SUPPLEMENT

Involvement of the brain (pro) renin receptor in cardiovascular homeostasis

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Short title: Increased PRR in the SHR brain

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Materials and Methods

Animals:

All Wistar–Kyoto rats (WKY), Spontaneously Hypertensive Rats (SHR) and Sprague-Dawley (SD) rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed individually and kept on a 12h: 12 h light–dark cycle in a climate-controlled room. Rat chow (Harlan Tekland) and water were provided *ad libitum*. All the animal experiments followed protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Measurement of PRR mRNA levels:

Thirteen-week-old male SHR (MAP ~160mmHg) and WKY rats (MAP ~ 100mmHg) were euthanized, the brains dissected, and regions containing the paraventricular nucleus (PVN), suproptic nucleus (SON), nucleus of the solitary tract (NTS), the rostral ventrolateral medulla (RVLM), organum vasculosum of lamina terminals (OVLT), median preoptic nucleus (MnPO), and central amygdala (CA) were punched out. These tissues were subjected to RNA isolation using RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA, ~500ng was reverse transcribed with iScript^{TM} cDNA synthesis kit (BioRad, Hercules, CA, USA) in a 20 µL reaction system. The PRR mRNA levels were analyzed by quantitative real-time PCR using PRR specific primers and Taqman probe Rn01430718_m1 (Applied Biosystems, Foster City, CA, USA) in the PRISM 7000 sequence detection system (Applied

Biosystems). All cDNA samples were assayed in triplicate. Data were normalized to 18S RNA.

PRR immunoreactivity in the brain:

Immunofluorescence staining was performed using 10 µm fresh brain sections with the following protocols. Male WKY rats (~250g) were euthanized, brains removed, and frozen. Coronal sections were cut from the brainstem or hypothalamus areas, and were air dried overnight at room temperature. Prior to staining, slides were submerged in -20^{o}C acetone for 5 minutes, permitted to air dry and then washed in TBS for 5 minutes. Sections were incubated with 3% horse serum in TBS for 20 minutes and then incubated overnight at 4℃ in a cocktail consisting of 1:200 dilution of goat anti-ATP6AP2 (a PRR specific antibody (Abcam, Cambridge, MA, USA), and either 1:250 dilution of anti-neuronal nuclear antibody (NeuN, Chemicon, Temecula, CA, USA) or 1:500 dilution of rabbit anti-arginine vasopressin (Millipore, Billerica, MA, USA). Afterwards, sections were washed in TBS twice for 5 minutes and incubated for one hour at room temperature in a mixture of secondary antibodies (Alexa Fluor 594 donkey anti-goat IgG and Alexa Fluor 488 donkey anti-mouse IgG; or Alexa Fluor 488 goat anti- rabbit IgG; all diluted 500 fold). The sections were mounted in Vectashield (Vector Labs, Burlingame, CA) and images were taken with a Leica TCS SP2, laser-disk scanning, confocal microscope.

Plasmid construction and production of AAV vectors containing human PRR or rat PRR-shRNA

Human PRR cDNA or GFP cDNA was cloned into the AAV vector, PTR-UF22, under the control of acytomegalovirus enhancer and chicken β-actin (CBA) promoter, generating construct, AAV-hPRR or control vector, AAV-GFP. Our previous evidence has indicated that AAV-GFP is an appropriate control for AAV vector containing a transgene because GFP itself has no effect on blood pressure.

A synthetic complementary DNA encoding shRNA targeted rat PRR mRNA at 989-1007 region (CCTACAACCTTGCGTATAA, gene accession number: XM_217592) or scrambled DNA, containing the same nucleotides as the PRR-shRNA DNA template but in a completely different arrangement and not targeted any rat gene, was designed and cloned into a AAV vector, PTR-UF11, under the control of human U6 promoter (Online Figure IV). A GFP reporter gene under the control of CBA promoter was cloned upstream of the shRNA expression cassette in order to directly visualize expression from the vector after delivery, generating construct AAV-PRR-shRNA or control vector, AAV-scrambled shRNA (AAV-Sc-shRNA).

