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Angiotensin II-Induced Mesangial Cell Apoptosis: Role of Oxidative Stress

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Abstract

Background: Angiotensin II (ANG II) has been shown to play a role in the induction of glomerular injury. In the present study, we evaluated the effects of ANG II on mesangial cell apoptosis and the involved molecular mechanism.
Materials and Methods: The effect of ANG II on apoptosis of mouse mesangial cells (MC) was evaluated by morphologic, DNA fragmentation and TUNEL assays. To evaluate the role of oxidative stress and involved mechanisms, we studied the effect of antioxidants, anti-TGF-β antibody, inhibitors of nitric oxide synthase and modulators of cytosolic calcium/heme oxygenase (HO) activity. In addition, we studied the effect of ANG II on the generation of reactive oxygen species (ROS) by MCs.
Results: ANG II promoted apoptosis of MCs in a dose dependent manner. This effect of ANG II was not only associated with ROS production, but also inhibited by antioxidants. Both Anti-TGF-β antibody and propranolol inhibited ANG II-induced ROS generation and apoptosis. BAPTA inhibited both ANG II- and TGF-β-induced apoptosis. On the other hand, thapsigargin stimulated MC apoptosis under basal as well as ANG II/TGF-β stimulated states. ANG II receptor types 1 and 2 antagonists attenuated the proapoptotic effect of ANG II. Hemin inhibited but zinc protoporphyrin enhanced the proapoptotic effect of ANG II. Propranolol increased HO activity; whereas pre-treatment with propranolol prevented ANG II-induced apoptosis.
Conclusions: ANG II promotes MC apoptosis. This effect of ANG II is mediated through downstream signaling involving TGF-β, phospholipase D, and Ca^{2+}, contributing to the activation of NADPH oxidase and generation of ROS. HO activity plays a modulatory role in ANG II-induced MC apoptosis.

Introduction

Angiotensin II (ANG II) plays an important role in the development and progression of renal injury (1–3). In humans as well as in experimental models of focal glomerulosclerosis (FGS), expansion of the mesangium precedes the development of FGS (4–6). The expanded mesangium is comprised of a large number of mesangial cells and increased amount of matrix. However, in the later course of the disease, there is an abundance of matrix and a limited number of mesangial cells (5,7,8). This process of initial mesangial cell hyperplasia followed by a paucity (loss) of mesangial cells is often associated with hyperfiltration and elevated intra-renal levels of ANG II and transforming growth factor β (TGF-β) (9,10). We asked whether there is a relationship between ANG II, TGF-β, and the loss (apoptosis) of mesangial cells.

ANG II plays an important role in the maintenance of glomerular filtration in both normal and diseased states (1). However, ANG II contributes to glomerular pathobiology through not only its hemodynamic effects but also via its nonhemodynamic effects on glomerular injury (2,9,11). In vitro, ANG II has been shown to stimulate the synthesis of matrix proteins in cultured mesangial cells (12). In vivo, administration of ANG II has also been shown to increase the production of mesangial matrix (12). Moreover, inhibition of ANG II production by angiotensin I converting enzyme inhibitors and ANG II receptor antagonists attenuates the progression of FGS (13,14). All these observations provide evidence of a causal relationship between ANG II and the accumulation of mesangial matrix. However, evidence for the role of ANG II in the induction of mesangial cell loss is lacking.

ANG II has been demonstrated to stimulate production of nitric oxide (NO) and oxygen radicals by endothelial cells in humans (15). Production of these two moieties in response to ANG II prevents unopposed vasoconstriction of smooth muscle cells in response to reactive oxygen species (ROS) (16). Sugiyama et al. (17) previously demonstrated that reactive oxygen species (ROS) trigger mesangial cell apoptosis. We and other investigators previously demonstrated that NO also triggers mesangial cell apoptosis (18,19). Because ANG II has the potential

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to stimulate production of both ROS and NO in other cells we hypothesize that both NO and ROS may be contributing to ANG II-induced mesangial cell apoptosis.

Apoptosis in glomerular cells has been shown to be an important process for normalizing the hypercellularity of injured glomeruli (20–22). In the majority of instances it is a physiologic process that brings the glomeruli back to normal cellularity; however, if it proceeds unregulated, it may lead to a pathologic lesion. The latter may happen either as a result of an altered environment or persistent up-regulation of cytokines such as TGF-β (23,24). Sugiyama et al. (25) suggested that occurrence of glomerular cell apoptosis is an important event in the progression of glomerulosclerosis. Glomerular cell apoptosis has been demonstrated in human diseases and animal experimental models of renal ablation, diabetes, and hypertensive nephrosclerosis; all these models have increased activity of ANG II (25–27). Although ANG II has been reported to induce apoptosis in a variety of cells, its effect on kidney cells has been limited to renal epithelial cells (28–34). There are scanty data on whether ANG II induces mesangial cell apoptosis in vivo or in vitro (4). Because paucity of mesangial cells is an important feature of FGS, it may be important to evaluate the role of ANG II in the induction of mesangial cell apoptosis.

We previously reported that TGF-β promotes mesangial cell apoptosis through NO and p53-dependent and independent pathways (18). Because many of the effects of ANG II on mesangial cells are mediated through the generation of TGF-β (12), it is possible that ANG II-induced mesangial cell apoptosis may also be mediated through TGF-β. Therefore, we studied the role of TGF-β in ANG II-induced mesangial cell apoptosis.

Because oxidative stress is known to modulate heme oxygenase (HO) activity, we evaluated the role of HO activity in ANG II-induced mesangial cell apoptosis.

Mesangial cells have been demonstrated to express ANG II type 1 (AT1) receptors (35,36). Many of the functions including contractility, proliferation, and matrix synthesis have been attributed to the stimulation of the AT1 receptors (35,36). However, the role of AT2 receptors in mesangial cells is not clear. Interestingly, ANG II-induced apoptosis in other cells is considered to be mediated by the ANG II type 2 (AT2) receptors, but there are reports of ANG II type 1 (AT1) receptors mediating apoptosis (32,37). In the present study, we also investigated the role of AT1 and AT2 receptors in mesangial cell apoptosis.

Material and Methods

Mesangial Cell Culture

Mouse mesangial cells (MMC, SV40 MES-13) were obtained from ATCC (Manassas, VA, USA). These mesangial cells showed typical phenotype and biochemical characteristics observed in wild-type mesangial cells (38–40). Mesangial cells (MC) were grown in culture medium, a 3:1 mixture of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) and Ham's F12 medium, 95% (Gibco), FCS, 5%, supplemented with 14 mM HEPES (Gibco), 50 U/ml penicillin, and 50 μg/ml of streptomycin (Gibco).

