Kidney International, Vol. 68 (2005), pp. 2143–2153

VASCULAR BIOLOGY – HEMODYNAMICS – HYPERTENSION

Up-regulation of glomerular COX-2 by angiotensin II: Role of reactive oxygen species

EDGAR A. JAIMES, RUN-XIA TIAN, DAMIEN PEARSE, and LEOPOLDO RAIJ

Nephrology Section, VA Medical Center, Miami, Florida; Renal Division, University of Miami, Miami, Florida; Vascular Biology Institute, University of Miami, Miami, Florida; and The Miami Project to Cure Paralysis, University of Miami, Miami, Florida

Up-regulation of glomerular COX-2 by angiotensin II: Role of reactive oxygen species.

Background. Prostaglandins such as prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) counteract the angiotensin II (Ang II)–induced vasoconstriction in the glomerular microcirculation. We have shown that Ang II promotes mesangial cell hypertrophy via reactive oxygen species (ROS), which originate from nicotinamide adenine dinucleotide phosphate and its reduced form (NADH/NADPH) oxidase. It has been reported that conditions associated with activation of the renin-angiotensin system result in increased glomerular cyclooxygenase-2 (COX-2) expression and activity.

Methods. We designed studies to determine (*1*) whether Ang II induces COX-2 in the glomerulus in vivo in the glomerulus as well as in vitro in mesangial cells, (*2*) whether ROS originated from Ang II are involved, and (*3*) whether COX-2–derived prostaglandins modulate the growth promoting effects of Ang II in mesangial cells. Rats were infused with Ang II (0.7 mg/kg/day) for 5 days and glomerular COX-2 expression and activity assessed in isolated glomeruli.

Results. Ang II increased glomerular PGE_2 production (100%) accompanied by a concomitant increase in glomerular COX-2 expression at the mRNA (1.7-fold) and protein level (sixfold). In mesangial cells, Ang II significantly increased mesangial cell PGE_2 (200%) and PGI_2 (100%) production as well as COX-2 mRNA that was prevented by the angiotensin type 1 (AT1) receptor blocker irbesartan and the COX-2 inhibitor NS-398. The NADPH oxidase inhibitor diphenyleneiodonium (DPI), the ROS scavenger tiron as well as catalase, inhibited Ang II–induced PGE_2 production suggesting that Ang II–induced ROS mediate COX-2 up-regulation. Strikingly, COX-2 inhibition as well as blockade of the type 1 PGE_2 receptor (EP1) prevented Ang II–induced mesangial cell hypertrophy suggesting that COX-2–derived prostaglandins, and specifically PGE_2, importantly contribute to the growth promoting effects of Ang II.

Conclusion. These studies suggest that blockade of specific PGE_2 receptors may be a novel strategy to modulate the pathologic effects of COX-2–derived prostaglandins without simultaneously affecting protective vasodilatory mechanisms.

Key words: angiotensin II, cycloxygenase-2, kidney glomerulus, reactive oxygen species, hypertrophy, prostaglandins, glomerular mesangium.

Received for publication February 9, 2003
and in revised form August 6, 2004, September 20, 2004, November 8, 2004, and April 25, 2005
Accepted for publication June 1, 2005

The countervailing interaction between angiotensin II (Ang II) and prostaglandins plays a critical role in the modulation of the renal microcirculation [1, 2]. Prostaglandins are produced in large amounts in the glomerulus in response to a variety of stimuli, including vasoactive agents such as Ang II [3–5], growth factors [6], and proinflammatory cytokines [7]. Prostaglandin E_2 (PGE_2) is quantitatively the major prostaglandin produced in the kidney and it has been shown to have both vasoconstrictory and vasodilatory actions on the renal microcirculation [8, 9]. Other prostaglandins produced in the kidney include prostaglandin I_2 (PGI_2) that has predominant vasodilatory effects and thomboxane A_2 (TxA_2), a vasoconstrictory prostanoid [10].

Ang II and prostaglandins have important nonhemodynamic effects that modulate the glomerular response to injury. Ang II promotes mesangial cell hypertrophy and proliferation [11–13] and increases extracellular matrix deposition [14]. We have shown that in mesangial cells these actions of Ang II are dependent to a great extent upon the activation of protein kinase C (PKC) and nicotinamide adenine dinucleotide phosphate and its reduced form (NADH/NADPH) oxidase which results in the generation of reactive oxygen species (ROS) and the activation of mitogen-activated protein kinases (MAPKs) [11]. PGE_2 has been reported to modulate mesangial cell proliferation in response to interleukin (IL)-1 [15] and platelet-derived growth factor (PDGF) [16] and to reduce extracellular matrix expression [17]; however, other studies have shown the opposite, namely that PGE_2 mediates mesangial cell proliferation [18]. PGI_2 has been shown to inhibit while TxA_2 promotes mesangial cell proliferation[19–21].

Two enzymes are rate limiting in the synthesis of prostaglandins: phospholipase A_2 (PLA_2), which catalyzes the release of arachidonic acid from phospholipids in cell membranes, and cyclooxygenase (COX), which

catalyzes the conversion of arachidonic acid to PGG_2 and to PGH_2 [22]. Two forms of COX have been identified, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types [23, 24]. COX-2 is induced in response to a variety of stimuli, including cytokines [7] and growth factors [25, 26]. However, some of the pathways involved in the glomerular synthesis of prostaglandins via COX-2 as well the biologic consequences of glomerular prostaglandins synthesis remain poorly understood. In combined in vivo and in vitro studies we tested the hypothesis that Ang II modulates the expression and activity of glomerular COX-2 and that in return prostanoids derived from COX-2 modulate Ang II actions. In the in vitro studies we focused on mesangial cells because of the critical role of the glomerular mesangium in modulating the glomerular microcirculation in response to vasoactive agents. Furthermore, the same vasoactive agents promote mesangial cell hypertrophy and proliferation, as well as increased matrix production [27]. Herein, we demonstrate that Ang II induces glomerular COX-2 expression and activity in vivo as well as in vitro in cultured mesangial cells. These effects were found to be dependent on Ang II–mediated ROS generation and MAPKs activation. We also demonstrate, for the first time, that COX-2–derived prostaglandins mediate in large part the growth promoting effects of Ang II in mesangial cells.

METHODS

In vivo studies

Six-week-old Sprague-Dawley male rats were purchased from Harlan (Indianapolis, IN, USA) and maintained under controlled conditions of light, temperature, and humidity. Rats were divided in two groups: (*1*) control ($N = 9$), implanted with vehicle and (*2*) Ang II ($N = 9$), infused with Ang II via osmotic minipump at 0.7 mg/kg/day for 5 days. Minipumps (Alzet model 1007D; Alzet, Cupertino, CA, USA) were implanted in the midscapular region under anesthesia with a cocktail of ketamine (45 mg/kg intraperitoneally), xylazine (8 mg/kg intraperitoneally), and acepromazine (1.5 mg/kg intraperitoneally). Rats were sacrificed on day 5 and kidney tissue saved for immunohistochemistry. Blood pressure was measured before sacrifice by the tail-cuff method. In addition glomeruli were isolated by sieving as described [28] and used for reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and PGE_2 measurements.

In separate experiments, rats were divided into three groups: (*1*) Control ($N = 6$), (*2*) Ang II ($N = 6$), infused with Ang II via osmotic minipump at 0.7 mg/kg/day for 5 days and injected with the specific COX-2 inhibitor NS-398 (1.5 mg/kg subcutaneously) 12 hours and 1 hour prior to sacrifice. Rats were sacrificed on day 5, and glomeruli isolated for PGE_2 measurements.

Glomerular PGE_2 measurements. Isolated glomeruli from the two experimental groups were incubated in RPMI 1640 with arachidonic acid 10^{-5} mol/L at 37°C for 1 hour with and without the COX-2 inhibitors NS-398 (10^{-7} mol/L), nimesulide (10^{-6} mol/L), or the nonspecific COX inhibitor indomethacin (10^{-5} mol/L). At the end of this incubation, the glomeruli were centrifuged, and protein assayed. PGE_2 was measured in the supernatant by enzyme immunoassay (EIA) (Cayman, Ann Arbor, MI, USA) and factored by glomerular protein content as measured by the Bio-Rad method (Hercules, CA, USA).

