Role of the Transcription Factor Erythroblastosis Virus E26 Oncogen Homolog-1 (ETS-1) as Mediator of the Renal Proinflammatory and Profibrotic Effects of Angiotensin II

Wenguang Feng, Phillip Chumley, Ping Hua, Gabriel Rezonzew, David Jaimes, Madison W. Duckworth, Dongqi Xing, Edgar A. Jaimes

Abstract—Angiotensin II (Ang II) plays a major role in the pathogenesis of end-organ injury in hypertension via its diverse hemodynamic and nonhemodynamic effects. Erythroblastosis virus E26 oncogen homolog-1 (ETS-1) is an important transcription factor recently recognized as an important mediator of cell proliferation, inflammation, and fibrosis. In the present studies, we tested the hypothesis that ETS-1 is a common mediator of the renal proinflammatory and profibrotic effects of Ang II. C57BL6 mice (n=6 per group) were infused with vehicle (control), Ang II (1.4 mg/kg per day), Ang II and an ETS-1 dominant-negative peptide (10 mg/kg per day), or Ang II and an ETS-1 mutant peptide (10 mg/kg per day) via osmotic minipump for 2 or 4 weeks. The infusion of Ang II resulted in significant increases in blood pressure and left ventricular hypertrophy, which were not modified by ETS-1 blockade. The administration of ETS-1 dominant-negative peptide significantly attenuated Ang II-induced renal injury as assessed by urinary protein excretion, mesangial matrix expansion, and cell proliferation. Furthermore, ETS-1 dominant-negative peptide but not ETS-1 mutant peptide significantly reduced Ang II–mediated upregulation of transforming growth factor-β, connective tissue growth factor, and α-smooth muscle actin. In addition, ETS-1 blockade reduced several proinflammatory effects of Ang II, including macrophage infiltration, nitrotyrosine expression, and NOX4 mRNA expression. Our studies suggest that ETS-1 is a common mediator of the proinflammatory and profibrotic effects of Ang II–induced hypertensive renal damage and may result in the development of novel strategies in the treatment and prevention of end-organ injury in hypertension. (Hypertension. 2012;60:1226-1233.) • Online Data Supplement

Key Words: angiotensin II ■ hypertension (kidney) ■ ETS-1 ■ extracellular matrix ■ physiology/pathophysiology ■ growth factors and cytokines ■ oxidative stress (kidney)

Maladaptive activation of the renin-angiotensin system plays a critical role in the pathogenesis of glomerulosclerosis and chronic kidney disease of different causes, including hypertension and diabetes mellitus. Angiotensin II (Ang II) is a potent systemic vasoconstrictor and modulator of renal microcirculation. In addition to these hemodynamic actions, Ang II activates downstream signaling cascades that trigger the increased production of several growth factors, cytokines, chemokines, and other mediators that stimulate mesangial cell hypertrophy and proliferation, extracellular matrix deposition, and inflammation. It is not clear, however, whether these effects of Ang II are mediated via the independent activation of multiple pathways or whether alternatively common mediators induce these diverse profibrotic and proinflammatory pathways in response to Ang II.

The ETS factors are a family of transcription factors that participate in the regulation of a wide variety of biological processes, including normal development and differentiation. Erythroblastosis virus E26 oncogen homolog-1 (ETS-1) is a member of the ETS transcription factor family involved in the expression of a variety of genes, including growth factors, chemokines, and adhesion molecules. ETS-1 is also a well-known proto-oncogene in the pathogenesis of several different types of cancer. Several studies have also supported the role of ETS-1 as a mediator of vascular inflammation, recruitment of inflammatory cells to the vessel wall, and proliferation and migration of vascular smooth muscle cells. In recent studies, we demonstrated that ETS-1 mediates the expression of proinflammatory cytokines and adhesion molecules that participate in the formation of neointima after balloon injury. In previous studies, we also demonstrated that ETS-1 in large part mediates the production of fibroinectin in response to Ang II in cultured mesangial cells.