Experimental Agents

ANG II was obtained from Sigma Chemical Co. (St. Louis, MO, USA). TGF-β1 was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, and was used in concentrations of 1.0 ng/ml. Anti-TGF-β1 antibody was obtained from Pharmingen (San Diego, CA, USA). N^G-nitro-L-arginine methyl ester (L-NAME; Calbiochem, La Jolla, CA, USA) was used in concentrations of 1 mM. Ascorbic acid, diphenyleneiodonium chloride (DPI), N-acetyl L-cysteine (NAC), propranolol, hemin, and zinc protoporphyrin were obtained from Sigma. Thapsigargin and 1,2-bis (o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid Tetra(acetoxymethyl) Ester (BAPTA) were obtained from Calbiochem.

Apoptosis Studies

Morphologic evaluation of MC apoptosis was performed by staining cells with H-33342 (Molecular Probes, Portland, OR, USA) and propidium iodide (PI; Sigma). Double staining by these two agents provides the percentage of live, apoptotic, and necrotic cells under control and experimental conditions (41). To determine the dose–response effects of ANG II, MMCs were grown to subconfluence in 24-well plates. Subsequently, cells were washed twice with phosphate buffered saline (PBS) and incubated in media (including 1% FCS) containing either vehicle or variable concentrations of ANG II (10^{-10} to 10^{-6} M) for 24 hr; four sets of experiments were carried out. At the end of the incubation period, cells were stained with H-33342 and PI. The percentage of live, apoptotic, and necrosed cells was recorded in eight random fields by two observers unaware of the experimental conditions.

To confirm the effect of ANG II on MC apoptosis, MC treated under control and experimental conditions were assayed by TUNEL method (kit supplied by Roche Applied Science, Indianapolis, IN, USA).

DNA Fragmentation Assay: Gel Electrophoresis

This is a simple method that is specific for isolation and confirmation of DNA fragments from apoptotic cells (42). Because this method only picks up DNA fragments, one will not visualize any loading of samples that do not contain any DNA fragments. Equal numbers (10^8 cells/Petri dish) of MMCs were prepared under control and experimental conditions (ANG II, 10^{-10} to 10^{-6} M; 24 hr). At the end of the incubation period, cells were centrifuged at 1600 g for 10 min at room temperature and the pellets were resuspended in DNA lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCL, pH 7.5; 10 μl per 10^6 cells).

After centrifugation, the supernatant was collected and the extraction was repeated. SDS, in a final concentration of 1%, was added to the supernatants before the samples were treated with RNAse A (final concentration 5 μg/μl) at 56°C. This was followed by digestion with proteinase K (Promega) for 2 hr at 37°C. After addition of 1/2 vol of 10 M ammonium acetate, the DNA was precipitated with 2.5 vol ethanol, dissolved in gel loading buffer, and separated by electrophoresis in 1.6% agarose gels.

Superoxide Assay

Equal numbers of MMCs 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 or ANG II (10^{-8} M) at 37°C for 2 hr. Supernatants were collected at 0, 30, 45, 60, and 120 min into precooled microcentrifuge test tubes. Superoxide assay was carried out subsequently. In brief, 50 μl of each supernatant was pipeted into a 96-well plate, kept on ice, and mixed with 100 μl of cytochrome C (160 μM final concentration, ICN Biomedicals Inc., Costa Mesa, CA, USA) diluted with Hanks balanced salt solution (HBSS, Gibco). Incubation was carried out at 37°C for 45, 90, and 150 min and OD read at 550 nm. Results are expressed in arbitrary units and experiments repeated four times each in triplicate.

Hydrogen Peroxide (H_2O_2) Assay

Equal numbers of MMCs 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 or ANG II (10^{-8} M) at 37°C for 2 hr. Supernatants were collected at 0, 30, 45, 60, and 120 min into precooled microcentrifuge test tubes. H_2O_2 assay was carried out. In brief, 50 μl of each supernatant was pipeted 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 horse radish peroxidase, and 100 μg/ml phorbol myrisate acetate. Incubation was carried out at 37°C for 45, 90, and 150 min and terminated by addition of 10 μl of 1M 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 H_2O_2. Results are expressed in arbitrary units and experiments repeated four times each in triplicate.

ELISA for Caspase-3 Activity

Caspase-3 activation initiates degradation phase and the beginning of irreversible stage of apoptosis. Thus it is important to evaluate the effect of ANG II on caspase-3 activation as well as the effect of blockade at different signal transduction pathways. MMCs were incubated under control and experimental conditions for 3 hr. At the end of the incubation period, cells were harvested and prepared for the measurement of caspase-3 activity with the use of Caspase-3 assay kit (Casp-3-C, Sigma).

Measurement of Heme Oxygenase Enzyme Activity

Heme oxygenase activity was measured by bilirubin generation method (43). In brief, MCs grown to confluence (in tissue flasks) were incubated in media containing either buffer or propranolol (100 μM) for 16 hr followed by reincubation in media containing either buffer or ANG II (10^{-8} M) for 16 hr. At the end of the incubation period, cells were washed, scraped and centrifuged (1000 $\times g$ for 10 min at 4°C). The cell pellet was suspended in $MgCl_2$ (2 mM) phosphate (100 mM) buffer (pH 7.4), sonicated on ice prior to centrifugation at 18,800 $\times g$ for 10 min at 4°C. The supernatant was added to the reaction mixture (400 μl) containing rat liver cytosol (2 mg), hemin (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 units), and NADPH (0.8 mM) for 1 hr at 37°C in the dark. The formed bilirubin was extracted with chloroform and Δ OD 464–530 was measured (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO activity is expressed as pmol of bilirubin formed/μg of mesangial cell protein/60 min.

Nitrotyrosine Immunostaining

MCs grown on chamber slides were incubated under control and ANG II-treated states and fixed with 4% paraformaldehyde followed by quenching of endogenous peroxidases (0.03% H_2O_2 in methanol for 30 min). Cells were preincubated in rabbit anti-nitrotyrosine antibody (1:100 dilution, Upstate Biotechnology, Lake Placid, NY, USA) overnight at 4°C followed by incubation with 1% goat biotinylated anti-rabbit IgG and visualized by reaction with avidin-biotin peroxidase (Vectastain Elite ABC kit; Vector Labs, Burlingame, CA, USA). The cells were then incubated with diaminobenzidine tetrahydrochloride (Sigma) for 7 min at room temperature.

Statistical Analysis

Comparison of MMC apoptosis between control and experimental conditions was carried out by an unpaired Student's *t*-test. When more than two groups were involved, intergroup comparisons were performed by analysis of variance. A Newman-Keuls multiple range test was used to calculate a *q* value. Results are presented as means $\pm$ SEM.

Results

Effect of ANG II on Mesangial Cell Apoptosis

To determine the effect of Ang II on mesangial cell apoptosis, equal numbers of MCs were incubated in media containing either vehicle (control) or variable concentrations of ANG II (10^{-10}, 10^{-8}, and 10^{-6} M) for 24 hr. Subsequently, cells were assayed for apoptosis (staining with H-33342 and PI/TUNEL method). Representative micrographs are shown in Figure 1. Cumulative data are shown in Figure 2A. ANG II promoted MC

apoptosis in a dose-dependent manner. ANG II in concentrations of 10^{-8} and 10^{-6} M also induced a mild degree of necrosis (Fig. 2A).