Western blot. After isolation, glomeruli were homogenized and protein separated on 6% sodium dodecyl sulfate (SDS) gels under reducing conditions and transferred to a nitrocellulose membrane (Hybond ECL) (Amersham Biosciences Corp., Piscataway, NJ, USA). The blots were incubated overnight with rabbit antimurine polyclonal antibody to COX-2 (Cayman; catalogue number 160126), rabbit monoclonal antibody to COX-1 (Cayman; catalogue number 160110), or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing the blots were incubated with goat antirabbit antibody (Santa Cruz Biotechnology) for 1 hour at a 1:2000 dilution and the signal detected by luminol chemiluminescence.

Cox mRNA expression. COX-1 and COX-2 mRNA expression in whole glomeruli was determined by RT-PCR. Total RNA was isolated by lysing the glomeruli in Trizol reagent (Life Technologies, Philadelphia, PA, USA) and by precipitation in isopropyl alcohol. A 5 μg aliquot of total RNA was used for cDNA synthesis with the Superscript Preamplification System (Life Technologies). An aliquot of cDNA was amplified using Taq polymerase in the presence of sense and antisense primers for rat COX-2: forward 5′-CAT TCT TTG CCC AGC ACT TCA C-3′, reverse 5′-GAC CAG GCA CCA GAC CAA AGA C-3′; rat COX-1: forward 5′-GTG AGC TAC TAT ACT CGC ATT C-3′, reverse 5′-GCC AAA CCT CTT TCG GTA TTC-3′; and glyderaldehyde-3-phosphate dehydrogenase (GAPDH); forward 5′-CCT TCA CTA CAT GGT CTA CAT G-3′, reverse 5′-GTT GAG CTC AGG GAT GAC CTT-3′.

Immunohistochemistry. Rats were sacrificed by decapitation, kidneys harvested and fixed in 10% paraformaldehyde. For antigen retrieval, slides were immersed in target retrieval solution (DakoCytomation, Carpinteria, CA, USA) for 30 minutes at 90°C. COX-2 immunoreactivity was localized with polyclonal rabbit antimurine COX-2 antibody (Cayman),polyclonal rabbit antimurine COX-1 antibody (Cayman) or polyclonal smooth muscle actin (Santa Cruz Biotechnology). As secondary antibodies we used goat antirabbit IgG (Santa Cruz Biotechnology). The first antibody was localized using Vectastain ABC-Elite (Vector, Burlingame, CA, USA) with either diaminobenzidine (DAB) or alkaline phosphatase as chromogen.

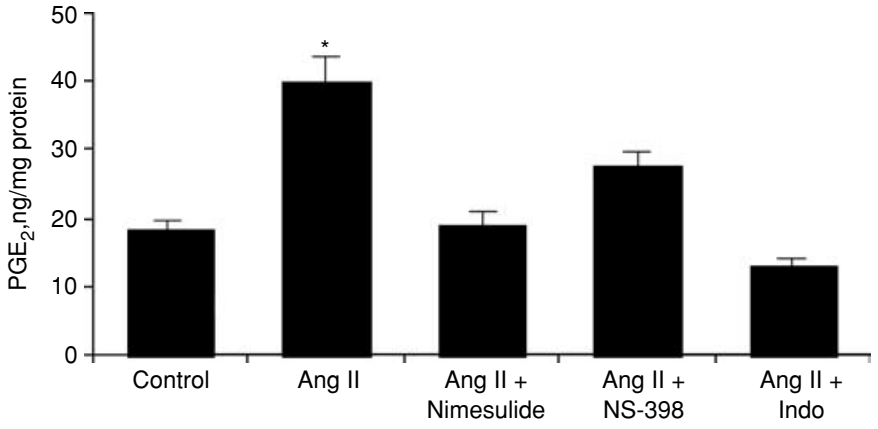

Fig. 1. Angiotensin II (Ang II) infusion increases glomerular production of prostaglandin E_2 (PGE_2) that is inhibited by two different cyclooxygenase (COX-2) inhibitors as well as by indomethacin (Indo). Results are expressed as mean ± SEM ($N = 6$). $^*P < 0.05$ compared to all other conditions.

In situ hybridization

In situ hybridization studies were performed in kidneys from control and Ang II–infused rats. For COX-2 probe preparation, a plasmid containing the COX-2 cDNA (Cayman) was used to generate sense and antisense riboprobes using a digoxigenin (DIG) RNA labeling kit (Boehinger-Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol. Prior to probe hybridization, tissue was formalin-fixed and embedded in paraffin and sectioned under RNAse-free conditions at 4 μm.

In vitro studies

Primary mesangial cell cultures were prepared from male Sprague-Dawley rats as previously described [28, 29]. Cells were grown in RPMI 1640 media (Gibco, Grand Island, NY, USA) supplemented with 17% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT, USA).

Mesangial cells were made quiescent in RPMI 1640/0.5% FCS for 72 hours and exposed to Ang II (10^{-5} to 10^{-9} mol/L) for 24 hours with and without the specific COX-2 inhibitor NS-398 (10^{-7} mol/L), the nonspecific COX inhibitor indomethacin (10^{-5} mol/L), the NADPH oxidase inhibitor dyphenilene iodinium (DPI) (10^{-5} mol/L), the ROS scavenger Tiron (5×10^{-3} mol/L), the angiotensin type 1 (AT1) receptor blocker irbesartan (10^{-6} mol/L) or the MAPK-1 inhibitor PD98059 (10^{-6} mol/L). At the end of the incubation period media was collected and saved for prostaglandin determinations. PGE_2, 6-keto-$PGF_{1\alpha}$, the stable PGI_2 metabolite, and TxB_2, the stable TxA_2 metabolite, were measured by EIA (Cayman), factored for cell protein content, and expressed as ng/mg protein.

In separate experiments we measured COX-2 and COX-1 mRNA expression by RT-PCR in mesangial cells exposed to Ang II (10^{-7} mol/L) for 1 hour with and without the NADPH oxidase inhibitor DPI.

3H-leucine incorporation. Mesangial cell protein synthesis was measured by ^{3}H-leucine incorporation as previously described [11] and used as an index of mesangial cell hypertrophy. Mesangial cells were grown in 6-well dishes, made quiescent in RPMI 1640/0.5% FCS for 72 hours. Mesangial cells were then incubated for 48 hours with Ang II (10^{-7} mol/L) with and without the specific COX-2 inhibitors NS-398 (10^{-6} to 10^{-7} mol/L) or nimesulide (10^{-6} to 10^{-7} mol/L), the nonspecific COX inhibitor indomethacin (10^{-5} mol/L), or the specific COX-1 inhibitor SC-560 (10^{-7} mol/L). Six hours before harvesting, mesangial cells were pulsed with ^{3}H-leucine 2 μCi/mL. After solubilization with SDS, duplicate aliquots were counted in a scintillation counter and the results were expressed as percent of change vs. control.

RESULTS

In vivo studies

Ang II induces glomerular COX-2 expression and activity. Infusion of Ang II for 5 days increased systolic blood pressure (controls 130 ± 5 mm Hg vs. Ang II 170 ± 7 mm Hg) ($P < 0.05$) and resulted in significant increases in the glomerular production of PGE_2 (Fig. 1) that was significantly inhibited by two different COX-2 inhibitors suggesting that increased COX-2 activity mediates in large part the glomerular production of PGE_2 in response to Ang II. In separate experiments we also determined the effect of COX-2 inhibition on the production of PGE_2 in glomeruli from control rats. COX-2 inhibition did not inhibit the glomerular production of PGE_2 suggesting that our glomerular preparation was devoid of structures known to have high COX-2 expression such as the macula densa [control 8.7 ± 0.8 ng/mg protein vs. NS-398 (10^{-6} mol/L) 7.91 ± 1.2 ng/mg protein] (P = NS) ($N = 3$). The nonspecific COX inhibitor indomethacin also reduced the glomerular production of PGE_2 to levels slightly below those from control glomeruli but without reaching statistical significance (Fig. 1).

