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[1]. The amino acid sequence of ETS-1 DN is RWGKRKNKPKMNYEKLSRGLRYYYDKNIIHKTAGKRYVYGRKKRRQRRRPPQ,[1] 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.[2] An inactive peptide ETS-1 mutant (ETS-1 MU) is generated by replacing two arginines for glycines as previously described. [1, 2]

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 phospate-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 IgG. 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 (μm^2) was measured in captured digital images by tracing around the perimeter of the glomerular capillary tuft. The mesangial matrix area (μm^2) 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 $4^{o}C$, 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

1. Ni W, Zhan Y, He H, Maynard E, Balschi J, Oettgen P. Ets-1 is a critical transcriptional regulator of reactive oxygen species and p47(phox) gene expression in response to angiotensin ii. *Circ Res*. 2007;101:985-994.
2. Feng W, Xing D, Hua P, Zhang Y, Chen Y, Oparil S, Jaimes E. The transcription factor ets-1 mediates proinflammatory responses and neointima formation in carotid artery endoluminal vascular injury. *Hypertension*. 2010;55:1381-1388.

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

Gene	Sense	Anti-sense
GAPDH	attcttccacctttgatgc	tggtccagggtttcttact
ETS-1	aggcattgtgggtaataaca	aagtccactgtcgctgtctc
TGF-β1	gcttcagctccacagagaag	acagaagttggcatggtagc
CTGF	cctccactagatgaggctga	gacaaggctctgactcctga
NOX4	gctgtgctatgccaaagaat	gattaaccatgccattttgc
NOX1	ctcgcttctgttctctccag	ctccagtaaggccagcaata
NOX2	gaagagaggcagaaccaaca	caaccacaccagaatgacaa

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

Group	LV (mg)	WT (g)	LV/Wt
Control	98.60±1.5	29.67±0.45	3.30±0.02
Ang II	139.87±14.65 *	29.07±0.55	4.82±0.41*
Ang II+ETS-1 DN	114.27±20.29	27.23±2.35	4.65±0.04*
Ang II+ETS-1 MU	129.23±4.13*	28.88±0.36	4.51±0.18*