To confirm the effect of ANG II on MC apoptosis, equal numbers of MCs were treated similarly followed by DNA extraction and gel electrophoresis. As shown in Figure 2B, ANG II in concentrations of 10^{-8} and 10^{-6} M induced multiple integers of 180 base pairs in the form of laddering. However, there were no visible DNA fragments in the lanes depicting control and ANG II, 10^{-10} M-treated cells. These findings confirmed the effect of ANG II on MCs.

Recently, Ding et al. (33) demonstrated that the effect of ANG II on glomerular epithelial cells is mediated through the generation of TGF-β. Therefore, we studied the role of TGF-β in ANG II-induced MC apoptosis. Equal numbers of MCs were incubated in media containing vehicle (control), ANG II (10^{-8} M), or anti–TGF-β antibody (1 μg/ml) + ANG II for 24 hr. Subsequently, cells were assayed for apoptosis. As shown in Figure 2C, ANG II promoted MC apoptosis. However, this effect of ANG II was inhibited ($p < 0.001$) by anti–TGF-β antibody. These findings suggest that ANG II-induced MC apoptosis may be mediated through TGF-β.

We previously reported that TGF-β induces MC apoptosis through the generation of NO (18). We hypothesized that if ANG II-induced MC apoptosis is mediated through TGF-β, then NO is also likely to play a role in the pro-apoptotic pathway of ANG II. Therefore, we studied the effect of L-NAME, an inhibitor of nitric oxide synthase, in ANG II-induced MC apoptosis. Equal numbers of MCs were incubated in media containing buffer (control), ANG II (10^{-8} M), L-NAME (1 mM), or L-NAME + ANG II for 24 hr. Subsequently cells were assayed for apoptosis. As shown in Figure 2D, ANG II promoted ($p < 0.001$) MC apoptosis; however, this

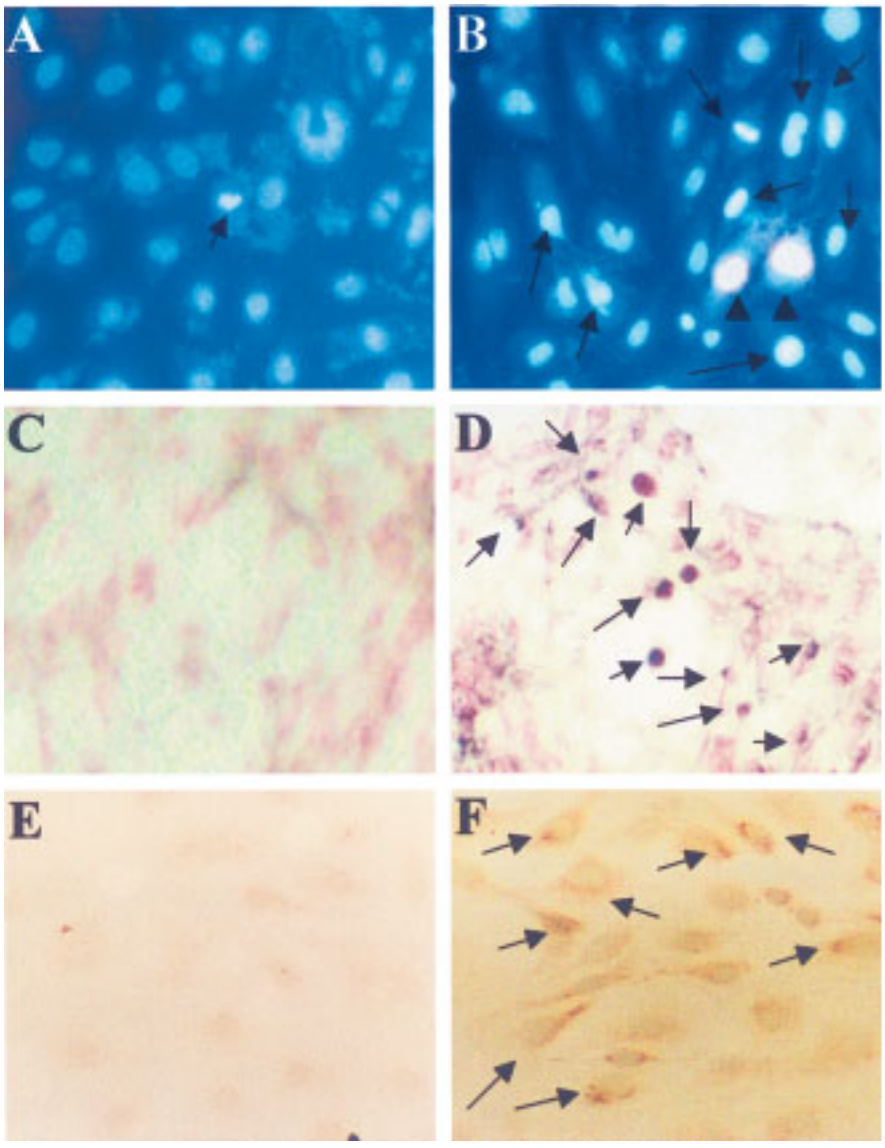

Fig. 1. (A and B) Effect of ANG II on MC apoptosis. Equal numbers of MCs were incubated in media containing either buffer or ANG II (10^{-6} M) for 24 hr. Subsequently, cells were stained with H-33342 and propidium iodide. **(A)** Control cells. An apoptotic cell shows bright fluorescence (*arrow*). **(B)** ANG II-treated cells. Apoptotic cells show bright fluorescence (*arrows*) and necrosed cells are stained pink (*arrowhead*). **(C and D)** Equal number of MCs were incubated in media containing either buffer (control) or ANG II (10^{-8} M) for 24 hr. Subsequently, cells were assayed for apoptosis by TUNEL method. **(C)** Control cells. **(D)** ANG II-treated cells. Nuclei of apoptotic cells are darkly stained (*arrows*). **(E and F)** Effect of ANG II on MC nitrotyrosine labeling. Equal number of cells, grown in chamber slides, incubated in media containing either buffer (control) or ANG II (10^{-8} M for 24 hr). Subsequently, cells were immunostained for nitrotyrosine. **(E)** Control cells. **(F)** ANG II-treated cells. Brown staining (*arrows*) indicates labeling for nitrotyrosine.