In a separate group of rats we evaluated the effect of the specific COX-2 inhibitor NS-398 administered systemically on the glomerular production of PGE_2 in

	COX-1	COX-2	Actin	COX-1/Actin	COX-2/Actin
Control ($N = 6$)	77.9 ± 6	29.4 ± 20	37.5 ± 0.2	2.1 ± 0.16	0.8 ± 05
Ang II ($N = 6$)	106.2 ± 1.9	173.2 ± 45	37.6 ± 0.1	2.8 ± 0.06*	4.6 ± 1.2*

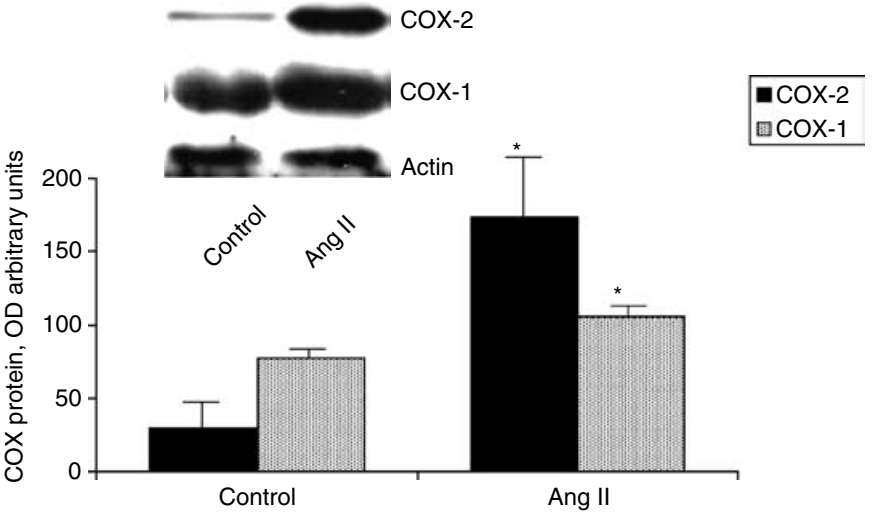

Fig. 2. Angiotensin II (Ang II) infusion increases glomerular cyclooxygenase (COX-2) protein expression and at a smaller degree COX-1 as assessed by Western blot. Table includes the densitometric numeric values adjusted and unadjusted for actin. Results are expressed as mean ± SEM ($N = 6$). *$P < 0.05$ vs. respective control. OD is optical density.

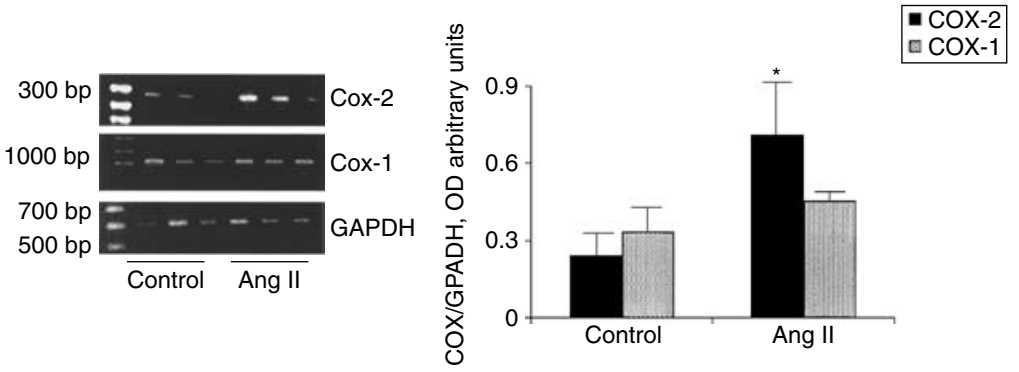

Fig. 3. Angiotensin II (Ang II) infusion increases glomerular cyclooxygenase (COX-2) mRNA expression without significantly modifying COX-1 mRNA expression. Results are expressed as mean ± SEM ($N = 3$). *$P < 0.05$. GAPDH is glyeraldehyde-3-phosphate dehydrogenase; OD is optical density.

rats infused with Ang II. Similar to our experiments utilizing the ex vivo incubation with NS-398, the systemic administration of NS-398 significantly reduced the glomerular production of PGE_2 (control 6.7 ± 0.7 ng/mg PGE_2, Ang II 10.7 ± 0.7 ng/mg PGE_2, Ang II + NS-398 8.2 ± 1.4 ng/mg PGE_2) ($N = 6$) (*$P < 0.05$ vs. control and Ang II + NS-398). Importantly, the systemic administration of NS-398 did significantly modify the increases in blood pressure induced by Ang II (control 130 ± 5 mm Hg, Ang II 168 ± 4 mm Hg, Ang II + NS-398 165 ± 1 mm Hg) (*$P < 0.05$ vs. control).

To determine whether these changes in COX-2 activity were the result of an increase in COX-2 protein expression, we measured COX-2 by Western blot in rats infused with Ang II. As shown in Figure 2, Ang II infusion significantly increased COX-2 protein expression (5.7-fold COX-2/actin ratio compared to control) in the glomerulus. These changes in COX-2 protein expression and activity were accompanied by a concomitant increase in COX-2 mRNA expression suggesting that Ang II increases COX-2 expression via transcriptional mechanisms (Fig. 3). In addition, we determined whether Ang II modified glomerular COX-1 protein and mRNA expression. As shown in Figure 2, Ang II infusion resulted in a significant increase in COX-1 protein expression (1.3-fold COX-1/actin ratio compared to control) that correlated with an increase in glomerular COX-1 mRNA expression (Fig. 3).

To determine the location of COX-2 in the glomerulus where COX-2 is up-regulated, we performed

immunohistochemistry studies in kidneys from control and Ang II–infused rats. Positive stain for COX-2 was clearly observed in the macula densas and thick ascending limb of both control as well as Ang II–infused rats (Fig. 4A). In control rats no COX-2 was detected in the glomerulus (Fig. 4B); however, in Ang II–infused rats we observed glomeruli with positive stain for COX-2 localized mostly at the level of the glomerular epithelium. (Fig. 4C). Given the critical role of the glomerular mesangium in the pathogenesis of glomerular injury [27], we performed dual labeling immunohistochemical studies to determine whether the glomerular mesangium expresses COX-2 in vivo after Ang II infusion. We used smooth muscle actin as a marker for the glomerular mesangium. By using this method we were not able to conclusively detect increases in mesangial COX-2 expression (not shown). In addition, we performed immunohistochemistry for COX-1 in controls as well as in rats infused with Ang II. We observed COX-1 baseline expression in the glomeruli of control rats, but were unable to appreciate any significant difference when compared with Ang II–infused rats (not shown).

By in situ hybridization we clearly detected significant increases in COX-2 mRNA expression in the glomerulus of rats infused with Ang II suggesting that transcriptional mechanisms are involved (Fig. 5).