AAV-hPRR, AAV-PRR-shRNA and their control vectors, AAV-GFP, AAV-scRNA were packaged into AAV virus. Virus production and titer determination were performed as previously described[1]. Briefly, HEK 293 cells were cotransfected

with the constructs and the helper plasmid pDG DNAs for 48-60 h. Cells were harvested, and the crude lysates purified through an iodixanol step gradient followed by Mono-Q FPLC chromatography. The vector genome (vg) titers of AAV particles were determined by real-time PCR.

Implantation of telemetry transducers, *In Vivo* PRR or PRR-shRNA delivery into the supraoptic nucleus (SON) and arginine vasopressin assay:

Six-week-old male SD rats, WKY, or SHR were anesthetized and telemetry transducers consisting of a fluid-filled catheter attached to a PA-C40 transmitter (DSI, St. Paul, MN, USA) were implanted into the abdominal aorta as described previously [2]. Following recovery for 10 days, animals were randomly divided into two groups. SD rats were used for bilateral injection into the SON with either experimental virus, AAV-hPRR or control virus, AAV-GFP (n=8 in each group). Similarly, SHR or WKY were injected with either AAV-PRR--shRNA or AAV-Sc-shRNA (n=6 in each group). 200 nl AAV viruses ($2x10^8$ genome containing virus particles) were injected into each injection site (A/P: 1.4mm, D/V: 9.2mm, M/L: 1.8mm). Mean arterial pressure (MAP) and heart rate (HR) were monitored 8 hours during day time before microinjection as baseline, and were sampled once a week at the same time each day after the microinjection at indicated time periods.

At twelve weeks post microinjection, SD rats were transferred into metabolic cages and allowed 72 h to get used to the environment. Basal 24 h water intake, urine excretion and urine osmolality were then assayed for 3 consecutive days.

Urine was collected and kept at -80^{o}C for AVP assay. All animals were euthanized one week after the metabolic experiment. Plasma was collected, and plasma AVP, plasma osmolality and urine AVP were then assayed.

AVP measurements from plasma and urine samples were performed using commercially available arg8-vasopressin EIA kits (Assay Designs, Michigan, USA) following the manufacturer's instructions.

Preparation of neuronal and astroglial cells in primary cultures from the WKY rat and SHR brains:

Neuronal cells in primary culture from the brainstem and hypothalamus of one-day-old WKY and SHR were established as described previously [3, 4]. Neuronal cultures contain more than 90% neurons (the remainder are primarily astroglia), while astroglial cultures contain>99% astroglial cells. The cultures were maintained for 12-14 days prior to their use in the experiments.

Angiotensin I and II conversion in neuronal cultures:

Fourteen day old primary neuronal cultures from the hypothalamus and brainstem of WKY rats were rinsed three times with 37℃ PBS containing 2mM $CaCl_2$, 2mM MgCl2 and 10mM glucose (PBSS). Cells were incubated in 0.5mL PBSS buffer with 2nM human prorenin and human angiotensinogen (AGT) for the indicated time periods and concentrations of AGT. Cultures incubated without AGT were used as control. Incubation media were collected to quantitate

Angiotensin I and Angiotensin II, which were determined by radio-immunoassay as described previously [5].

Measurement of phosphorylation of ERK1/2 MAP kinases:

Western blot analysis was used to measure phosphorylated and total ERK1/2 MAP kinases using the neuronal cultures from WKY rats and SHR as described previously[4]. Briefly, neuronal cultures were incubated with or without recombinant human renin (Sigma-Aldrich, St Louis, MO, USA) in the presence of 2µM losartan, an AT1 receptor antagonist. Proteins were isolated, separated with 10% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with mouse monoclonal antibody to phosphorylated ERK1/2. Hybrid protein bands were detected with ECL™ Western Blotting Detection Reagents. The membrane was stripped of phosphorylated ERK1/2 antibody and re-probed with ERK1 (C-16) and ERK2 (C-14) antibody to assay total ERK1/2 protein levels. Protein bands representing ERK1/2 MAP kinase were quantified using the NIH ImageJ program (Bethseda, Maryland, USA; http://rsb.info.nih.gov/ij) phosphorylated ERK1/2 were normalized to total ERK1/2.

