Angiotensin II increases the expression of the transcription factor ETS-1 in mesangial cells

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Maladaptive activation of the renin-angiotensin system (RAS) has been shown to play a critical role in the pathogenesis of chronic kidney disease. Reactive oxygen species (ROS) are critical signals for many of the nonhemodynamic effects of angiotensin II (ANG II). We have demonstrated that ANG II increases mesangial and cortical cyclooxygenase-2 (COX-2) expression and activity via NADPH oxidase-derived ROS. The transcription factor ETS-1 (E26 transformation-specific sequence) has been identified as a critical regulator of growth-related responses and inflammation. The present studies were designed to determine: 1) whether ANG II induces ETS-1 expression in vitro in cultured rat mesangial cells and in vivo in rats infused with ANG II; and 2) whether ROS and COX-2 are mediators of ETS-1 induction in response to ANG II. Mesangial cells stimulated with ANG II (10⁻⁷ M) exhibited a significant increase in ETS-1 expression that was prevented by the angiotensin type I receptor blocker candesartan. NADPH oxidase inhibition with diphenylene iodinium or apocynin also prevented ETS-1 induction, establishing the role of ROS as mediators of ETS-1 expression in response to ANG II. COX-2 inhibition prevented ETS-1 expression in response to ANG II, suggesting that COX-2 is required for ETS-1 induction. By utilizing short interfering RNAs against ETS-1, we have also determined that ETS-1 is required to induce the production of fibronectin in response to ANG II. Furthermore, rats infused with ANG II manifested increased glomerular expression of ETS-1. These studies unveil novel pathways that may play an important role in the pathogenesis of renal injury when RAS is activated.

Maladaptive activation of the renin-angiotensin system (RAS) has been shown to play a critical role in the pathogenesis of chronic kidney disease (CKD) of different etiologies, including hypertension (34) and diabetes (1). Angiotensin II (ANG II), produced as a result of RAS activation, is a potent systemic vasoconstrictor and modulator of the renal microcirculation (11). In addition, ANG II has important nonhemodynamic effects that have been implicated in the pathogenesis of CKD by promoting mesangial cell hypertrophy and proliferation (12), increasing extracellular matrix deposition (29) and promoting inflammation (16). As our laboratory and others have shown, reactive oxygen species (ROS) are critical intracellular signals for these nonhemodynamic effects of ANG II (12). As a result of RAS activation, downstream signaling cascades trigger the increased production of a variety of growth factors (7), cytokines (15), chemokines (17), and other mediators that may be involved in the pathogenesis of end-organ injury in hypertension. Models of CKD that are associated with increased RAS activation, including renal ablation (30) and obstructive uropathy, exhibit increased expression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the synthesis of prostaglandins. Our laboratory has recently shown that ANG II increases glomerular COX-2 expression in vivo and in vitro in rat mesangial cells (14). Although it is clear that RAS activation has a multifarious nature in the pathogenesis of end-organ injury in hypertension, whether these effects occur via activation of a common convergent or multiple independent pathways has not been established. Recently, ETS-1 (E26 transformation-specific sequence) has been identified as a critical transcription factor involved in regulating the expression of a variety of genes that include growth factors, chemokines, and adhesion molecules (22). Importantly, recent studies have shown that ETS-1 is required for vascular inflammation and tissue remodeling in response to ANG II, suggesting that ETS-1 may be a common mediator for many of its actions (33). Whether ANG II increases ETS-1 expression in the glomerulus, however, has not been investigated. In the present study, we investigated whether: 1) ANG II increases ETS-1 expression in cultured rat mesangial cells and in vivo in rats infused with ANG II; and 2) ROS and COX-2 play a role in the induction of ETS-1 in response to ANG II.

Methods

Cell culture. Rat mesangial cells (ATCC) were grown in DMEM media (GIBCO, Grand Island, NY), supplemented with 17% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT). These studies were approved by the Institutional Animal Care and Use sub-Committee at the Miami VA Medical Center.