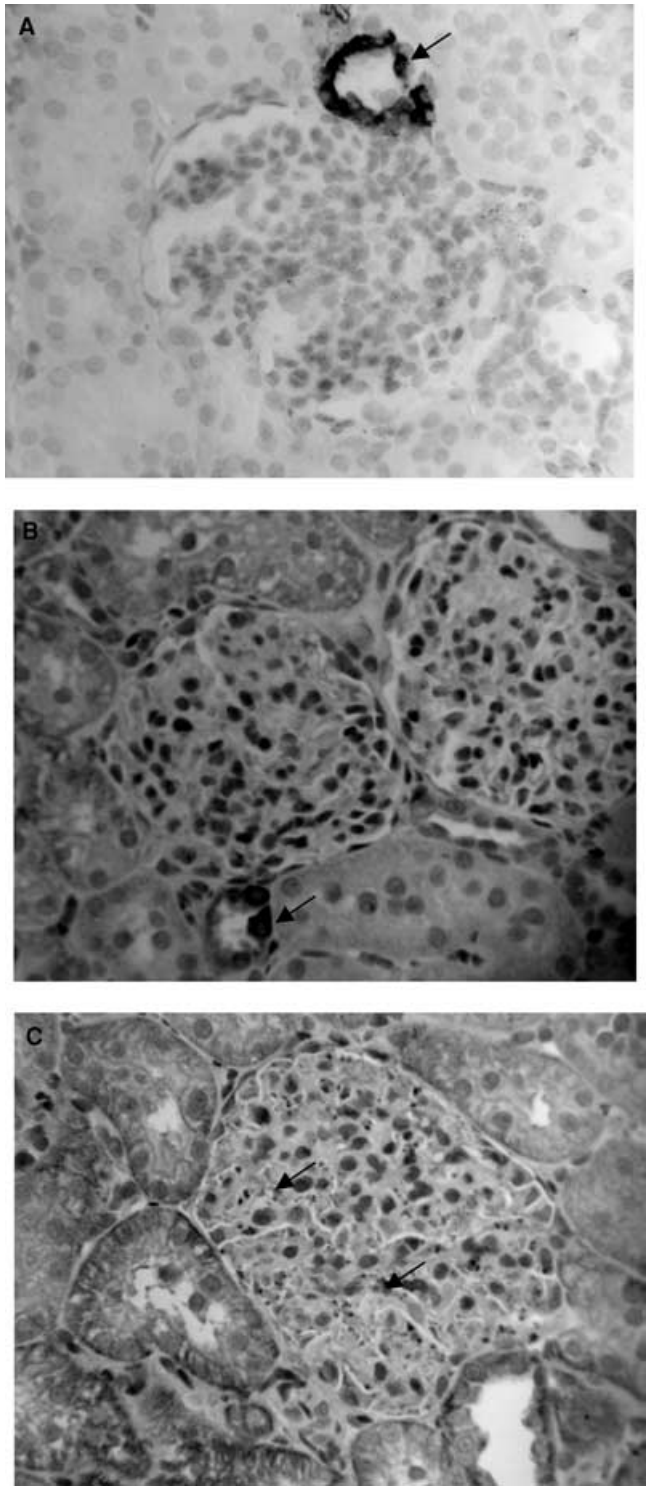

Fig. 4. Angiotensin II (Ang II) increases the glomerular expression of cyclooxygenase (COX-2). On basal conditions positive stain for COX-2 was observed at the level of the macula densa (*A*, arrow) but no significant COX-2 expression was observed in the glomerulus (A and *B*). In the glomeruli from rats infused with Ang II, a significant number of cells were found positive for COX-2 (*C*, arrows).

In vitro studies

Ang II induces COX-2 expression and activity in mesangial cells. To better explore the mechanisms by which Ang II induces COX-2 expression and activity in the glomerulus, we performed the following experiments in cultured mesangial cells. Mesangial cells exposed to Ang II (10^{-9} to 10^{-5} mol/L) for 24 hours significantly increased PGE_2 production in a dose-dependent manner (Fig. 6). This increase in PGE_2 production was blocked by the AT1 receptor blocker irbesartan, suggesting that these effects of Ang II on PGE_2 production were mediated through AT1 receptor activation (Fig. 7). In a similar manner the Ang II–stimulated production of PGI_2 was also inhibited by AT1 receptor blockade (Fig. 7). In contrast, Ang II did not significantly increase TxA_2 production (control 6.4 ± 0.3 ng/mg protein vs. Ang II 6.6 ± 0.2 ng/mg protein) ($N = 3$) ($P =$ NS) which support previous studies suggesting that cultured mesangial cells lack the isomerase that coverts PGH_2 into TxA_2 [30, 31]. Similar to our findings in vivo, the specific COX-2 inhibitor NS-398 prevented the production of PGE_2 and PGI_2 in mesangial cells stimulated with Ang II (Fig. 7). Furthermore, these increases in COX-2 activity were accompanied by concomitant increases in COX-2 mRNA expression (Fig. 8). In contrast, mesangial cell COX-1 mRNA expression was not modified by Ang II stimulation (Fig. 8) and COX-1

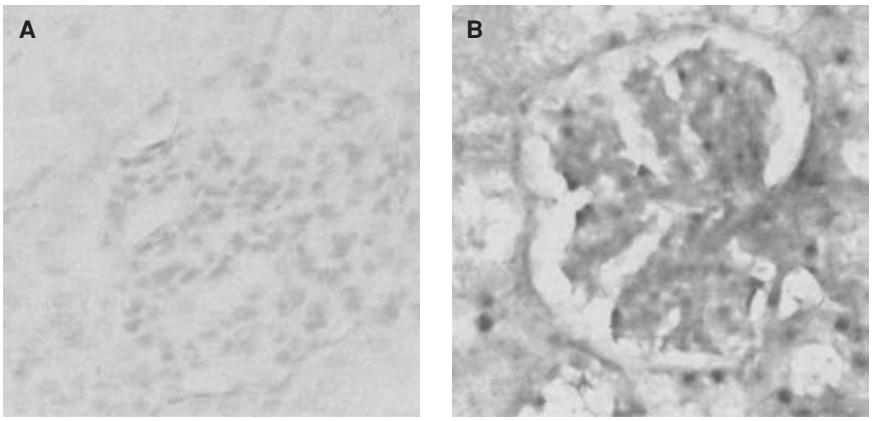

Fig. 5. Angiotensin II (Ang II) increases glomerular cyclooxygenase (COX-2) mRNA expression. On basal conditions no glomerular COX-2 mRNA expression was observed (*A*). In glomeruli from Ang II–infused rats a significant number of glomerular cells are positive for COX-2 mRNA expression (*B*).

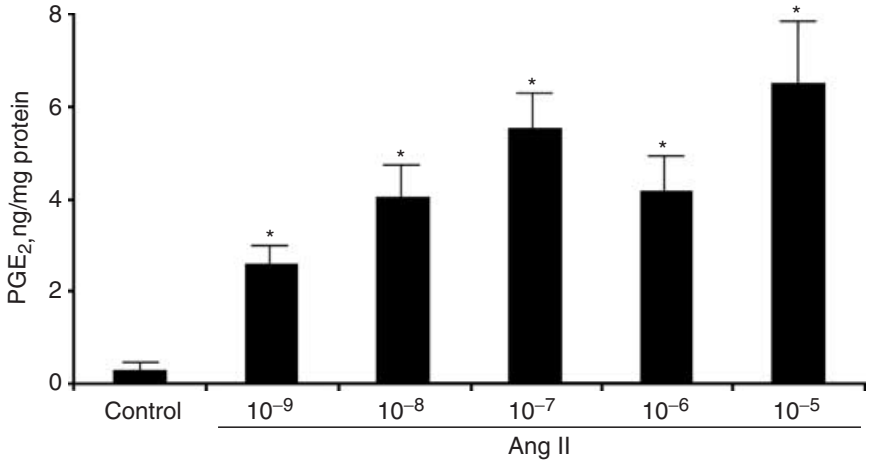

Fig. 6. Angiotensin II (Ang II) increased prostaglandin E_2 (PGE_2) production in cultured mesangial cells in dose-dependent manner. Results are expressed as mean ± SEM ($N = 6$). $^*P < 0.05$ vs. control.