Statistical analysis:

All data are expressed as means ± S.E.M. Statistical significance was evaluated with the use of one-way ANOVA and unpaired Students t test. Differences were considered to be significant at $p < 0.05$.

Figure Legends:

Online Figure I: Levels of PRR mRNA are increased in neuronal cultures from SHR brain

Neuronal and astroglial cells in primary cultures derived from the hypothalamus and brainstem regions of WKY and SHR were established as described previously [3]. RNA was isolated from 12-14 day old cultures for quantitation of PRR mRNA. *p < 0.05 vs. corresponding astroglial sample. †p < 0 .05 vs. WKY neurons

Online Figure II: PRR immunoreactivity colocalizes with AVP in the SON of SD rats

Representative fluorescence images of PRR (left) and AVP (middle) immunostaining, and merged image (right), respectively.

Online Figure III : Increased PRR expression in the SON of SD rats does not influence mean arterial pressure or heart rate

MAP and HR were monitored for 8 hours via telemetry transducers, one day before microinjection of AAV-hPRR or AAV-GFP, in order to provide baseline values. After a one week period to recover from the procedure, MAP and HR were monitored twice a week at the same time, for up to twelve weeks, as described in the Methods. Data are means ± S.E.M from 8 rats in each group.

Online Figure IV: PRR-shRNA and its effect in WKY rat brain neurons

a. Map of AAV-PRR-shRNA (top) and control vector, AAV-Sc-shRNA (bottom).

Both PRR-shRNA and Sc-shRNA are under the control of human U6 promoter. A GFP reporter gene driven by CBA promoter was used to visualize expression from the vector after delivery.

b. PRR-shRNA decreases PRR mRNA level in neuronal cultures

Six-day-old neuronal cultures derived from hypothalamus and brainstem regions of WKY were treated with either AAV-PRR-shRNA (MOI=1:500) or AAV-Sc-shRNA (MOI=1:500). PRR mRNA level of neuronal cells were investigated at five days after virus infection. Data are means ± S.E.M (n=3 for each group). Experiments were repeated for twice. *P<0.05 compared to control and Sc-shRNA.

c. PRR-shRNA attenuates ERK1/2 phosphorylation in neuronal cultures.

Six-day-old neuronal cultures derived from hypothalamus and brainstem regions of WKY were infected with either AAV-PRR-shRNA or AAV-Sc-shRNA as described as Figure 4b. Cells were treated with 20nM of renin in the presence of 2 μM losartan for 2 minutes at seven days after infection. Western blotting was performed to evaluate p-ERK1/2 expression. Top is a representative image of Western blot. Bottom is ratio of phosphorylated ERK1/2 to total ERK1/2. ShRNA represent PRR-shRNA, ScRNA represent Sc-shRNA. *P<0.05 compared to control and Sc-shRNA.

Online Figure V: Effect of PRR on angiotensin I and II formation in WKY brain neurons

Fourteen-day old neuronal cultures were washed and incubated in PBS for 1 h. They were then treated with 2nM human prorenin in the presence or absence of increasing concentrations of human angiotensinogen substrate for 3 h (a) or for the indicated time period with 100μM angiotensinogen (b), media collected and used for measurement of angiotensin I and II by radioimmunoassay. Data are means ± S.E.M (n=3 for each group). This experiment was repeated for 3 times.