In vitro experiments. Mesangial cells grown in six-well dishes were made quiescent in DMEM/0.5% FCS for 72 h and exposed to ANG II (10⁻⁷ M), with and without the NADPH oxidase inhibitors diphenylene iodinium (DPI, 10⁻⁵ M) or apocynin (10⁻⁴ M), the specific COX-2 inhibitor NS-398 (10⁻⁷ M) or nimesulide (10⁻⁷ M), the nonspecific COX inhibitor indomethacin (10⁻⁵ M), the angiotensin type 1 receptor (AT₁R) blocker candesartan (10⁻⁶ M), the ERK1/2 inhibitor PD98059 (10⁻⁷ M), or the PKC inhibitor H-7 (10⁻⁷ M). At the end of the incubation period, cells were saved for Western blot analysis.

Western blotting. Western blots were performed as previously described (13, 14). Briefly, cell lysates were washed once with

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phosphate-buffered saline, resuspended in 300 μl homogenization buffer (50 mM Tris·HCl, pH 7.6, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% Triton X-100), and then incubated on ice for 30 min. Thereafter, lysates were centrifuged for 30 min at 10,000 g at 4°C. Supernatants were collected, and the protein content determined by using the Bio-Rad protein estimation kit. Thirty micrograms of protein were separated by SDS-PAGE (6% acrylamide gel) and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-ETS-1 antibody (Cayman), anti-fibronectin antibody (Sigma), or anti-α-actin antibody (Santa Cruz). After washing, the blots were incubated with goat anti-rabbit antibody (Santa Cruz Biotechnology) for 1 h, and the signal was detected by luminol chemiluminescence.

**Short interfering RNA knockdown of ETS-1.** Rat mesangial cells were transfected with three different short interfering RNAs (siRNAs) specific for ETS-1 (Ambion ID: 47814, cat. nos. AM199898, AM16704, and AM199897). Briefly, rat mesangial cells were grown to 70% confluence; 1 day before transfection, cells were washed and media changed to DMEM/10% FCS without antibiotics. Cells were transfected using Lipofectamine 2000 and 1.5–5 nmol of each siRNA. The cells were harvested after 24 h, and ETS-1 expression determined by Western blot. Once the most effective siRNA was determined, mesangial cells were transfected as described above, made quiescent for 24 h in DMEM/0.5% FCS, and stimulated with ANG II (10⁻⁷ M) for 24 h. Cells were harvested and saved for Western blot analysis.

**Real-time PCR.** ETS-1 and fibronectin mRNA expression was determined by real-time PCR. Total RNA was isolated utilizing the RNeasy mini kit (Qiagen, Valencia, CA). A 5-μg aliquot of total RNA was used for cDNA synthesis using the Superscript premultiplication system (Life Technologies). Primers and probes for ETS-1 and fibronectin were designed using Primer Express software 101 (ABI). As an active reference, endogenous 18S ribosomal RNA (r18S) was amplified using specific primers and probes labeled with VIC (ABI). The comparative threshold cycle (Ct) method was used for relative quantification.

**Fig. 1.** Angiotensin II (ANG II) increases mesangial ETS-1 (E26 transformation-specific sequence) expression in a time-dependent manner. Rat mesangial cells were stimulated with ANG II (10⁻⁷ M) for different time periods as shown. ANG II increased ETS-1 expression after 4-h incubation, and levels remained significantly elevated through 24 h. A: representative Western blots for ETS-1 and actin, which was used to control for unequal loading. C, control. B: densitometry data analysis performed after reprobing the blot with an α-actin antibody. Values are means ± SE; N = 3. *P < 0.05 vs. respective time control.

**Fig. 2.** Candesartan (CAN) prevents ETS-1 expression in response to ANG II in rat mesangial cells. B and C: representative Western blots for ETS-1 and actin, which was used to control for unequal loading. A: densitometry data analysis performed after reprobing the blot with an α-actin antibody. Values are means ± SE; N = 3–6. *P < 0.05 vs. control, ANG + CAN, and CAN. #P < 0.05 vs. control.
quantification and statistical analysis. A unit difference in cycle value represents a twofold change in mRNA abundance.