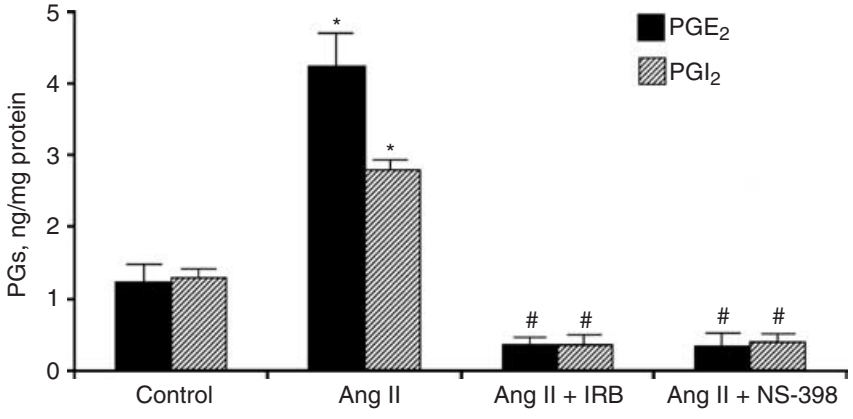

Fig. 7. The angiotensin type 1 (AT1) receptor blocker irbesartan (IRB) (10^{-6} mol/L) and the cyclooxygenase (COX-2) inhibitor NS-398 (10^{-7} mol/L) inhibit prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) production in mesangial cells stimulated with angiotensin II (Ang II). Results are expressed as mean ± SEM ($N = 6$). $^*P < 0.05$ vs. all other conditions; $\#P < 0.05$ vs. control.

inhibition did not modify Ang II–stimulated PGE_2 production (not shown).

ROS mediate COX-2 induction by Ang II. We have previously demonstrated that Ang II stimulates NADH/NADPH oxidase activity in mesangial cells resulting in the generation of ROS [11]. To investigate whether NADH/NADPH oxidase–derived ROS participate in COX-2 induction by Ang II, mesangial cells were exposed to DPI, a compound that by tightly binding to flavoproteins is an effective inhibitor of flavin-containing oxidases such as NADH/NADPH oxidase, (10^{-5} mol/L) prior to Ang II. Treatment with DPI prevented the increase in PGE_2 and PGI_2 production (Fig. 9), as well as COX-2 mRNA expression (Fig. 8) increase in response to Ang II, demonstrating that NADH/NADPH oxidase–derived ROS are important intracellular signals responsible for the initiation of COX-2 induction by Ang II.

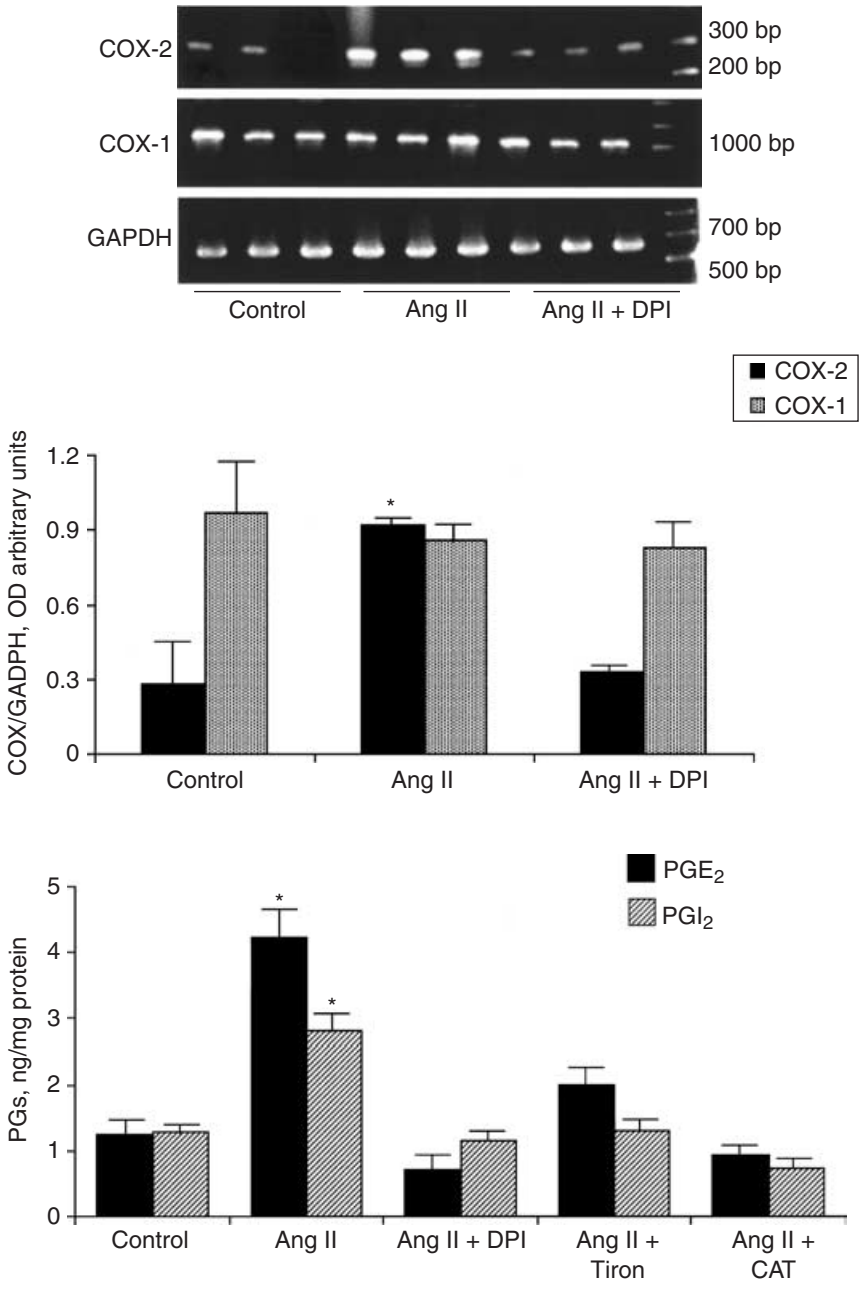

Fig. 8. Cyclooxygenase (COX) mRNA expression in mesangial cells. Angiotensin II (Ang II) (10^{-7} mol/L) ($N = 3$) increased COX-2 mRNA expression that was prevented by pretreatment with the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium (DPI) (10^{-5} mol/L) ($N = 3$). Ang II did not modify baseline COX-1 expression. GAPDH, glyderaldehyde-3-phosphate dehydrogenase.

Fig. 9. The reactive oxygen species (ROS) scavenger Tiron, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium (DPI) as well as catalase (CAT) inhibit mesangial cell production of prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) in response to angiotensin II (Ang II). Results are expressed as mean ± SEM ($N = 6$). *$P < 0.05$ vs. control.

To more directly assess the role of ROS on COX-2 induction, mesangial cells were treated with the cell-permeable superoxide scavenger Tiron (5×10^{-3} mol/L) prior to Ang II stimulation. Tiron prevented the increase in PGE_2 and PGI_2 production by mesangial cells in response to Ang II further suggesting that ROS are mediators of COX-2 induction in response to Ang II (Fig. 9). Superoxide anion produced as a result of increased NADH/NADPH oxidase activity rapidly dismutates to H_2O_2 spontaneously or via superoxide dismutase. As shown in Figure 9, catalase, an enzyme that decomposes H_2O_2, inhibited Ang II–stimulated PGE_2 production, suggesting that H_2O_2 is in fact the main ROS responsible for Ang II–stimulated PGE_2 production. Similar findings were observed when PGI_2 production was measured (Fig. 9).