* $P < 0.05$ vs Control, N=6

Figure S1

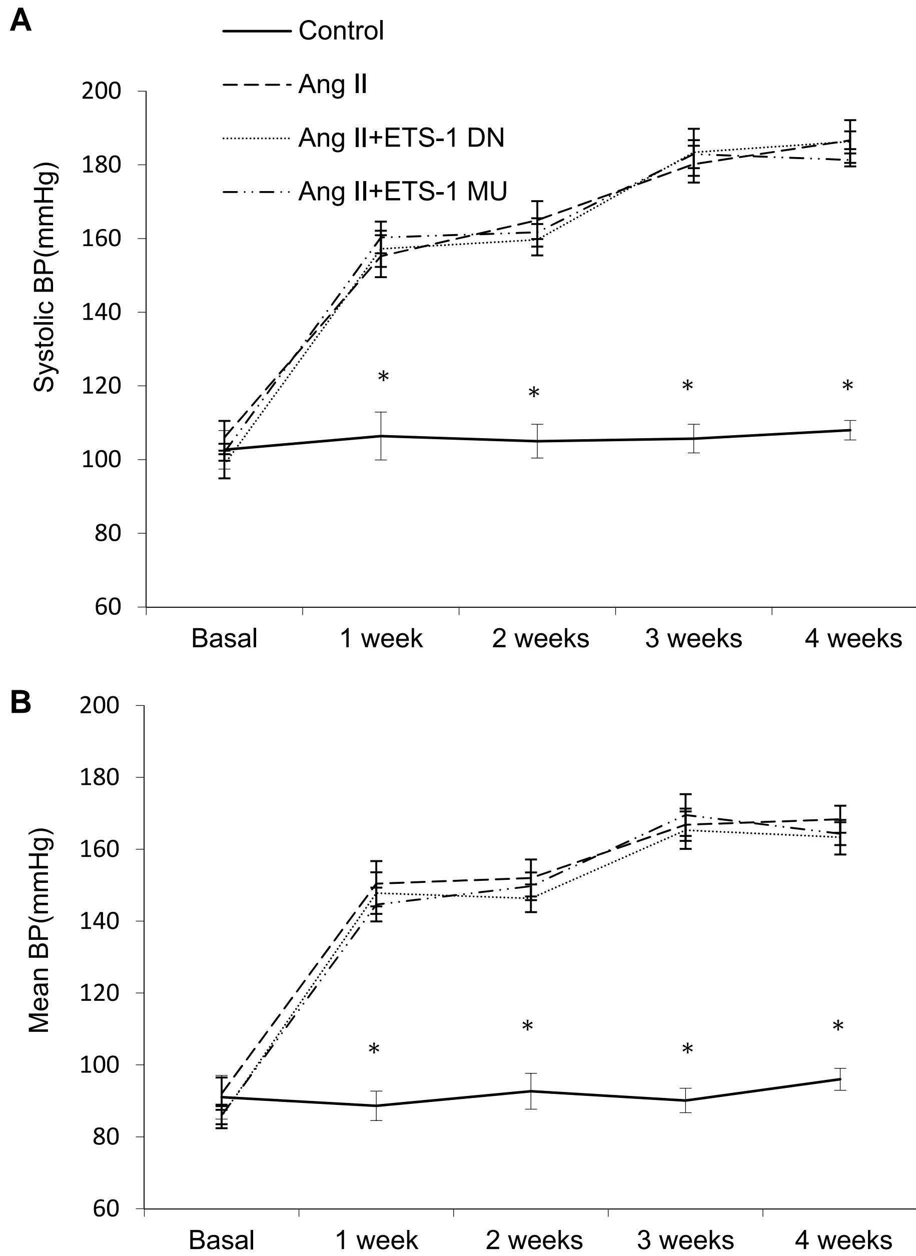

Figure S1. **Effects of ETS-1 blockade on blood pressure**.
The infusion of Ang II resulted in significant elevations in blood pressure that were not modified by the administration of the active ETS-1 DN peptide and the inactive ETS-1 MU peptide. (N=6 per group, * $P < 0.05$ vs. Ang II, Ang II + DN and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

Figure S2

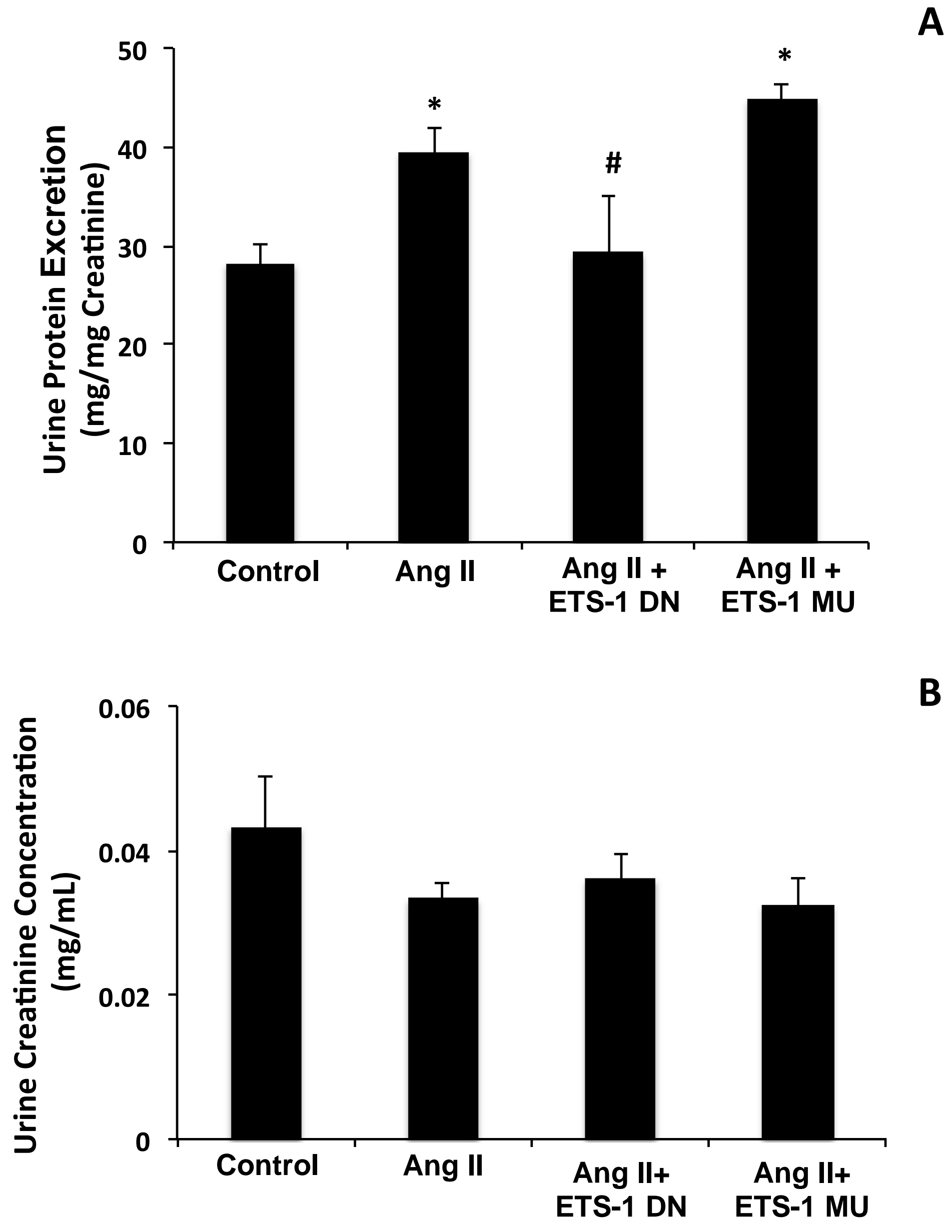

Figure S2. Effects of ETS-1 Blockade on Proteinuria

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).