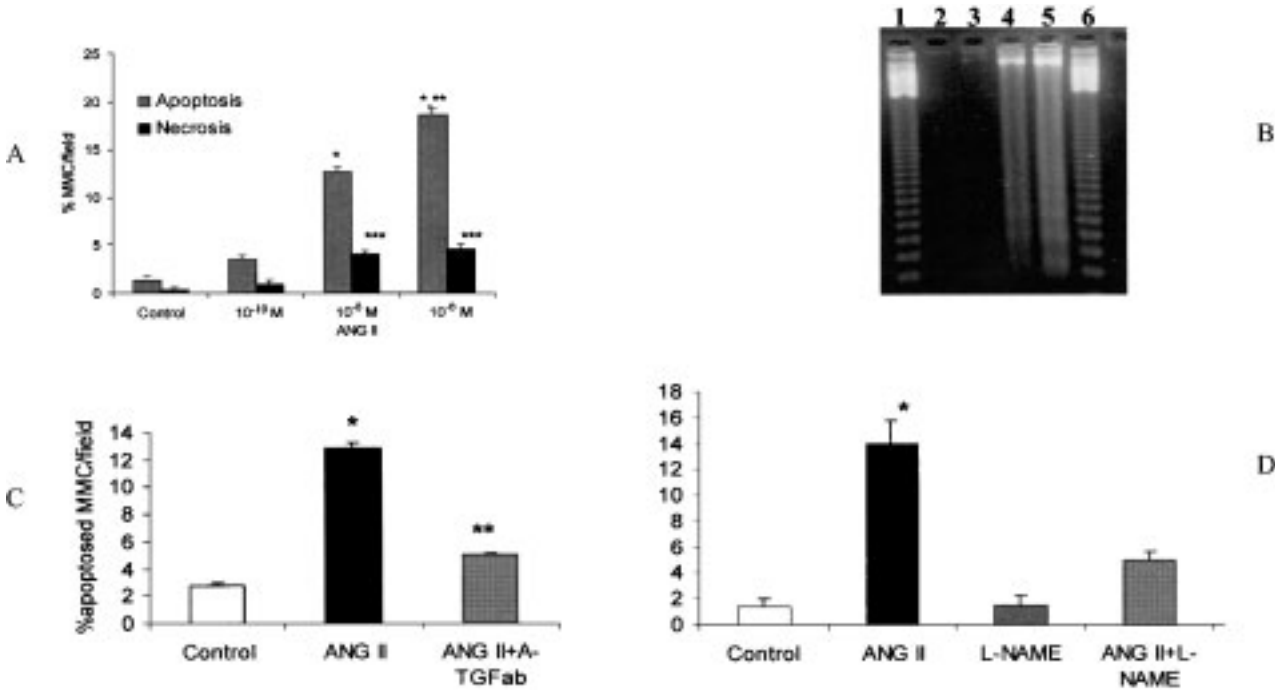

Fig. 2. (A) Dose-response effect of Ang II on mesangial cell apoptosis. Equal numbers of MCs were incubated in media containing either vehicle (control) or variable concentrations of ANG II (10^{-10}, 10^{-8}, and 10^{-6} M) for 24 hr. At the end of the incubation, cells were stained with H-33342 and PI. Results (means ± SEM) are from four sets of experiments, each carried out in triplicate. Intergroup comparisons were performed by analysis of variance. A Newman-Keuls multiple range test was used to calculate a q value. *$p < 0.001$ compared with respective control and ANG II (10^{-10} M); **$p < 0.001$ compared with respective ANG II (10^{-8} M); ***$p < 0.001$ compared with respective control and ANG II (10^{-10} M). **(B)** Effect of ANG II on mesangial cell DNA fragmentation. Equal numbers of MCs were incubated in media containing either vehicle (control) or variable concentrations of ANG II (10^{-10}, 10^{-8}, and 10^{-6} M) for 24 hr. Subsequently, cells were washed, DNA extracted, and electrophoresed. Lanes 1 and 6 are molecular markers. Lanes 2 and 3 are control and ANG II (10^{-10} M), respectively. ANG II 10^{-8} and 10^{-6} M are shown in lanes 4 and 5, respectively. ANG II in concentrations 10^{-8} and 10^{-6} M induced DNA fragmentation in the form of a ladder pattern. **(C)** Effect of anti–TGF-β antibody on ANG II-induced mesangial cell apoptosis. Equal numbers of MMCs were incubated in media containing either vehicle (control), ANG II (10–8 M), or anti–TGF-β antibody (1 μg/ml) + ANG II for 24 hr. Cells were then assayed for apoptosis. Results (means ± SEM) are from four series of experiments, each carried out in triplicate. *$p < 0.001$ compared with control and ANG II + anti–TGF-β antibody; **$p < 0.01$ compared with control. **(D)** Evaluation of the effect of L-NAME on ANG II-induced mesangial cell apoptosis. Equal numbers of MMCs were incubated in media containing buffer (control), ANG II (10^{-8}M), L-NAME (1 mM), or L-NAME + ANG II for 24 hr. Cells were then stained for apoptosis. Results (means ± SEM) are from four series of experiments, each carried out in triplicate. *$p < 0.001$ compared with control and ANG II + L-NAME.

effect of ANG II was partly attenuated ($p < 0.001$) by L-NAME.

Role of Oxidative Stress in Ang II-Induced MC Apoptosis

To determine the role of oxidative stress in ANG II-induced MC apoptosis, we studied the effect of DPI, ascorbic acid, and NAC on ANG II-induced MC apoptosis. Equal numbers of MCs were incubated in media containing buffer (control), DPI (10 μM), ascorbic acid (100 μM), or NAC (50 μM) with or without ANG II (10^{-8} M) for 24 hr. At the end of the incubation period, cells were assayed for apoptosis. Both DPI and NAC partially inhibited ($p < 0.01$) the effect of ANG II (Fig. 3A). Ascorbic acid showed more pronounced inhibition ($p < 0.001$) of ANG II-induced apoptosis when compared with the effect of DPI and NAC. These findings suggest that oxidative stress plays a causal role in ANG II-induced MC apoptosis.

We previously reported that TGF-β promotes MC apoptosis through NO generation (18). Because NO and superoxide/H_2O_2 contribute to the production of peroxynitrite, a toxic and stable compound, we studied whether ANG II can promote mesangial cell nitrotyrosine production, a marker of peroxynitrite formation. Equal numbers of mesangial cells were incubated in media containing vehicle (control) or ANG II (10^{-8} M) for 24 hr. Subsequently cells were immunostained for nitrotyrosine as described in Material and Methods. As shown in Figure 1F, ANG II-treated MCs showed increased labeling for nitrotyrosine.

In vascular smooth muscle cells, ANG II has been demonstrated to generate diacylglycerol formation through phospholipase D-induced phosphatidylcholine hydrolysis (44). We evaluated the effect of propranolol, an inhibitor of phosphatidic acid phosphohydrolase (45), on the ANG II-induced mesangial cell apoptosis. Equal numbers of MCs were preincubated in media containing either buffer or propranolol (100 μM) for 16 hr followed by incubation with either buffer or ANG II (10^{-8} M) for 16 hr.

Subsequently cells were assayed for apoptosis. As shown in Figure 3B, pretreatment with propranolol attenuated ($p < 0.001$) the pro-apoptotic effect of ANG II.