**In vivo studies.** Six-week-old Sprague-Dawley male rats were purchased from Harlan (Indianapolis, IN) and maintained under controlled conditions of light, temperature, and humidity. Rats were divided into two groups: control (N = 6), and implanted with vehicle (PBS) and ANG II (N = 6) and infused with ANG II via osmotic minipump at 0.7 mg·kg⁻¹·day⁻¹ for 7 days. Minipumps (Alzet model 1007D) were implanted in the midscapular region under anesthesia with a cocktail of ketamine (45 mg/kg ip), xylazine (8 mg/kg ip), and acepromazine (1.5 mg/kg ip). Rats were killed on day 7, and kidney tissue was saved for immunohistochemistry.

Rats were killed by decapitation, and kidneys were harvested and fixed in 10% paraformaldehyde. For antigen retrieval, slides were immersed in target retrieval solution (DakoCytomation, Carpinteria, CA) for 30 min at 90°C. ETS-1 immunoreactivity was localized with polyclonal rabbit anti-murine ETS-1 antibody (Cayman, Ann Arbor, MI). The secondary antibody used was a biotin-conjugated goat anti-rabbit IgG (Santa Cruz, Santa Cruz, CA), and immunocytochemical detection was performed using the Vectastain ABC-Elite kit (Vector, Burlingame, CA) with diaminobenzidine as chromogen.

**Statistical analysis.** Data are expressed as means ± SE. For statistical comparisons involving two groups, an unpaired Student t-test was used, whereas, for comparisons involving more than two groups, an ANOVA (StatView, BrainPower, Calabasas, CA) was used. Significance was considered when P < 0.05.

**RESULTS**

**ANG II increases ETS-1 expression in rat mesangial cells.** To determine the effects of ANG II on ETS-1 expression, rat mesangial cells were exposed to ANG II (10⁻⁷ M) for 2, 4, 8, 16, and 24 h. At the end of the different incubation time points, cells were harvested, and ETS-1 expression assessed by Western blot. As shown in Fig. 1, ANG II increased the levels of ETS-1 protein compared with nonstimulated controls from 4- through 24-h stimulation with a peak induction at 4 h. To determine if AT₁R was involved in ETS-1 induction by ANG II, rat mesangial cells were stimulated with ANG II (10⁻⁷ M) for 4 h, with or without the AT₁R blocker candesartan (10⁻⁶ M). As shown in Fig. 2, AT₁R blockade significantly reduced ETS-1 expression in response to ANG II, demonstrating that the effects of ANG II on ETS-1 expression are mediated via the AT₁R. In separate experiments, we also determined the effects of AT₁R blockade on ETS expression in unstimulated mesangial cells. As shown in Fig. 2, A and C, AT₁R blockade did not modify ETS-1 expression in control cells.

**ROS mediate ETS-1 expression in response to ANG II.** To determine the role of ROS in ANG II-mediated ETS-1 expression, mesangial cells were stimulated with ANG II (10⁻⁷ M), with or without the NADPH inhibitors DPI or apocynin. As shown in Fig. 3, both DPI and apocynin significantly reduced ETS-1 expression in response to ANG II, suggesting that NADPH oxidase-derived ROS are involved in ANG II-induced ETS-1 expression. In separate experiments, we also determined the effects of NADPH oxidase inhibition on ETS-1 expression in unstimulated mesangial cells. As shown in Fig. 3, C and D, NADPH oxidase inhibition with either DPI or apocynin did not modify ETS-1 expression in control cells.

