretreatment of RPTECs with ZnP, an inhibitor of HO-1 activity, exacerbated the proapoptotic effect of ANG II. Only pretreatment and not the
simultaneous treatment of tubular cells with hemin provided protection against the proapoptotic effect of ANG II.

Fas and the FasL pathway have been reported to mediate apoptosis in the kidney (19). These proapoptotic factors have been found to be attenuated by ANG II inhibition (32). In addition, captopril, an angiotensin-converting enzyme inhibitor, has been shown to inhibit Fas-induced apoptosis and FasL expression in human activated peripheral T cells (22). Our finding of enhanced expression of tubular Fas and FasL is consistent with these observations. Moreover, inhibition of ANG II-induced tubular cell apoptosis by anti-FasL antibody established a causal relationship. We also

Fig. 8. Effect of ANG II on tubular cell Bax and Bcl-2 expression. Equal nos. of RPTECs were incubated in media containing either buffer (control) or 10^{-8} and 10^{-6} M ANG II for 16 h. Subsequently, cells were harvested, protein was extracted, and blots were probed for Bax and Bcl-2. A: representative gel showing the effect of ANG II on tubular cell expression of Bax. B: cumulative data from 3 sets of experiments showing the effect of ANG II on tubular cell Bax expression. *P < 0.01 compared with control. **P < 0.001 compared with control and 10^{-8} M ANG II. C: representative gel showing the effect of ANG II on RPTEC expression of Bcl-2. D: cumulative data from 3 series of experiments showing the effect of ANG II on tubular cell Bcl-2 expression. *P < 0.01 compared with control. **P < 0.001 compared with control and 10^{-8} M ANG II.

Fig. 9. A: effect of ANG II on RPTEC HO-1 induction. Equal nos. of cells were incubated in media containing either buffer (control) or 10^{-8} and 10^{-6} M ANG II for 16 h. Subsequently, Western blots were generated and probed for HO-1. B: cumulative data from 3 series of experiments showing the effect of ANG II on tubular cell HO-1 expression. *P < 0.001 compared with control. **P < 0.001 compared with control and 10^{-8} M ANG II. C: effect of A-TGF-ab on ANG II-induced HO-1 expression. Equal nos. of cells were treated with either buffer (control) or 10^{-8} and 10^{-6} M ANG II with or without 10 ng/ml A-TGF-ab for 16 h. Subsequently, Western blots were generated and probed for HO-1. D: cumulative data from 3 series of experiments showing the effect of A-TGF-ab on ANG II-induced tubular cell HO-1 expression. *P < 0.01 compared with control and A-TGF-ab+10^{-8} M ANG II. **P < 0.01 compared with control and A-TGF-ab+10^{-8} M ANG II. E: effect of curcumin and hemin on HO-1 expression. Equal nos. of RPTECs were incubated in media containing either buffer (control), 5 \mu M hemin, 15 \mu M curcumin, or hemin+curcumin for 16 h. Subsequently, cells were prepared for Western blotting and probed for HO-1. F: cumulative data from 3 sets of experiments showing the effect of hemin and curcumin on tubular cell HO-1 expression. *P < 0.001 compared with control. **P < 0.01 compared with control. ***P < 0.001 compared with control.
observed the alteration in the expression of Bax and Bcl-2 in ANG II-treated cells, which may have tilted the balance toward apoptosis. In the present study, there was apparent variability in β-actin expression in control and ANG II-treated tubular cells. This may have partly contributed to the altered Bax/actin ratio. Aizawa et al. (2) found increased expression of Bax in the kidneys of ANG II-infused rats without any decrease in Bcl-2. Because these investigators studied Bcl-2 expression in whole kidney lysates, their finding may not necessarily represent Bcl-2 expression by proximal tubular cells.

AT₁ receptors are responsible for most of the reported actions of ANG II (25). AT₂ receptors are predominantly expressed during fetal development and are considered to be critical for ontogenesis. Expression of AT₂ receptors declines rapidly after cessation of developmental apoptosis. Conversely, Cao et al. (7) demonstrated that proximal tubular cells in adult rats express AT₂ receptors. These investigators demonstrated that infusion of ANG II for 14 days in 8-wk-old rats induced the proliferation and apoptosis of proximal tubular cells. The administration of the AT₂ antagonist PD-123319 or the AT₁ antagonist valsartan was associated with attenuation of the increase in both PCNA- and TUNEL-positive cells after ANG II infusion. These findings suggest that both AT₁ and AT₂ receptors are involved in both proliferation and apoptotic processes in proximal tubular cells. In the present study, both the AT₁ antagonist (losartan) and AT₂ antagonist (PD-123319) inhibited ANGII-induced apoptosis of tubular cells. These findings suggest that downstream signaling mediated by both AT₁ and AT₂ receptors is needed for the activation of ANG II-induced tubular cell apoptosis.