To determine the effect of HO-1 modulation in ANG II-induced mesangial cell apoptosis, equal numbers of MCs were preincubated in media containing either buffer, hemin (5 μM, an inducer of HO-1), or zinc protoporphyrin (50 μM, an inhibitor of HO-1 activity) for 16 hr followed by incubation in media containing either buffer or ANG II (10^{-8} M) for 16 hr. At the end of the incubation period, cells were evaluated for apoptosis. As shown in Figure 3C, hemin inhibited pro-apoptotic effect of ANG II; zinc protoporphyrin enhanced the pro-apoptotic effect of ANG II. These studies suggest that pre-induction of HO activity may attenuate the ANG II-induced apoptosis.

To determine whether propranolol may also be modulating HO activity, equal numbers of MCs were preincubated in media containing either buffer or propranolol (100 μM) for 16 hr followed by incubation in media containing either buffer or ANG II (10^{-8} M) for 16 hr. At the end of the incubation period, cells were harvested and HO activity measured. Propranolol increased ($p < 0.001$) HO activity when compared with control cells (control, 265 $\pm$ 15 versus propranolol, 700 $\pm$ 21 pmol bilirubin/(μg protein/60 min). Because pretreatment of propranolol was associated with attenuation of pro-apoptotic effect of ANG II, it appears that propranolol may also be inhibiting the ANG II-induced oxidative stress by increasing HO activity.

To confirm the role of oxidative stress we studied the effect of ANG II on the generation of ROS such as superoxide and hydrogen peroxide. 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. ANG II promoted MC generation of both superoxide (control, 14.8 $\pm$ 0.9 versus ANG II, 25.5 $\pm$ 0.8 arbitrary units, at 45 min) and hydrogen peroxide (control, 7.5 $\pm$ 0.5 versus ANG II 17.8 $\pm$ 0.8 arbitrary units, at 45 min) at 45 and 90 min. The generation of ROS plateaued at 120 min.

ANG II-induced increase in cytosolic Ca^{2+} has been demonstrated to contribute to mesangial cell

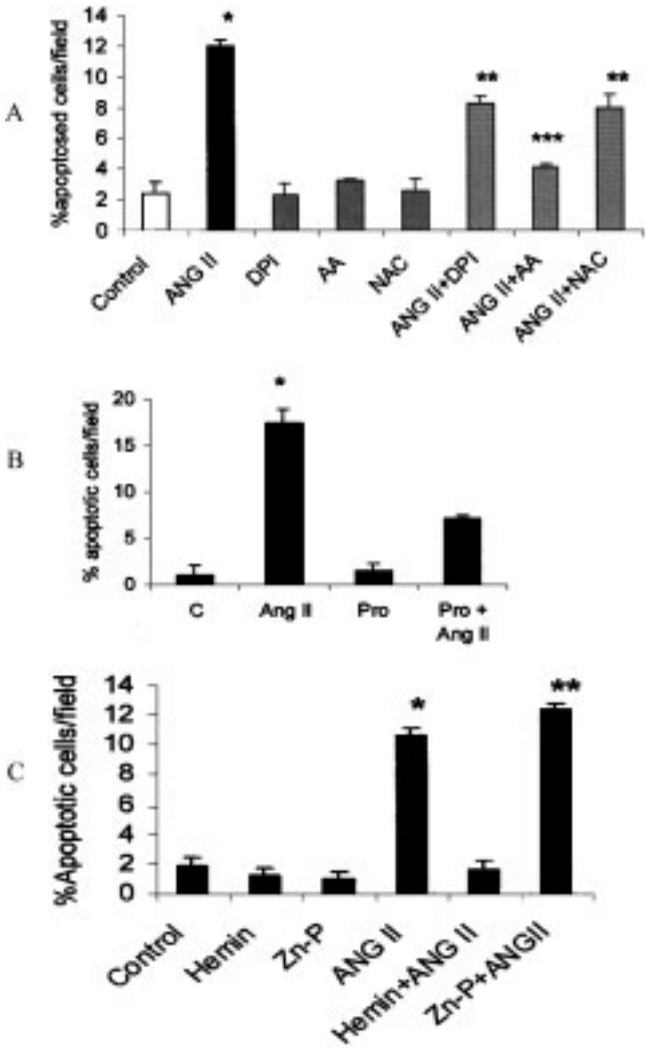

Fig. 3. Role of oxidative stress in ANG II-induced mesangial cell apoptosis. (A) Effect of antioxidants on ANG II-induced mesangial cell apoptosis. Equal numbers of MMCs were incubated in media containing buffer (control), DPI (10 μM), ascorbic acid (AA, 100 μM), or NAC (50 μM) with or without ANG II (10^{-8} M) for 24 hr. Subsequently, cells were stained with H-33342 and PI. Results (means $\pm$ SEM) are from four series of experiments, each carried out in triplicate. *$p < 0.001$ compared with control, ANG II + DPI, ANG II + AA, or ANG II + NAC; **$p < 0.001$ compared with control and respective DPI and NAC; ***$p < 0.001$ compared with ANG II + DPI and ANG II + NAC. **(B)** Effect of propranolol on ANG II-induced mesangial cell apoptosis. Equal numbers of MCs were preincubated in media containing either buffer or propranolol (100 μM) for 16 hr followed by incubation with either buffer or ANG II (10^{-8} M) for 16 hr. Subsequently cells were assayed for apoptosis. *$p < 0.001$ compared with all other variables. **(C)** Effect of hemin and zinc protoporphyrin on ANG II-induced MC apoptosis. Equal numbers of MCs were preincubated in media containing either buffer (control), hemin (5 μM), or zinc protoporphyrin (Zn-P, 50 μM) for 16 hr followed by incubation in media containing either buffer or ANG II (10^{-8} M) for 16 hr. Subsequently, cells were assayed for apoptosis. Results (means $\pm$ SEM) are from eight series of experiments. *$p < 0.001$ compared with control and hemin + ANG II; **$p < 0.05$ compared with ANG II alone.

contractility (46). We studied whether ANG II-induced elevation of Ca^{2+} plays a role in ANG II-induced MC apoptosis. Equal numbers of cells were incubated in media containing either buffer (control), ANG II (10^{-8} M), thapsigargin (THPS, 10 nM; increases cytosolic Ca^{2+}), BAPTA (50 μM; a chelator of Ca^{2+}), ANG II + THPS, or ANG II + BAPTA for 24 hr. Subsequently, cells were assayed for apoptosis. As shown in Figure 4A, ANG II promoted ($p < 0.001$) MC apoptosis. Similarly, THPS also promoted ($p < 0.001$) MC apoptosis. However, BAPTA inhibited the effect of ANG II. On the other hand, THPS in combination with ANG II induced greater MC apoptosis when compared with ANG II or THPS alone (Fig. 4A). These results suggest that ANG II-induced MC apoptosis has a direct relationship with agents that modulate cytosolic calcium.