![Fig. 3. NADPH oxidase inhibition and cyclooxygenase-2 (COX-2) inhibition prevent ETS-1 induction in response to ANG II. A and C: representative Western blots for ETS-1 and actin, which was used to control for unequal loading. B and D: densitometry data analysis performed after reprobing the blot with an α-actin antibody. DPI, diphenylene iodonium; Apo, apocynin; NS, NS-398; Nime, nimesulide; Indo, indomethacin. Values are means ± SE; N = 3–6. *P < 0.05 vs. control and ANG + all inhibitors.](http://ajprenal.physiology.org/)

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COX-2 is required for ETS-1 expression in response to ANG II. Our laboratory has previously demonstrated that ANG II increases COX-2 expression and activity in mesangial cells and that COX-2-derived prostaglandins mediate in a large part the growth-promoting effects of ANG II in these cells (14). To determine COX-2 involvement in ANG II-induced ETS-1 expression, mesangial cells were stimulated with ANG II (10^{-7} M), with or without the COX-2 inhibitors NS-398 (10^{-7} M) and nimesulide (10^{-7} M), or the nonspecific COX inhibitor indomethacin (10^{-5} M). As shown in Fig. 3, specific COX-2 inhibition, as well as nonspecific COX inhibition, significantly reduced ETS-1 expression in response to ANG II, demonstrating the requirement of COX-2-derived prostaglandins for this induction. In separate experiments, we also determined the effects of COX inhibition on ETS-1 expression in unstimulated mesangial cells. As shown in Fig. 3, C and D, COX-2 inhibition or nonspecific COX inhibition did not modify ETS-1 expression in control cells.

PKC is required for ETS-1 expression in response to ANG II. To determine the role of PKC activation in ANG II-mediated ETS-1 expression, rat mesangial cells were stimulated with ANG II (10^{-7} M) and nimesulide (10^{-7} M), or the nonspecific COX inhibitor indomethacin (10^{-5} M). As shown in Fig. 4, specific COX-2 inhibition, as well as nonspecific COX inhibition, significantly reduced ETS-1 expression in ANG II-stimulated mesangial cells. As shown in Fig. 4, A: densitometry data analysis performed after reprobing the blot with an α-actin antibody. Values are means ± SE; N = 3–6. *P < 0.05 vs. ANG II and ANG II + PD98059 and H-7. B, C, and D: representative Western blots for ETS-1 and actin, which was used to control for unequal loading.

Fig. 4. MAPK inhibition and PKC inhibition prevent ETS-1 induction in response to ANG II. A: densitometry data analysis performed after reprobing the blot with an α-actin antibody. Values are means ± SE; N = 3–6. *P < 0.05 vs. ANG II and ANG II + PD98059 and H-7. B, C, and D: representative Western blots for ETS-1 and actin, which was used to control for unequal loading.

Fig. 5. ETS-1 knockdown by specific short interfering RNAs (siRNAs) against ETS-1. Transfection of specific siRNAs sequences (1.5–5 nmol) reduces ETS-1 protein expression in rat mesangial cells compared with control. A: representative Western blots for ETS-1 and actin, which was used to control for unequal loading. B: densitometry data analysis performed after reprobing the blot with an α-actin antibody. Values are means ± SE; N = 3. *P < 0.05 vs. the respective control.
this process. In separate experiments, we also determined the effects of PKC inhibition on ETS-1 expression in unstimulated mesangial cells. As shown in Fig. 3, A and D, PKC inhibition with H-7 did not modify ETS-1 expression in control cells.

MAPK activation is required for ETS-1 expression in response to ANG II. To determine the necessity of MAPK activation for ANG II-induced ETS-1 expression, mesangial cells were exposed to ANG II, with and without the ERK1/2 inhibitor PD98056 (10^{-6} M), for 4 h. After incubation, cells were harvested, and ETS-1 expression determined by Western blot. As shown in Fig. 3, ERK1/2 inhibition prevented the ANG II-induced increases in ETS-1 expression, demonstrating the requirement for MAPK activation. In separate experiments, we also determined the effects of ERK1/2 inhibition on ETS-1 expression in unstimulated mesangial cells. As shown in Fig. 3, A and D, ERK1/2 inhibition with PD98056 did not modify ETS-1 expression in control cells. ETS-1 is required for ANG II-stimulated fibronectin production.