Herein, we performed a series of studies to determine the role of ETS-1 in the proinflammatory and profibrotic effects of Ang II in the kidney in vivo, including cell proliferation, macrophage infiltration, mesangial expansion, oxidative stress, and fibrosis. We took advantage of the availability of an

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ETS-1 dominant-negative (ETS-1 DN) peptide, which competes with ETS-1 for binding to the target genes.20

Methods

Animals and Treatments

Eight-week-old male C57BL6 mice (Jackson Labs, Bar Harbor, ME) were divided into the following groups (n=6 per group): group 1, control mice infused with vehicle; group 2, mice treated with Ang II (1.4 mg/kg per day) for 28 days via osmotic minipump; group 3, mice treated with Ang II and ETS-1 DN (10 mg/kg per day) for 28 days via osmotic minipump; and group 4, mice treated with Ang II and ETS-1 mutant peptide (ETS-1 MU, 10 mg/kg per day) for 28 days via osmotic minipump. Additional groups of mice were also studied after 2 weeks of infusion of Ang II only or Ang II plus the ETS-1 DN or ETS-1 MU peptide. Details on the peptide sequences and synthesis are available in the online-only Data Supplement. A urine sample was collected before euthanasia and saved for protein and creatinine measurements. The mice were euthanized by exsanguinations and kidneys and hearts collected for subsequent analysis. The animal protocols were approved by the institutional animal care and use committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Blood Pressure Measurements

We used a radio telemetry system (DSI, St Paul, MN) to monitor blood pressure in conscious mice. Blood pressure was measured at baseline and then weekly for 6 hours continuously until euthanasia. See the online-only Data Supplement for expanded methods.

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis of mRNA Expression

Total RNA was extracted from renal cortices with TRIzol (Invitrogen, Carlsbad, CA), treated with DNase I and then purified with an RNA purification kit (Invitrogen). The protein- and DNA-free RNA was reverse-transcribed to cDNA (Invitrogen), amplified by PCR with specific primers (Table S1 in the online-only Data Supplement), and quantified using SYBR Green and a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA), as previously described.21 Levels of specific mRNAs were normalized using GAPDH as an internal control.

Immunofluorescence

Five-micrometer sections of kidney cortex were prepared from paraffin-embedded tissues. Sections were incubated with a rabbit antibody to ETS-1 (sc-350, Santa Cruz Biotechnology) or a rabbit antibody to nitrotyrosine (06–284, Upstate) at 4°C overnight. See the online-only Data Supplement for expanded methods.

Figure 1. Angiotensin II (Ang II) increases cortical ETS-1 expression. A, Representative confocal photomicrographs showing low basal expression of ETS-1 (green) in control kidney cortex, which is predominantly glomerular and increased by Ang II. B, The renal cortical ETS-1 protein expression increases after 2 weeks of Ang II as assessed by Western blot (n=6, P<0.05 vs control) and returns to baseline after 4 weeks of Ang II. The expression of ETS-1 was not significantly modified by treatment with either ETS-1 dominant-negative (ETS-1 DN) or ETS-1 mutant (ETS-1 MU) peptide. C, Densitometric analysis for ETS-1 showing significant increases in ETS-1 protein expression after 2 but not after 4 weeks of Ang II. Neither ETS-1 DN nor ETS-1 MU modified ETS-1 protein expression. Data expressed as mean±SEM are normalized to GAPDH (*P<0.05 vs control; #P<0.05 vs Ang II group; n=6). D, The infusion of Ang II for 2 weeks resulted in increases in cortical ETS-1 mRNA expression as assessed by real-time reverse transcriptase polymerase chain reaction (n=6; *P<0.05 vs control) and returns to baseline levels after 4 weeks of Ang II.
Immunohistochemistry

The avidin-biotin-peroxidase immunohistochemical technique (ABC kit; Vector) was used to detect microphage infiltration, cell proliferation, and α-smooth muscle actin expression, using primary antibodies against macrophages (F4/80, Ab6640; Abcam), Ki67 (m7249; Dako), and α-smooth muscle actin (A2547; Sigma). See the online-only Data Supplement for expanded methods.

Morphometric Analysis

Light microscopy of periodic acid Schiff–stained kidney sections from the 4 experimental groups was used for morphometric analysis. See the online-only Data Supplement for expanded methods.