MAPKs activation has been shown to participate in COX-2 induction [32, 33]. We have previously demonstrated that ROS produced as a result of Ang II stimulation activate MAPK in mesangial cells [11]. Hence, we treated mesangial cells with the specific MAPK-1 inhibitor PD98059 (50 μmol/L) prior to Ang II stimulation. Pretreatment with PD98059 prevented Ang II induced PGE_2 (Ang II 4.22 ± 0.7 ng/mg vs. Ang II + PD98059 0.69 ± 0.2 ng/mg protein) ($N = 6$) ($P < 0.05$) and PGI_2 production (Ang II 2.8 ± 0.2 ng/mg vs. 1.45 ± 0.1 ng/mg)

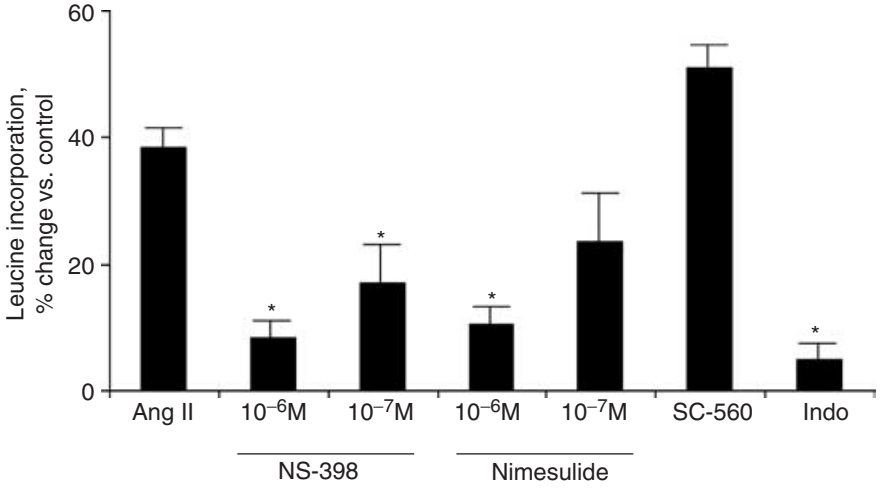

Fig. 10. The cyclooxygenase (COX-2) inhibitors NS-398 (10^{-6} to 10^{-7} mol/L) ($N = 15$), nimesulide (10^{-6} to 10^{-7} mol/L) ($N = 6$) as well as indomethacin (Indo) (10^{-5} mol/L) ($N = 6$), but not the COX-1 inhibitor SC-560 (10^{-7} mol/L) ($N = 6$) inhibit ^{3}H-leucine incorporation in mesangial cells stimulated with angiotensin II (Ang II) (10^{-7} mol/L). Results are expressed as mean ± SEM. *$P < 0.05$ vs. Ang II.

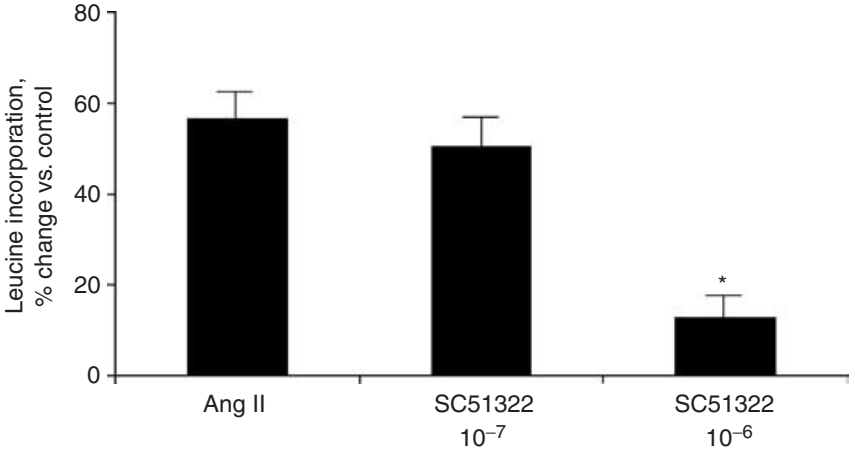

Fig. 11. The EP1 receptor blocker SC51322 significantly inhibited ^{3}H-leucine incorporation in mesangial cells stimulated with angiotensin II (Ang II). Results are expressed as mean ± SEM. *$P < 0.05$ vs. control.

($N = 6$) ($P < 0.05$) demonstrating that MAPK activation is necessary for COX-2 induction by Ang II. Treatment with PD98059 was not accompanied by increased cell lethality as assessed by trypan blue exclusion (trypan blue exclusion > 90%).

COX-2–derived prostaglandins mediate Ang II–stimulated mesangial cell hypertrophy. To determine the effect of prostaglandins produced in response to COX-2 induction by Ang II on mesangial cell hypertrophy, mesangial cells were exposed to Ang II (10^{-7} mol/L) for 48 hours and mesangial cell hypertrophy measured by ^{3}H-leucine incorporation in the presence of the COX-2 inhibitors NS-398 (10^{-7} to 10^{-6} mol/L), nimesulide (10^{-7} to 10^{-6} mol/L), the COX-1 inhibitor SC-560 (10^{-7} mol/L) or the nonspecific COX inhibitor indomethacin (10^{-5} mol/L). As shown in Figure 10, Ang II–induced mesangial cell hypertrophy was inhibited by indomethacin as well as by the COX-2 inhibitors NS-398 and nimesulide but not by the COX-1 inhibitor SC-560 suggesting that prostaglandins originating from COX-2–derived prostaglandins play an important role in mediating the growth promoting effects of Ang II. Higher concentrations of the COX-1 inhibitor were associated with significant cell lethality. To determine whether PGE_2 was the prostaglandin responsible for the growth-promoting effects of COX-2–derived prostaglandins, mesangial cells were pretreated with the type 1 PGE_2 receptor (EP1) blocker SC12311 (10^{-6} to 10^{-7} mol/L) prior to Ang II (10^{-7} mol/L) stimulation. EP1 blockade dose-dependently inhibited leucine incorporation in response to Ang II demonstrating that PGE_2 acting via the EP1 receptor mediates the growth-promoting effects of Ang II (Fig. 11).

DISCUSSION

In glomeruli isolated from rats infused with Ang II for 5 days, PGE_2 production was increased twofold and was significantly inhibited by two different COX-2 inhibitors. The increase in glomerular PGE_2 production was accompanied by a concomitant 1.7-fold increase in COX-2 mRNA and sixfold increase in protein expression suggesting that the effects of Ang II on COX-2 occur at a transcriptional level. In agreement with other

studies we observed a higher glomerular baseline expression of COX-1 [34] compared to COX-2. COX-1 is widely expressed in most cell types and although constitutively expressed, its expression can be regulated [24]. In contrast, COX-2 is induced in response to a variety of stimuli, including growth factors and cytokines such as IL-1 and tumor necrosis factor-α (TNF-α) [7], fibroblast growth factor (FGF) [35], PDGF, and transforming growth factor-β (TGF-β) [36]. Although COX-2 is considered to be the COX-inducible isoform, there is evidence for constitutive COX-2 expression in several segments of the nephron, including the cortical macula densa and the medullary interstitium [37]. Previous studies demonstrated that Ang II activates PLA_2 and induces the release of prostaglandins by mesangial cells [38] and vascular smooth muscle cells [39, 40]. In the current studies we demonstrate that COX-2 induction is a major pathway by which Ang II induces the production of prostaglandins in the glomerulus. Previous studies have shown increased glomerular COX-2 expression and activity in animal models of diabetes [41], ureteral obstruction [42], and renal insufficiency [37, 43] as well as in humans with activated renin-angiotensin system due to renal artery stenosis [44]. Importantly, in these conditions the increase in COX-2 expression and activity is not accompanied by changes in COX-1 activity [37, 41, 42, 44]. In our studies, we observed a small increase in the glomerular expression of COX-1 after Ang II infusion. Moreover, Ang II induces COX-2 expression and activity in cultured vascular smooth muscle cells [45, 46] as well as in human endothelial cells [47].