ETS-1 has been shown to be a critical mediator of the growth-promoting effects of ANG II in the vasculature (33). To examine the requirement of ETS-1 in the profibrotic effects of ANG II in mesangial cells, we employed ETS-1-specific siRNA knockdown during ANG II stimulation. Initial experiments screened three rat ETS-1-specific sequences in rat mesangial cells: one siRNA sequence, AM199897, was determined to be most effective for ETS-1 knockdown at a concentration of 5 nmol (Fig. 5). All subsequent experiments were performed utilizing this specific siRNA sequence and concentration. Rat mesangial cells were transfected with ETS-1 siRNA, starved for 48 h in DMEM/0.5% FCS, and then stimulated with ANG II (10^{-7} M) for 24 h. Cells were harvested, and fibronectin expression measured by Western blot. As shown in Fig. 6, ETS-1 knockdown significantly reduced mesangial cell fibronectin expression, suggesting a critical role of ETS-1 in the growth-promoting effects of ANG II within the glomerular mesangium. To confirm these results, we measured ETS-1 and fibronectin mRNA expression by real-time PCR in mesangial cells stimulated with ANG II (10^{-7} M) and transfected with ETS-1 siRNA. ANG II stimulation resulted in a significant increase in fibronectin mRNA expression that was completely inhibited by transfection with ETS-1 siRNA: control ΔCT 5.8 ± 0.14; ANG II ΔCT 1.9 ± 1.5; ANG II + ETS-1 siRNA ΔCT 5.5 ± 0.15 (P < 0.05 vs. control and ANG II + ETS siRNA; a one-point reduction in ΔCT equals a twofold increase in mRNA expression). ANG II also increased ETS-1 mRNA expression that was prevented by ETS-1 siRNA: control ΔCT 17.7 ± 0.19; ANG II ΔCT 13.5 ± 2.9; ANG II + ETS siRNA ΔCT 16.1 ± 0.5 (P < 0.05 vs. control and ANG II + ETS siRNA; a one-point reduction in ΔCT equals a twofold increase in mRNA expression).

ANG II increases ETS-1 expression in vivo. To determine whether ANG II increases ETS-1 expression in vivo, Sprague-Dawley rats were infused with ANG II (0.7 mg·kg^{-1}·day^{-1}) for 7 days, and renal ETS-1 expression was determined by immunohistochemistry. As shown in Fig. 7, rats infused with ANG II had a significant increase in glomerular ETS-1 expression.

DISCUSSION

In these studies, we have demonstrated for the first time that ANG II increases the expression of the transcription factor ETS-1 in vitro, in cultured rat mesangial cells, as well as in vivo, in the glomerulus of rats infused with ANG II. In addition, we have identified several of the signaling intermediaries that participate in ANG II-induced ETS-1 expression and determined that ETS-1 is required for ANG II-stimulated fibronectin production in mesangial cells.

Fig. 6. ETS-1 knockdown reduces fibronectin production in response to ANG II. A: representative Western blots for fibronectin and actin, which was used to control for unequal loading. B: densitometry data analysis performed after reprobing the blot with an α-actin antibody. OD, optical density. Values are means ± SE; N = 3. *P < 0.05 vs. ANG II and ANG II + siRNA.
ETS-1 is a member of the ETS family of transcription factors that share a highly conserved DNA-binding domain (ETS domain) (22). The “ETS” originates from the original sequence described in the E26 avian erythroblastosis virus (E26 transformation-specific sequence) (21). Recently, ETS-1 has emerged as a critical ETS transcription factor involved in the regulation of normal development and differentiation and as a protooncogene (6, 31, 32). ETS-1 has been implicated in the pathogenesis of different types of cancer (27).