Western Blot

Western blot analysis was performed as described before.22 The blots were incubated with the primary antibodies against ETS-1 (sc-350; Santa Cruz Biotechnology), transforming growth factor-β (TGF-β, MAB1835; R&D), or connective tissue growth factor (CTGF, sc-14940; Santa Cruz Biotechnology) at 4°C for 24 or 48 hours. The blots were washed and incubated with secondary antibodies and the signal detected by luminol chemiluminescence. See the online-only Data Supplement for expanded methods.

Statistical Analysis

Results are expressed as mean±SEM. The data were evaluated by 1-way or 2-way ANOVA. When the overall $F$ test result of ANOVA was significant, a multiple-comparison Dunnett test was applied. Student $t$ test was used in 2-mean comparisons. Differences were reported as significant when $P$ was <0.05.

Results

Blood Pressure and Left Ventricle

Blood pressure as measured by radio telemetry was increased by Ang II and not modified by the administration of either ETS-DN (Figure S1 in the online-only Data Supplement) or ETS-1 MU, an inactive peptide used as control. The administration of Ang II resulted in significant increases in left ventricular weight, which were not significantly changed by the administration of either ETS-1 DN or ETS-1 MU peptide (Table S2 in the online-only Data Supplement).
Ang II Increases Cortical ETS-1 Expression
To determine whether ETS-1 expression is increased in the renal cortex of mice infused with Ang II, we measured ETS-1 mRNA expression by real-time quantitative reverse transcriptase PCR and protein expression by Western blot. As shown in Figure 1, 12 weeks of Ang II infusion resulted in a 3-fold increase in ETS-1 mRNA expression and a 4-fold increase in ETS-1 protein expression compared with control mice. By immunofluorescence we determined that the increase in cortical ETS-1 expression was mostly glomerular (Figure 1). After 4 weeks of Ang II, the levels of ETS-1 mRNA and protein returned to baseline (Figure 1). The administration of either ETS-DN or ETS-1 MU peptide had no effect on ETS-1 expression.

ETS-1 Blockade Reduces Proteinuria and Matrix Expansion in Mice Infused With Ang II
As previously shown by others,23 the infusion of Ang II resulted in modest albeit significant increases in urinary protein excretion, which were reduced by ETS-1 DN but not by ETS-1 MU (Figure S2). As also previously shown by others,23 the administration of Ang II for 4 weeks resulted in significant increases in glomerular matrix expansion as assessed by morphometric analysis of periodic acid Schiff–stained kidney sections, which was significantly reduced by the administration of ETS-1 DN but not ETS-1 MU (Figure 2). By immunohistochemistry, we determined that the cortical staining of α-smooth muscle actin, a well-validated marker of renal fibrosis,24 was significantly increased by the infusion of Ang II and reduced by the administration of ETS-1 DN but not ETS-1 MU (Figure 3).

ETS-1 Blockade Inhibited Profibrotic Gene Expression in Mice Infused With Ang II
To determine whether these changes were linked to changes in known mediators of mesangial matrix expansion, we measured the expression of TGF-β and CTGF,25 2 profibrotic cytokines with promoter sequences that indicate the existence of several ETS-1 binding sites. As shown in Figures 4 and 5, the administration of Ang II resulted in a significant increase in the mRNA expression of TGF-β and CTGF after 2 and 4 weeks of Ang II, which were significantly reduced by the administration of ETS-1 DN but not ETS-1 MU. These changes in mRNA expression were accompanied by similar directional changes in the protein expression of TGF-β and CTGF after 4 weeks of Ang II as assessed by Western blot (Figures 4 and 5).

ETS-1 Mediates Proinflammatory Effects of Ang II
To evaluate the role of ETS-1 on the proinflammatory effects of Ang II, we measured macrophage infiltration as assessed by the number of F4/80-positive cells in kidney sections. In the control group, there were scattered low numbers of F4/80-positive cells, which increased significantly after 2 and 4 weeks of infusion with Ang II. The number of F4/80-positive cells was higher at 2 weeks compared with 4 weeks of Ang II infusion and was reduced by treatment with ETS-1 DN peptide but not by ETS-1 MU peptide. F4/80-positive cells were present in the tubulointerstitium, as well as in glomerular spaces (Figure 6).
The number of Ki-67 cells, a marker of cell proliferation, was assessed by immunohistochemistry. The infusion of Ang II for either 2 or 4 weeks resulted in significant increases in cell proliferation (Figure 7), which were ameliorated by the administration of ETS-1 DN peptide but not ETS-1 MU peptide. Ki-67–positive cells were found in the glomerular and tubulointerstitial areas.