PGE_2, the major prostanoid produced in the kidney, has been shown to have either vasodilatory or vasoconstrictive effects in the glomerular microcirculation depending on binding to specific PGE_2 receptors (EP). Four distinct EP subtypes have been identified [48]. In the glomerular microcirculation, EP4 is the most abundant EP receptor and mediates PGE_2-induced vasodilation, while EP3 promotes vasoconstriction [8, 9]. In addition to PGE_2, other prostaglandins have significant hemodynamic effects, including PGI_2 which has a predominantly vasodilatory effect and TxA_2 and $PGF_{2\alpha}$ which are vasoconstrictors [10]. Prostaglandins produced by COX-2 in the glomerulus may have a critical role counterbalancing the effects of Ang II upon vasoconstriction and tubuloglomerular feedback. Recent studies suggest that Ang II up-regulates COX-2 in the glomerular afferent arteriole which is associated with increased TxA_2 production and increased constriction in response to vasoactive agents [49]. These effects of COX-2–derived prostaglandins may explain the deleterious effects of acute COX inhibition on the glomerular microcirculation when the renin-angiotensin system is activated. Our studies indicate that Ang II induces COX-2 expression in the glomerulus particularly at the level of the glomerular epithelium. In the current studies we clearly demonstrate that Ang II induces COX-2 mRNA and activity (PGE_2 increased 200% and PGI_2 increased 100%) in cultured mesangial cells in a process that is dependent on both ROS generation and MAPK activation.

In contrast, Ang II stimulation did not modify mesangial cell COX-1 mRNA expression. We also demonstrate that the ROS are derived from NADH/NADPH oxidase and that H_2O_2 produced as a result of O_2^- dismutation is the principal mediator. In support of this notion, ROS scavengers have been shown to reduce TNF-α–stimulated COX-2 mRNA and prostaglandin production [7]. Our laboratory was the first to demonstrate in mesangial cells that Ang II up-regulates NADH/NADPH oxidase, stimulates the generation of O_2^- via PKC activation resulting in MAPK activation thereby promoting mesangial cell hypertrophy and proliferation [11]. Although we observed a rather strong increase in COX-2 expression in mesangial cells in vitro, we could not conclusively demonstrate increases in COX-2 expression in the glomerular mesangium in vivo. These findings would suggest that factors present under cell culture conditions might favor strong COX-2 induction in mesangial cells in response to Ang II, or alternatively, that the surrounding glomerular environment has a modulatory effect on mesangial COX-2 expression [50, 51]. PGI_2 has growth inhibitory effects upon mesangial cells [20] and vascular smooth muscle cells [52]. PGE_2, on the other hand, has been shown to either promote or to inhibit growth related responses [15–18]. Our studies, show for the first time, that COX-2 inhibition abolishes Ang II induced mesangial cell hypertrophy, thereby supporting the notion that prostaglandins produced as a result of COX-2 induction are important mediators of the growth-promoting effects of Ang II. Mesangial cells are known to express the EP1 and the EP4 receptors [53]. In our studies the EP1 receptor blocker SC12111 prevented the growth-promoting effects of Ang II in mesangial cells suggesting that PGE_2 via activation of the EP1 receptor mediates the growth promoting effects of COX-2 in mesangial cells.

Our findings would suggest that COX-2 blockade might be potentially beneficial in mitigating pathologic glomerular growth related responses associated with activation of the renin-angiotensin system and progressive renal disease. However, recent studies have shown that renal COX-2, but not COX-1, is involved in the production of prostaglandins that antagonize the glomerular vasoconstrictor effects of Ang II [54]. Indeed, acute selective and nonselective COX-2 inhibition results in significant declines in glomerular filtration rate (GFR) associated with blunting of the vasodilatory effects of endogenous prostaglandins in the glomerular microcirculation [54]. Furthermore, with aging there is evidence for increased COX-2 expression in the kidney suggesting that in the elderly there may be a higher dependence

on endogenous prostaglandins for maintaining a normal GFR [55]. The complexity of effects of COX-2–derived prostaglandins that result in a variety of hemodynamic and nonhemodynamic actions suggests that novel strategies are needed in order to selectively modulate the actions of specific prostaglandins. Our studies support the notion that targeting specific prostaglandin receptors instead of blocking COX may be a suitable strategy to modulate the pathologic effects of COX-2–derived prostaglandins without negatively affecting glomerular hemodynamics.

ACKNOWLEDGMENTS

This work was supported by Merit Review Awards from the Veterans Affairs Administration to Leopoldo Raij and Edgar Jaimes. We thank the technical assistance of Brian Johnston, David Alzamora, and Raisa Puzis.

Reprint requests to Edgar A. Jaimes, M.D., VA Medical Center, 1201 NW 16th Street, Nephrology Section, Room A-1009, Miami, FL 33125. E-mail: ejaimes@med.miami.edu

REFERENCES

1. Ito S, Johnson CS, Carretero OA: Modulation of angiotensin II-induced vasoconstriction by endothelium-derived relaxing factor in the isolated microperfused rabbit afferent arteriole. *J Clin Invest* 87:1656–1663, 1991
2. Navar LG, Inscho EW, Majid SA, *et al*: Paracrine regulation of the renal microcirculation. *Physiol Rev* 76:425–536, 1996
3. Schlondorff D, Roczniak S, Satriano JA, Folkert VW: Prostaglandin synthesis by isolated rat glomeruli: effect of angiotensin II. *Am J Physiol* 239:F486–F495, 1980
4. Kreisberg JI, Karnovsky MJ, Levine L: Prostaglandin production by homogeneous cultures of rat glomerular epithelial and mesangial cells. *Kidney Int* 22:355–359, 1982
5. Stahl RA, Paravicini M, Schollmeyer P: Angiotensin II stimulation of prostaglandin E_2 and 6-keto-F_{1alpha} formation by isolated human glomeruli. *Kidney Int* 26:30–34, 1984
6. Goppelt-Struebe M, Stroebel M, Hoppe J: Regulation of platelet-derived growth factor isoform-mediated expression of prostaglandin G/H synthase in mesangial cells. *Kidney Int* 50:71–78, 1996
7. Feng L, Xia Y, Garcia GE, *et al*: Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. *J Clin Invest* 95:1669–1675, 1995
8. Purdy KE, Arendshorst WJ: EP(1) and EP(4) receptors mediate prostaglandin E(2) actions in the microcirculation of rat kidney. *Am J Physiol Renal Physiol* 279:F755–F764, 2000
9. Tang L, Loutzenhiser K, Loutzenhiser R: Biphasic actions of prostaglandin E(2) on the renal afferent arteriole: Role of EP(3) and EP(4) receptors. *Circ Res* 86:663–670, 2000
10. Nath KA, Chmielewski DH, Hostetter TH: Regulatory role of prostanoids in glomerular microcirculation of remnant nephrons. *Am J Physiol* 252:F829–F837, 1987
11. Jaimes EA, Galceran JM, Raij L: Angiotensin II induces superoxide anion production by mesangial cells. *Kidney Int* 54:775–784, 1998
12. Johnson RJ, Alpers CE, Yoshimura A, *et al*: Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19:464–474, 1992
13. Kagami S, Border WA, Miller DE, Noble NA: Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest* 93:2431–2437, 1994
14. Tharaux PL, Chatziantoniou C, Fakhouri F, Dussaule JC: Angiotensin II activates collagen I gene through a mechanism involving the MAP/ER kinase pathway. *Hypertension* 36:330–336, 2000
15. Stahl RA, Thaiss F, Haberstroh U, *et al*: Cyclooxygenase inhibition enhances rat interleukin 1 beta-induced growth of rat mesangial cells in culture. *Am J Physiol* 259:F419–F424, 1990
16. Nakahama K, Morita I, Murota S: Effects of endogenously produced arachidonic acid metabolites on rat mesangial cell proliferation. *Prostaglandins Leukot Essent Fatty Acids* 51:177–182, 1994
17. Ardaillou N, Nivez MP, Bellon G, *et al*: Effect of prostaglandin E_2 on proline uptake and protein synthesis by cultured human mesangial cells. *Kidney Int* 38:1151–1158, 1990
18. Lovett DH, Haensch GM, Goppelt M, *et al*: Activation of glomerular mesangial cells by the terminal membrane attack complex of complement. *J Immunol* 138:2473–2480, 1987
19. Schmitz PG, Zhang K, Dalal R: Eicosapentaenoic acid suppresses PDGF-induced DNA synthesis in rat mesangial cells: involvement of thromboxane A_2. *Kidney Int* 57:1041–1051, 2000
20. Mene P, Abboud HE, Dunn MJ: Regulation of human mesangial cell growth in culture by thromboxane A_2 and prostacyclin. *Kidney Int* 38:232–239, 1990
21. Kelefiotis D, Bresnahan BA, Stratidakis I, Lianos EA: Eicosanoid-induced growth and signaling events in rat glomerular mesangial cells. *Prostaglandins* 49:269–283, 1995
22. Han R, Tsui S, Smith TJ: Up-regulation of prostaglandin E_2 synthesis by interleukin-1beta in human orbital fibroblasts involves coordinate induction of prostaglandin-endoperoxide H synthase-2 and glutathione-dependent prostaglandin E_2 synthase expression. *J Biol Chem* 277:16355–16364, 2002
23. Smith CJ, Morrow JD, Roberts LJ, 2nd, Marnett LJ: Induction of prostaglandin endoperoxide synthase-1 (COX-1) in a human promonocytic cell line by treatment with the differentiating agent TPA. *Adv Exp Med Biol* 400A:99–106, 1997
24. Rocca B, Spain LM, Ciabattoni G, *et al*: Differential expression and regulation of cyclooxygenase isozymes in thymic stromal cells. *J Immunol* 162:4589–4597, 1999
25. Sato T, Nakajima H, Fujio K, Mori Y: Enhancement of prostaglandin E_2 production by epidermal growth factor requires the coordinate activation of cytosolic phospholipase A_2 and cyclooxygenase 2 in human squamous carcinoma A431 cells. *Prostaglandins* 53:355–369, 1997
26. Sakamoto C, Matsuda K, Nakano O, *et al*: EGF stimulates both cyclooxygenase activity and cell proliferation of cultured guinea pig gastric mucous cells. *J Gastroenterol* 29 (Suppl 7):73–76, 1994
27. Shultz PJ, Raij L: The glomerular mesangium: Role in initiation and progression of renal injury. *Am J Kidney Dis* 17:8–14, 1991
28. Jaimes EA, Nath KA, Raij L: Hydrogen peroxide downregulates IL-1-driven mesangial iNOS activity: Implications for glomerulonephritis. *Am J Physiol* 272:F721–F728, 1997
29. Shultz PJ, Tayeh MA, Marletta MA, Raij L: Synthesis and action of nitric oxide in rat glomerular mesangial cells. *Am J Physiol* 261:F600–F606, 1991
30. Lovett DH, Resch K, Gemsa D: Interleukin I and the glomerular mesangium. II. Monokine stimulation of mesangial cell prostanoid secretion. *Am J Pathol* 129:543–551, 1987
31. Soler M, Camacho M, Sola R, Vila L: Mesangial cells release untransformed prostaglandin H_2 as a major prostanoid. *Kidney Int* 59:1283–1289, 2001
32. Guan Z, Baier LD, Morrison AR: p38 mitogen-activated protein kinase down-regulates nitric oxide and up-regulates prostaglandin E_2 biosynthesis stimulated by interleukin-1beta. *J Biol Chem* 272:8083–8089, 1997
33. Goppelt-Struebe M, Hahn A, Stroebel M, Reiser CO: Independent regulation of cyclo-oxygenase 2 expression by p42/44 mitogen-activated protein kinases and Ca2+/calmodulin-dependent kinase. *Biochem J* 339 (Pt 2):329–334, 1999
34. Campean V, Theilig F, Paliege A, *et al*: Key enzymes for renal prostaglandin synthesis: Site-specific expression in rodent kidney (rat, mouse). *Am J Physiol Renal Physiol* 285:F19–F32, 2003
35. Sasaki E, Pai R, Halter F, *et al*: Induction of cyclooxygenase-2 in a rat gastric epithelial cell line by epiregulin and basic fibroblast growth factor. *J Clin Gastroenterol* 27 (Suppl 1):S21–S27, 1998