To determine the direct effect of cytosolic Ca^{2+} on MC apoptosis, we studied the dose–response effect of THPS. Equal numbers of MCs were incubated in media containing either vehicle or variable concentrations of THPS (1, 10, 100 nM) for 24 hr. Subsequently, MCs were assayed for apoptosis. As shown in Figure 4B, THPS induced MC apoptosis in a dose-dependent manner.

To determine whether the effect of TGF-β on MC can also be modulated by altering cytosolic Ca^{2+},

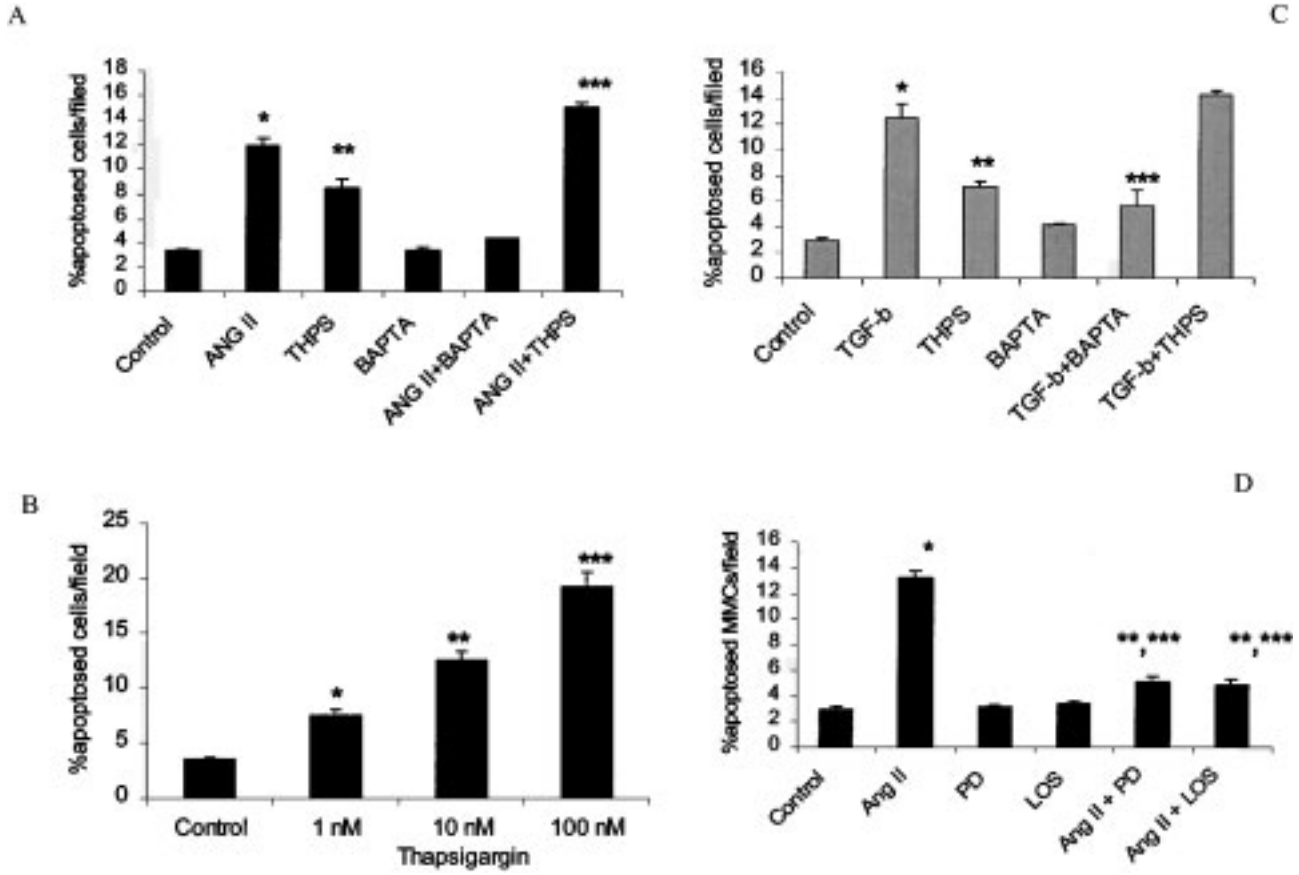

Fig. 4. (A) Effect of THPS and BAPTA on ANG II-induced mesangial cell apoptosis. Equal numbers of cells were incubated in media containing either buffer (control), ANG II (10–8 M), THPS (50 μM), BAPTA (50 μM), ANG II + THPS, or ANG II + BAPTA for 24 hr. Subsequently, cells were assayed for apoptosis. Results (means ± SEM) are from four series of experiments, each carried out in triplicate. *$p < 0.001$ compared with control, THPS, BAPTA, ANG II + BAPTA, ANG II + THPS; **$p < 0.001$ compared with control, ANG II and Ang II + THPS; ***$p < 0.001$ compared with ANG II and THPS alone. **(B)** Dose–response effect of thapsigargin (THPS) on mesangial cell apoptosis. Equal numbers of MMCs were incubated in media containing either vehicle or variable concentrations of THPS (1, 10, 100 nM) for 24 hr. Subsequently, MMCs were stained with H-33342 and PI. Results (means ± SEM) are from four series of experiments, each carried out in triplicate. *$p < 0.01$ compared with control; **$p < 0.001$ compared with control and THPS, 1 nM; ***$p < 0.001$ compared with control and THPS, 1–10 nM. **(C)** Effect of THPS and BAPTA on TGF-β–induced mesangial cell apoptosis. Equal numbers of MMCs were incubated in media containing buffer (control), TGF-β (1 ng/ml), THPS (10 nM), BAPTA (50 μM), TGF-β +BAPTA, or TGF-β + THPS for 24 hr. At the end of the incubation period, cells were assayed for apoptosis. Results (means ± SEM) are from four series of experiments, each carried out in triplicate. *$p < 0.001$ compared with control, THPS, BAPTA, TGF-b + BAPTA; **$p < 0.001$ compared with control; ***$p < 0.05$ compared with control. **(D)** Effect of PD-123319 and losartan on ANG II-induced mesangial cell apoptosis. Equal numbers of MMCs were incubated in media containing either buffer (control) or ANG II (10^{-8} M) in the absence or presence of PD-123319 (PD) or losaratan (LOS) for 24 hr. Cells were then stained for apoptosis. Results (means ± SEM) are from four series of experiments, each carried out in triplicate. *$p < 0.001$ compared with control, ANG II + PD and Ang II + LOS; **$p < 0.01$ compared with control; ***$p < 0.05$ compared with respective PD and LOS alone.

equal numbers of MCs were incubated in media containing either buffer (control), TGF-β (1 ng/ml), THPS (10 nM), BAPTA (50 μM), TGF-β + BAPTA, or TGF-β + THPS for 24 hr. At the end of the incubation period, cells were assayed for apoptosis. TGF-β promoted ($p < 0.001$) apoptosis (Fig. 4C). Similarly, THPS promoted MC apoptosis. The effect of TGF-β was partly inhibited by BAPTA. On the other hand, TGF-β + THPS induced greater apoptosis when compared with THPS alone. These findings suggest that cytosolic Ca^{2+} modulating agents determine pro-apoptotic effect of TGF-β.