Nitrotyrosine Formation

Nitrotyrosine, a marker of oxidative stress, was detected by immunofluorescence. Kidneys from mice infused with Ang II for 4 weeks had increased nitrotyrosine immunofluorescence, both interstitial and glomerular (Figure 8). Treatment with ETS-1 DN but not with ETS-1 MU resulted in significant reductions in the intensity of immunofluorescence. These changes in nitrotyrosine formation were accompanied by concomitant increases in NOX4 mRNA expression, which were reduced by treatment with ETS-1 DN but not ETS-1 MU peptide after 2 and 4 weeks of Ang II (Figure 8). The infusion of Ang II for 2 weeks resulted in significant increases in NOX2 mRNA that were not modified by ETS-1 DN: vehicle, 1.05±0.21; Ang II, 1.86±0.23; Ang II+ETS-DN, 2.24±0.47 (mRNA relative expression, n=6; P<0.05 versus vehicle). After 4 weeks of Ang II, the mRNA levels of NOX2 were no different from the control: vehicle 1.17±0.2 (mRNA relative expression, n=6; P value was not significant). The mRNA expression for NOX1 was detected only after 35 cycles of PCR amplification in both vehicle and Ang II–infused mice, indicating low expression of this NOX isoform.

Discussion

ETS-1 is a member of the ETS family of transcription factors that share a highly conserved DNA-binding domain.
ETS-1 and Angiotensin II–Induced Hypertension

ETS-1 (ETS domain). The ETS originates from the sequence described in the E26 avian erythroblastosis virus (E26 transformation-specific sequence). ETS-1 is involved in the regulation of normal development and differentiation and as a proto-oncogene is implicated in the pathogenesis of different types of cancer. Several studies have shown that ETS-1 is required for the normal development of the mammalian kidney and for the maintenance of glomerular integrity. Indeed, ETS-1 knockout animals have fewer glomeruli, and among the existing glomeruli, a higher percentage are immature. The renal expression of ETS-1 is increased in several models of acute kidney injury, including ischemia-reperfusion and cisplatin toxicity, and in models of glomerular injury, including the anti-Thy model of glomerulonephritis and antiglomerular basement-induced glomerulonephritis. In previous studies, we also demonstrated that Ang II increases the cortical expression of ETS-1 in rats and that knockdown of ETS-1 reduces Ang II–stimulated fibronectin production in rat mesangial cells. In the current studies, we observed that the expressions of ETS-1 mRNA and protein were increased after 2 weeks but returned to baseline at week 4. Similarly, studies by others have demonstrated that some of the
proinflammatory effects of Ang II are more pronounced during the first 2 weeks of infusion with Ang II and then reduced at later time points.\textsuperscript{3,3} We hypothesize that ETS-1 functions as an initiator of proinflammatory and profibrotic pathways, which at the same time trigger feedback mechanisms that modulate these responses.

In our studies, ETS-1 blockade in mice infused with Ang II did not modify blood pressure or left ventricular hypertrophy, but the severity of kidney damage was significantly reduced compared with Ang II alone, suggesting that these effects were independent of the hemodynamic effects of Ang II. As we and others have demonstrated, Ang II has important proinflammatory effects by promoting the expression of several proinflammatory mediators, including monocyte chemotactic protein 1, tumor necrosis factor-\textgreek{\alpha}, interleukin 1, reactive oxygen species (ROS), and cyclooxygenase 2 among others.\textsuperscript{2,2,33,34} In addition, Ang II has profibrotic effects that are mediated via increases in the expression of growth factors such as TGF-\textbeta\textsuperscript{37} and CTGF.\textsuperscript{38} In agreement with previous reports by others, the infusion of pressor doses of Ang II for 4 weeks resulted in significant increases in proteinuria, mesangial expansion,\textsuperscript{2,35} and \textalpha-smooth muscle actin expression,\textsuperscript{24} an important marker of renal fibrosis. These renal effects of Ang II were significantly reduced by the administration of an ETS-1 DN peptide but not by an ETS-1 MU peptide, suggesting that ETS-1 regulates and initiates the activation of pathways involved in the development of fibrosis in response to Ang II. TGF-\textbeta and CTGF are 2 important growth factors that mediate the proinflammatory effects of Ang II.\textsuperscript{2,33,34} Analysis of the TGF-\textbeta and CTGF promoter sequences reveals the existence of several ETS-1 binding sites. Based on our results, we hypothesize that ETS-1 may be directly regulating the expression of these growth factors.