In the vasculature, ETS-1 is induced in response to a variety of stimuli, including ANG II (33), platelet-derived growth factor (18), thrombin (10), IL-1 (24), and TNF-α (8). In our studies, we have now demonstrated that ETS-1 expression is increased in response to ANG II in vitro in rat mesangial cells, as well as in the glomerulus in vivo. Target genes identified to be downstream of ETS-1 include the chemokine monocyte chemoattractant protein-1, the adhesion molecule vascular cellular adhesion molecule, and plasminogen activator inhibitor-1 (33). ETS-1 expression in vascular smooth muscle cells (VSMC) also induces the expression of platelet-derived growth factor, thereby promoting VSMC proliferation (25). Indeed, VSMC isolated from ETS-1-deficient mice have been shown to exhibit a decreased proliferative response to ANG II (33). Additionally, the systemic administration of ANG II to ETS-1-deficient mice is associated with marked reductions in medial hypertrophy, even though similar increases in blood pressure are observed in wild-type and ETS-1-deficient mice (33). In our studies, we also demonstrate that ETS-1 is required for ANG II-induced fibronectin production in rat mesangial cells. In VSMCs, ROS have been shown to be involved in ETS-1 expression (2). Our laboratory was the first to demonstrate that ANG II upregulates NADPH oxidase in mesangial cells, resulting in a PKC-dependent generation of ROS (12). As we and others have demonstrated, ROS play a critical role as mediators of the growth-promoting effects of ANG II (12). In the present study, we show that NADPH oxidase inhibition with two different pharmacological inhibitors, as well as PKC inhibition with H-7, prevented ANG II-induced ETS-1, demonstrating that, in mesangial cells, NADPH oxidase-derived ROS mediate ETS-1 induction in response to ANG II.

Studies using other cell types have demonstrated that ETS-1 is a target of the Ras-Raf-MAPKs pathway (31), a signaling cascade that is activated in response to several types of stimuli, including growth factors (5), stress signaling pathways (26), and ANG II (29). In the present investigation, ERK1/2 inhibition with PD98059 prevented ANG II-induced ETS-1, demonstrating that ANG II-stimulated MAPK activation is required for ETS-1 induction.

Our laboratory has recently demonstrated that ANG II increases COX-2 expression and activity in mesangial cells and that COX-2-derived prostaglandins mediate, in large part, the growth-promoting effects of ANG II in mesangial cells (14). In the present study, we show that specific COX-2 inhibition, as well as nonspecific COX inhibition, prevented ANG II-induced ETS-1 expression that COX-2 is required for ETS-1 induction. These findings would also suggest that the growth-promoting effects of COX-2-derived prostaglandins may be mediated, at least in part, via ETS-1 induction.

Several studies have shown that the ETS-1 gene is essential for the normal development of the mammalian kidney and for the maintenance of glomerular integrity (3, 4). Indeed, ETS-1 knockout animals have been found to have fewer and more immature glomeruli (9). The renal expression of ETS-1 is increased in a variety of models of renal injury. In the anti-Thy model of glomerulonephritis, ETS-1 expression was found to be increased fourfold, with the strongest expression found in the glomerular mesangium and to a lesser degree in podocytes and glomerular endothelial cells (23). In an ischemic model of acute renal failure, the tubular expression of ETS-1 was found to be increased and associated with an increased expression of cyclin D, suggesting a role for ETS-1 in the control of tubular regeneration in acute renal failure (28). In rats with anti-glomerular basement-induced glomerulonephritis, there is also an upregulation of ETS-1 in the glomeruli and in the interstitial (19). ETS-1 expression has also been found to be increased in models of cisplatin toxicity and to be associated with increased matrix metalloproteinase-1 expression (20). In our present work, we demonstrate for the first time that ANG II infusion increases the glomerular expression of ETS-1 and that knockdown of ETS-1 in ANG II-stimulated mesangial cells reduces subsequent fibronectin production; these results suggest that ETS-1 may be playing an important role as a mediator of the processes of injury and repair mediated by ANG II.

In conclusion, we have shown that ANG II increases ETS-1 expression in mesangial cells, as well as in the glomerulus in vivo. We have also demonstrated that ANG II-induced ETS-1 requires NADPH oxidase-derived ROS, COX-2, and MAPK. In addition, we have identified that ETS-1 is a critical transcription factor involved in the growth-promoting effects of ANG II. These studies unveil novel mechanistic pathways that may help to identify new targets for the treatments of kidney diseases that are accompanied by activation of the RAS.

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**REFERENCES**

ANGIOTENSIN II INDUCES MESANGIAL ETS-1