To determine the role of AT1 and AT2 receptors, we evaluated the effect of losartan (LOS), an AT1 antagonist, and PD-123319, an antagonist of AT2 receptor, on ANG II-induced MC apoptosis. Equal numbers of MCs were incubated in media containing either vehicle (control), ANG II (10^{-8} M), losartan (LOS, 10^{-7} M), PD-123319 (PD, 10^{-7}M), PD + ANG II, or LOS + ANG II for 24 hr. Subsequently, cells were assayed for apoptosis. ANG II promoted ($p < 0.001$) MC apoptosis (Fig. 4D). However, both PD and LOS attenuated ($p < 0.001$) the apoptotic effect of ANG II.

To determine the role of various inhibitors of NADPH oxidase on ANG II-induced mesangial cell superoxide production, equal numbers of MCs were incubated in media containing either buffer (control), DPI (10 μM), propranolol (100 μM), BAPTA (50 μM), anti–TGF-β antibody in the presence or absence of ANG II (10^{-8} M) for 2 hr. Supernatants were collected at 0, 30, 45, 60, 90, and 120 min and evaluated for superoxide content. As shown in Figure 5A, ANG II enhanced MC production of superoxide. DPI, propranolol, BAPTA, and anti–TGF-β antibody attenuated this effect of ANG II.

Because apoptosis proceeds via caspase-3 activation, we evaluated the effect of blockade of various postulated signal transduction pathways on ANG II-induced caspase-3 activation. Equal numbers of cells were incubated in media containing either buffer (control), anti–TGF-β antibody (1 μg/ml), DPI (10 μM), L-NAME (1 mM) in the presence or absence of ANG II (10^{-8} and 10^{-6} M) for 3 hr. Subsequently, caspase-3 activity was determined. As shown in Figure 5B, ANG II promoted ($p < 0.001$) MC caspase-3 activities. Anti–TGF-β partially inhibited this effect of ANG II. Similarly, both DPI and L-NAME partially inhibited ANG II-induced caspase-3 activity. These findings suggest that ANG II-induced pro-apoptotic

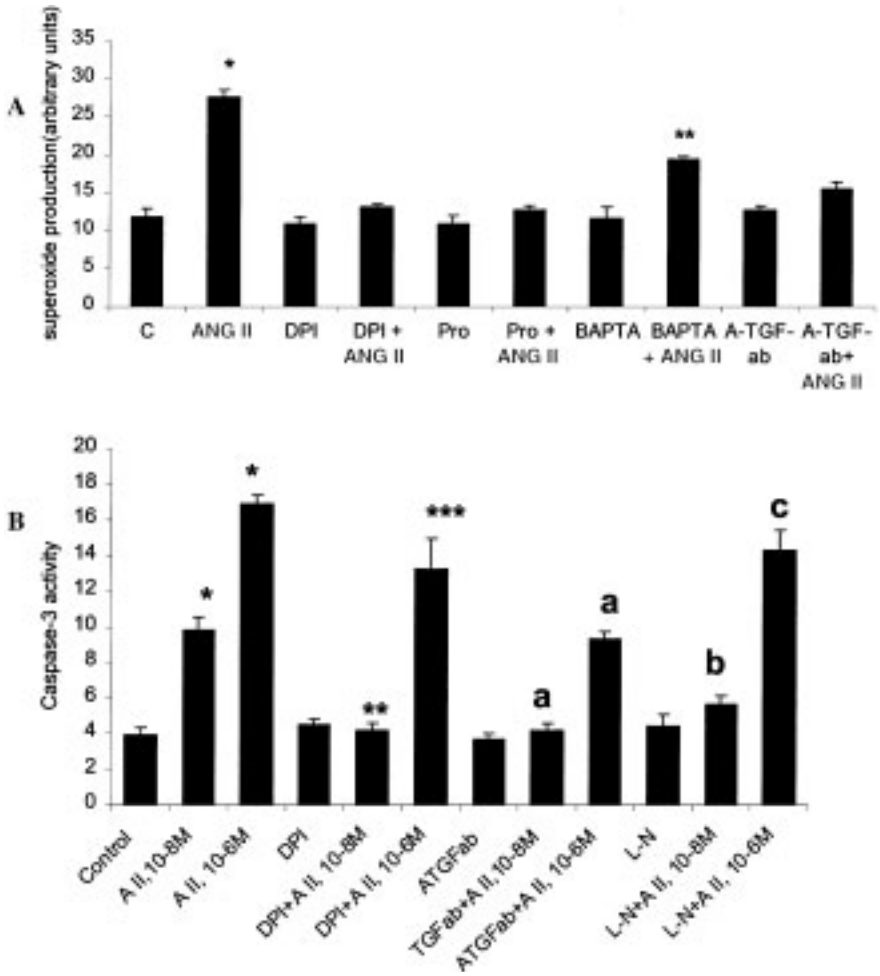

Fig. 5. (A) The effects of various inhibitors of NADPH oxidase on ANG II-induced mesangial cell superoxide production. Equal numbers of MCs were incubated in media containing either buffer (control), DPI (10 μM), propranolol (Pro, 100 μM), BAPTA (50 μM), anti–TGF-β antibody (A-TGF-ab, 1 μg/ml) in the presence or absence of ANG II (10^{-8} M) for 2 hr. Supernatants were collected at 30, 60, 90, and 120 min and evaluated for superoxide content. Results (means ± SEM) are from four series of experiments. *$p < 0.001$ compared with control, DPI + ANG II, propranolol + ANG II, and anti-TGF-β antibody + ANG II; **$p < 0.01$ compared with ANG II alone. **(B)** Effect of anti–TGF-β antibody, DPI and L-NAME on ANG II-induced caspase-3 activity. Equal numbers of cells were incubated in media containing either buffer (control), anti–TGF-β antibody (ATGFab, 1 μg/ml), DPI (10 μM), L-NAME (L-N, 1 mM) in the presence or absence of ANG II (10^{-8} and 10^{-6} M) for 3 hr. Subsequently, caspase-3 activity was determined. Results are four series of experiments. *$p < 0.001$ compared with control, DPI alone, ATGFab alone, and L-N alone; **$p < 0.001$ compared with ANG II, 10^{-8} M alone; ***$p < 0.01$ compared with ANG II, 10^{-6} M alone; [a]$p < 0.001$ compared with respective concentrations of ANG II; [b]$p < 0.001$ compared with ANG II, 10^{-8} M alone; [c]$p < 0.05$ compared with ANG II, 10^{-6} M alone.

effect is mediated through NADPH oxidase, iNOS, and TGF-β.