The infusion of Ang II resulted in significant increases in the number of macrophages in the renal cortex, which were also reduced by ETS-1 blockade. Most of the macrophage infiltration was found in the tubulointerstitial space and to a lesser degree in the glomeruli. In the kidney, Ang II induces inflammation by promoting the production of ROS and proinflammatory cytokines and chemokines, leading to increased chemotaxis and macrophage infiltration.\textsuperscript{3,34} Macrophages subsequently secrete a variety of mediators that participate in the pathogenesis of renal damage in response to Ang II.\textsuperscript{2,3} Our results indicate that ETS-1 is an important mediator of the proinflammatory effects of Ang II in the kidney cortex, likely by regulating the expression of chemokines and cytokines involved in macrophage infiltration.\textsuperscript{13,34}

The administration of Ang II also resulted in significant increases in renal cell proliferation, which was predominantly tubulointerstitial and significantly reduced by ETS-1 blockade. As we and others have shown, ROS are important mediators of cell proliferation in response to Ang II.\textsuperscript{4,41} In our studies, we observed significant increases in nitrotyrosine expression, a well-validated marker of oxidative stress.\textsuperscript{42} The expression of nitrotyrosine was accompanied by concomitant increases in the mRNA expression of NOX4, the most abundant NOX isoform and source of ROS in response to Ang II in the kidney.\textsuperscript{4,41} Blockade of ETS-1 resulted in significant reductions in nitrotyrosine and NOX4 mRNA expression, suggesting that ETS-1 may be directly regulating NOX4 expression or alternatively regulating the expression of proinflammatory mediators that stimulate the production of ROS in response to Ang II. In contrast, although the administration of Ang II increased NOX2 mRNA expression, treatment with ETS-DN did not modify its expression, suggesting that ETS-1 does not regulate this NOX isoform.

Perspectives

In these studies, we have unveiled the role of the transcription factor ETS-1 as a common mediator in the simultaneous activation of profibrotic and proinflammatory pathways in the presence of increased activation of the renin-angiotensin system. Given its a role as a common mediator of multiple pathways, we postulate that ETS-1 may be a target for novel strategies in the prevention and treatment of end-organ injury in hypertension, as well as other conditions characterized by increased activation of profibrotic and proinflammatory pathways, including chronic kidney disease of different causes, glomerulonephritis, acute kidney injury, and diabetic nephropathy, among others.

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Sources of Funding

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Disclosures

None.

References

ETS-1 and Angiotensin II—Induced Hypertension

What Is Relevant?

- In these studies, we have identified ETS-1 as a potential new target for the treatment and prevention of renal injury in hypertension.

What Is New?

- These studies establish the role of the transcription factor ETS-1 as a mediator of the proinflammatory and profibrotic effects of angiotensin II.

Summary

In conclusion, we have characterized the transcription factor ETS-1 as a mediator of several effects of angiotensin II involved in the pathogenesis of renal injury in hypertension. These studies may result in the development of novel strategies in the treatment and prevention of end-organ injury in hypertension.
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Role of the Transcription Factor ETS-1 as Mediator of the Renal Pro-inflammatory And Pro-fibrotic Effects of Angiotensin II

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On line data supplement

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Methods:
ETS-1 DN and ETS-1 MU peptides:
The ETS-1 DN peptide was synthesized (CPC Scientific Inc, San Jose, Calif) following the sequences described by Ni et al. The amino acid sequence of ETS-1 DN is RWGKRKNKPKMNYYEKLKRNYDKHHTAGKRYVYGRKRKRRQQRRPPQ, which competes for binding to target genes with ETS-1 but does not initiate transcription. An HIV-1 TAT sequence is added to the carboxyl terminus to facilitate intracellular delivery and the amino terminus is biotinylated. An inactive peptide ETS-1 mutant (ETS-1 MU) is generated by replacing two arginines for glycines as previously described.