Haugen et al. (13) studied the occurrence of oxidative stress in two models of hypertension, i.e., ANG II- and DOCA salt-treated rats. However, only ANG II-treated rats showed evidence of renal oxidative stress in the form of lipid peroxidation, protein carbonyl content, and induction of HO-1 (13). These investigators further localized the induction of proximal tubular cell HO-1 in ANG II-treated animals (13). Because DOCA salt-treated rats did not show renal oxidative stress despite having identical levels of blood pressure, these investigators suggested that ANG II induces oxidative stress independently of its hemodynamic effects. Similarly, Aizawa et al. (3), in two models of hypertension, i.e., ANG II- and norepinephrine-infused rats, showed that only ANG II-infused rats developed a decrease in glomerular filtration rate and proteinuria. Interestingly, tubular cell HO-1 was upregulated only in ANG II-treated rats (not in norepinephrine-treated rats). Pretreatment of rats with HO-1 expression modulators, i.e., hemin, an inducer, and ZnP, an inhibitor, modulated not only tubular cell HO-1 expression but also proteinuria (hemin decreased and ZnP increased ANG II-induced proteinuria) (3). These investigators also studied the occurrence of apoptosis and proliferation of tubular cells in these models (2). Only ANG II-infused rats showed increased numbers of both PCNA- and TUNEL-positive cells. Pretreatment of these rats with HO-1 inducers and inhibitors modulated the severity of ANG II-induced tubular cell apoptosis and proliferation. Moreover, only ANG II-treated rats showed tubular cell upregulation of HO-1 and Bax expression. The effect of ANG II partially persisted despite normalization of blood pressure with hydralazine, again suggesting a nonhemodynamic effect of ANG II. The in vitro observations in the present study are consistent with the in vivo findings of Aizawa et al. (2).

In the present study, ANG II promoted HO-1 expression, thus indicating the occurrence of oxidative stress. Because antioxidants such as DPI, NAC, and AA inhibited the proapoptotic effect of ANG II, it appears that ANG II-induced tubular cell apoptosis is mediated through oxidative stress. Moreover, enhanced production of superoxide and H₂O₂ by tubular cells in response to ANG II further delineates the molecular mechanism involved. Both superoxide and H₂O₂ have been demonstrated to promote HO-1 expression in a variety of cells (1). Thus it appears that ANG II-induced HO-1 expression may be mediated through the generation of superoxide and H₂O₂ by tubular cells.

Induction of HO-1 may be looked at from two perspectives. On one hand, this may occur as a cellular reflex response to ongoing or acute oxidative stress, of which it is a marker. On the other hand, if preinduced, it may act as part of the armamentarium against incoming injury. Preinduction of HO-1 may generate enough antioxidants to neutralize the effect of oxidative stress. Therefore, preinduction of HO-1 has been used as a tool in preventing damage in various models of oxidative injury (8, 10, 17, 23, 31). Because ANG II induces oxidative stress, it is likely to promote HO-1 expression. Because ANG II could induce tubular cell injury despite ongoing expression of HO-1, it appears that expression of HO-1 may have been a futile attempt to contain oxidative stress. This hypothesis is further supported by our data showing that the proapoptotic effect of ANG II was attenuated in tubular cells pretreated with hemin but not in tubular cells that were treated simultaneously with hemin. On the other hand, ZnP, an inhibitor of HO-1 activity, enhanced the proapoptotic effect of ANG II. These findings suggest that ANG II-induced HO-1 expression may be providing at best limited protection against ANG II-induced oxidative stress.

We conclude that ANG II induces proximal renal tubular cell apoptosis through the generation of TGF-β and ROS, p38 MAPK phosphorylation, expression of Fas, FasL, and Bax, and activation of caspase 3. This effect of ANG II seems to be mediated by both AT₁ and AT₂ receptors. Modulation of tubular cell HO-1 expression inversely affects ANG II-induced tubular cell apoptosis. These findings may provide a basis for a hypothesis that nonhemodynamic effects of ANG II may be playing a role in the development and progression of tubular cell injury in conditions associated with elevated levels of ANG II.
REFERENCES