Discussion

The present study demonstrates that ANG II induces mesangial cell apoptosis. Because ANG II stimulated MC superoxide and H_2O_2 formation and antioxidants such as ascorbic acid, DPI, and NAC prevented ANG II-induced MC apoptosis, this effect of ANG II appears to be mediated through oxidative stress. Interestingly, anti–TGF-β antibody not only inhibited the pro-apoptotic effect of ANG II but TGF-β also induced MC apoptosis; thus, it appears that ANG II-induced MC apoptosis may be mediated through TGF-β. Moreover, anti–TGF-β inhibited ANG II-induced MC superoxide production. Because propranolol inhibited ANG II-induced MC superoxide production it appears that ANG II-induced activation of NADPH oxidase activation occurs through the activation of phospholipse D. BAPTA inhibited the pro-apoptotic effect of ANG II as well as TGF-β. On the other hand, thapsigargin promoted MC apoptosis under basal as well as ANG II/TGF-β stimulated states. These findings suggest that alteration of cytosolic Ca^{2+} directly correlates with the pro-apoptotic effects of ANG II and TGF-β. Because L-NAME partially inhibited ANG II-induced MC apoptosis, it appears that NO also plays a role in the pro-apoptotic effect of ANG II. In addition, HO-1 activity modulates the pro-apoptotic effect of ANG II.

ANG II has been demonstrated to play an important role in the development of FGS (1,8). The mechanism by which ANG II contributes to this lesion involves both direct and hemodynamic effects (2,9,11). Continuous infusion of ANG II in the isolated perfused kidney resulted in a loss of glomerular size permselectivity and an increase in urinary protein excretion rate (47). Similarly, transfection of genes for rennin and angiotensinogen into the rat kidney resulted in phenotypic changes of the mesangial cells and development of glomerulosclerosis (48). In in vivo studies, ANG II has also been demonstrated to promote renal cell growth and accumulation of extracellular matrix proteins (2,9).

Loss of resident mesangial cells and accumulation of mesangial matrix is a predominant characteristic of FGS (5,7,8). It has been suggested that an imbalance between cell survival and cell death may result in a loss of glomerular cells that may be responsible for the development as well as progression of FGS (25,27,32,49). ANG II, in addition to its growth-promoting effects, has been demonstrated to trigger apoptosis in several renal and nonrenal cells in culture (30–34,50). In the present study, we evaluated whether ANG II can directly affect the survival of mesangial cells irrespective of its hemodynamic effects. Our data clearly indicate that ANG II induces mesangial cell apoptosis and thus is able to contribute directly to the loss of mesangial cells. However, in vivo, ANG II may also be contributing to mesangial cell apoptosis by elevating glomerular capillary pressure (41).

Saleh et al. (51) previously demonstrated that thapsigargin promoted mesangial cell apoptosis through the elevation of cytosolic calcium. In the present study, thapsigargin also induced mesangial cell apoptosis in a dose-dependent manner. Interestingly, thapsigargin when used in combination with ANG II showed greater apoptosis when compared with thapsigargin or ANG II alone. On the other hand BAPTA, which decreases cytosolic calcium, attenuated ANG II-induced mesangial cell apoptosis. These results suggest that modulation of cytosolic calcium has direct correlation with the occurrence of apoptosis.

TGF-β has been demonstrated to have a bimodal effect on mesangial cell growth (18). We previously reported that TGF-β–induced suppression of MC growth is partly contributed by the induction of apoptosis (18). Similarly, TGF-β is also involved in the apoptosis of cultured tubular and glomerular epithelial cells (33,34). In the present study, we found that ANG II-induced apoptosis was prevented by anti–TGF-β antibody. Because many of the effects of ANG II are mediated through the generation of TGF-β by MCs, we suggest that the pro-apoptotic effect of ANG II may also be contributed by TGF-β.

There is increasing evidence that ANG II enhances oxidative stress (52). Activation of both systemic and tissue renin-angiotensin enhances the production of ROS primarily through the activation of membrane-bound NADH and NADPH oxidase (53,54). These oxidase enzymes are present in mesangial cells, endothelial cells, fibroblasts, vascular smooth cells, and phagocytic mononuclear cells (53,54). The increased cellular activity of NADH and NADPH oxidase enhances the production of reactive oxygen species by multiple pathways, including the increased activation of xanthine oxidase, the autooxidation of NADH, and the inactivation of superoxide dismutase. In addition, the interaction of ROS with NO leads to the production of peroxynitrite, a potent and relatively stable oxidant that may also induce mesangial cell injury (54,55). Studies of a number of cell types and organs have shown that oxidative stress leads to putative evidence of peroxynitrite formation and tissue damage (56,57). Peroxynitrite has been shown to induce apoptosis in a wide variety of cells, including renal mesangial and epithelial cells (57).

We propose the role of ANG II in the induction of oxidative stress in the activation of caspase-3 and subsequent apoptosis as outlined in Figure 6. ANG II enhances the production of superoxide and hydrogen peroxide. Because DPI (an inhibitor of NADPH oxidase) inhibited MC superoxide production as well as apoptosis it appears that ANG II-induced

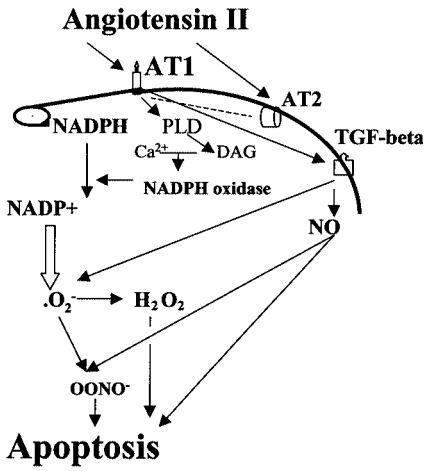

Fig. 6. Proposed hypothesis for ANG II-induced MC apoptosis.

MC superoxide production and apoptosis are mediated through the activation of NADPH oxidase. Similarly, propranolol, an inhibitor of phospholipase D pathway (44), also inhibited ANG II-induced MC superoxide production as well as apoptosis thus, suggesting the role of phospholipase D activation in the ANG II-induced oxidative stress. Because agents that decrease cytosolic Ca^{2+} also induced a reduction in ANG II-mediated apoptosis, it appears that Ca^{2+} is also playing a role in ANG II mediated oxidative stress. Because anti–TGF-β antibody attenuated ANG II-induced ROS generation as well as apoptosis, it appears that ANG II-mediated downstream signaling is mediated thorough TGF-β. In addition, L-NAME, an inhibitor of iNOS, partially inhibited ANG II-induced MC apoptosis. Therefore, NO also seems to be playing a role perhaps through peroxynitrite formation as suggested by immunocytochemical studies. In addition, we previously demonstrated that TGF-β promotes NO production by MCs.

We conclude that ANG II can induce MC apoptosis in culture and that pro-apoptotic effects are mediated by both AT_1 and AT_2 receptors. This effect of ANG II involves downstream signaling through TGF-β, NADPH oxidase, iNOS, and casapse-3. These findings suggest the role of ANG II contributing to pathobiology of glomerulosclerosis.

Acknowledgment

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