Blood Pressure Measurements by Radiotelemetry
After mice were anesthetized, the left common carotid artery was isolated, the catheter connected to the transducer (DSI, Saint Paul, MN) was introduced into the carotid artery and advanced until the tip was just inside the thoracic aorta. The transmitter was positioned along the right flank, close to the hind limb. Mice were allowed to recover for 10 days before the Ang II treatment started. Blood pressure recordings from unrestrained mice were collected and analyzed using Dataquest A.R.T. software (version 2.2; Transoma Medical).

Immunofluorescence
After deparaffinization, the sections were rinsed in phosphate-buffered saline. Nonspecific sites were blocked with 10% serum of the same species as the secondary antibody. Sections were washed, then incubated with secondary antibodies Alexa Flour 488 conjugated goat anti rabbit Ig G. Controls for antibody, in which we omitted the primary or secondary antibody, and control for nitrotyrosine using a nitrotyrosine pre-absorbed primary antibody, were included in each experiment. Images were acquired using a Leica DM6000 epifluorescence microscope (Leica Microsystems, Bannockburn, IL) with a Hamamatsu ORCA ER cooled CCD camera and SimplePCI software (Compix, Inc., Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence. Relative Fluorescent intensities were measured using Simple PCI software. For the nitrotyrosine measurements, the final intensity values were calculated after subtracting the pre-absorbed primary antibody intensity values.

Immunohistochemistry
In brief, after deparaffinization and heat mediated antigen retrieval, F4/80 and Ki67-positive cells and α-SMA positive areas were immunolocalized by incubation with respective primary antibody, followed by application of a biotinylated goat anti-rabbit or horse anti-goat secondary antibody (1:200) for 30 minutes. F4/80, Ki67 positive cells and α-SMA positive areas were quantitated by an observer unaware of the experimental conditions (Image-Pro, Media Cybernetics, Bethesda, MD).

Morphometric analysis
The glomerular surface and mesangial matrix area (PAS-positive areas) were measured in a minimum of 30 glomeruli from each animal in digital images (Image-Pro). The glomerular surface area (µm²) was measured in captured digital images by tracing around the perimeter of the glomerular capillary tuft. The mesangial matrix area (µm²) was determined by measuring the glomerular PAS-positive areas utilizing the same
software. Measurements were expressed as the percent of glomerular area occupied by PAS-positive areas.

Western blot:

Briefly, 100 mg of kidney cortex were homogenized in 500µl lysis buffer (Pro# 78510, Thermo Scientific, Rockford, IL). The resulting lysates were centrifuged for 30 min at 10,000 g at 4°C, the supernatants collected and protein concentration quantitated by Bio-Rad assay. For immunoblotting 30 µg of protein were separated by SDS-PAGE (10 or 15% acrylamide gel) and transferred to a PVDF membrane.

References

Table S1. Primers Used for Real-Time RT-PCR Analysis

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Table S2. ETS-1 blockade has no effect on Ang II induced LVH.

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<td>Ang II+ETS-1 MU</td>
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* P < 0.05 vs Control, N=6
Figure S1. Effects of ETS-1 blockade on blood pressure.
The infusion of Ang II resulted in significant elevations in blood pressure that were not modified by the administration of the active ETS-1 DN peptide and the inactive ETS-1 MU peptide. (N=6 per group, * P < 0.05 vs. Ang II, Ang II + DN and Ang II + MU)
Figure S2. Effects of ETS-1 Blockade on Proteinuria

A. The infusion of Ang II resulted in significant increases in urinary protein excretion that were normalized by ETS-1 blockade with ETS-1 DN but not by the administration of an inactive mutant peptide (ETS-1 MU) (* P < 0.05 vs. Control, # P < 0.05 vs. Ang II, N=6).

B. These changes in urinary excretion of protein were not secondary to changes in the urinary concentration of creatinine as they were not significantly different among all groups (P=NS, N=6).