36. Fong CY, Pang L, Holland E, Knox AJ: TGF-beta1 stimulates IL-8 release, COX-2 expression, and PGE(2) release in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 279:L201–L207, 2000
37. Wang JL, Cheng HF, Zhang MZ, *et al*: Selective increase of cyclooxygenase-2 expression in a model of renal ablation. *Am J Physiol* 275:F613–F622, 1998
38. Schlondorff D, DeCandido S, Satriano JA: Angiotensin II stimulates phospholipases C and A_2 in cultured rat mesangial cells. *Am J Physiol* 253:C113–C120, 1987
39. Freeman EJ, Ruehr ML, Dorman RV: Ang II-induced translocation of cytosolic PLA_2 to the nucleus in vascular smooth muscle cells. *Am J Physiol* 274:C282–C288, 1998
40. Rao GN, Griendling KK, Frederickson RM, *et al*: Angiotensin II induces phosphorylation of eukaryotic protein synthesis initiation factor 4E in vascular smooth muscle cells. *J Biol Chem* 269:7180–7184, 1994
41. Komers R, Lindsley JN, Oyama TT, *et al*: Immunohistochemical and functional correlations of renal cyclooxygenase-2 in experimental diabetes. *J Clin Invest* 107:889–898, 2001
42. Salvemini D, Seibert K, Masferrer JL, *et al*: Endogenous nitric oxide enhances prostaglandin production in a model of renal inflammation. *J Clin Invest* 93:1940–1947, 1994
43. Hernandez J, Astudillo H, Escalante B: Angiotensin II stimulates cyclooxygenase-2 mRNA expression in renal tissue from rats with kidney failure. *Am J Physiol Renal Physiol* 282:F592–F598, 2002
44. Mann B, Hartner A, Jensen BL, *et al*: Acute upregulation of COX-2 by renal artery stenosis. *Am J Physiol Renal Physiol* 280:F119–F125, 2001
45. Ohnaka K, Numaguchi K, Yamakawa T, Inagami T: Induction of cyclooxygenase-2 by angiotensin II in cultured rat vascular smooth muscle cells. *Hypertension* 35:68–75, 2000
46. Young W, Mahboubi K, Haider A, *et al*: Cyclooxygenase-2 is required for tumor necrosis factor-alpha- and angiotensin II-mediated proliferation of vascular smooth muscle cells. *Circ Res* 86:906–914, 2000
47. Li Volti G, Seta F, Schwartzman ML, *et al*: Heme oxygenase attenuates angiotensin II-mediated increase in cyclooxygenase-2 activity in human femoral endothelial cells. *Hypertension* 41:715–719, 2003
48. Smith GC, Coleman RA, McGrath JC: Characterization of dilator prostanoid receptors in the fetal rabbit ductus arteriosus. *J Pharmacol Exp Ther* 271:390–396, 1994
49. Wang D, Chabrashvili T, Wilcox CS: Enhanced contractility of renal afferent arterioles from angiotensin-infused rabbits: Roles of oxidative stress, thromboxane prostanoid receptors, and endothelium. *Circ Res* 94:1436–1442, 2004
50. Michael AF, Keane WF, Raij L, *et al*: The glomerular mesangium. *Kidney Int* 17:141–154, 1980
51. Sweeney C, Shultz PJ, Raij L: Interactions of the endothelium and mesangium in glomerular injury. *J Am Soc Nephrol* 1 (Suppl 1):S13–S20, 1990
52. Uehara Y, Ishimitsu T, Kimura K, *et al*: Regulatory effects of eicosanoids on thymidine uptake by vascular smooth muscle cells of rats. *Prostaglandins* 36:847–857, 1988
53. Ishibashi R, Tanaka I, Kotani M, *et al*: Roles of prostaglandin E receptors in mesangial cells under high-glucose conditions. *Kidney Int* 56:589–600, 1999
54. Qi Z, Hao CM, Langenbach RI, *et al*: Opposite effects of cyclooxygenase-1 and -2 activity on the pressor response to angiotensin II. *J Clin Invest* 110:61–69, 2002
55. Swan SK, Rudy DW, Lasseter KC, *et al*: Effect of cyclooxygenase-2 inhibition on renal function in elderly persons receiving a low-salt diet. A randomized, controlled trial. *Ann Intern Med* 133:1–9, 2000