Online Figure VI: Renin stimulates a time-dependent increase in ERK1/2 activation in WKY rat and SHR neurons

WKY rat or SHR neuronal cultures were incubated with 20nM recombinant human renin in the presence of 2μM losartan for the indicated time periods. Proteins were separated and probed with total (T) ERK1/2 and phosphorylated (P) ERK1/2 antibodies. A. Top panel are representative Western blots showing P-ERK in neurons from WKY rats (left) and SHR (right). Bottom panel are representative Western blots showing T-ERK in the same neurons from WKY rats (left) and SHR (right). B. Quantification of P-ERK1/2 that has been normalized against T-ERK1/2 and compared with control, normalized to unity. Data are means ± S.E.M. *P < 0.05 vs. SHR control, # P<0.05 vs. WKY control; *$ P$< 0.05 vs. WKY in the same treatment.

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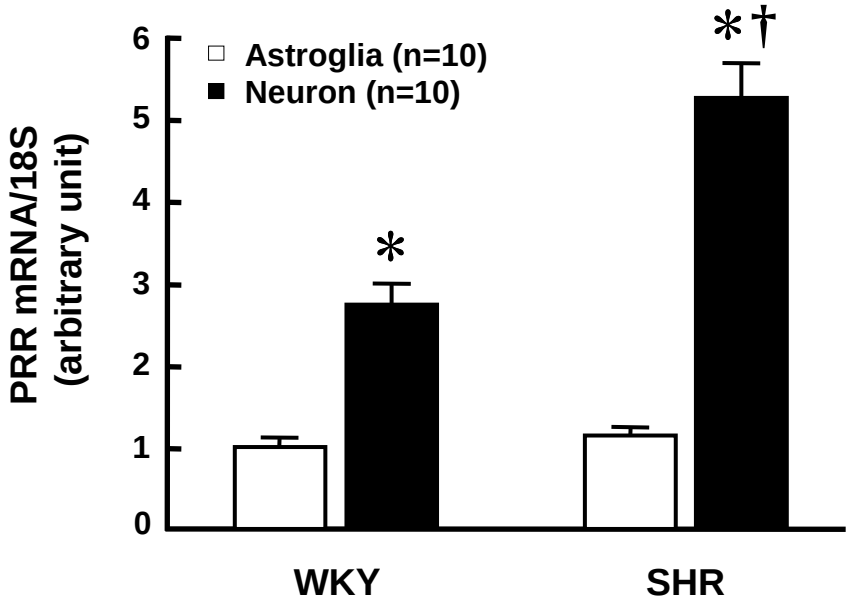
Online Figure I



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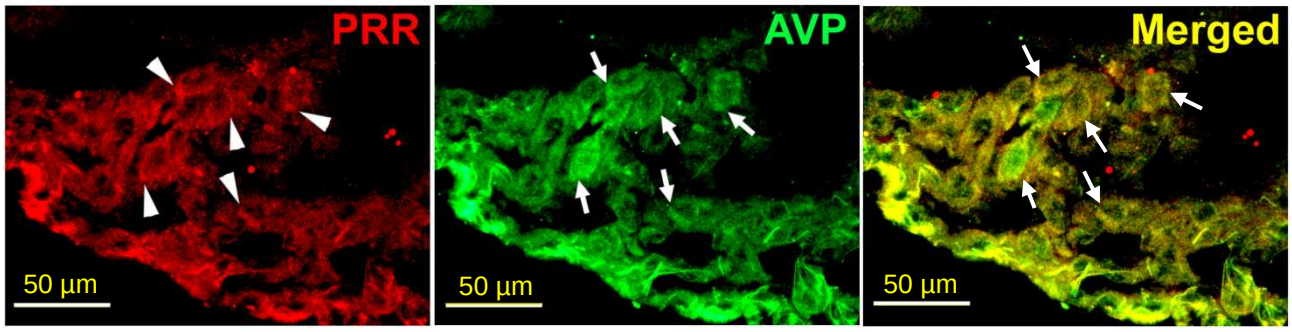

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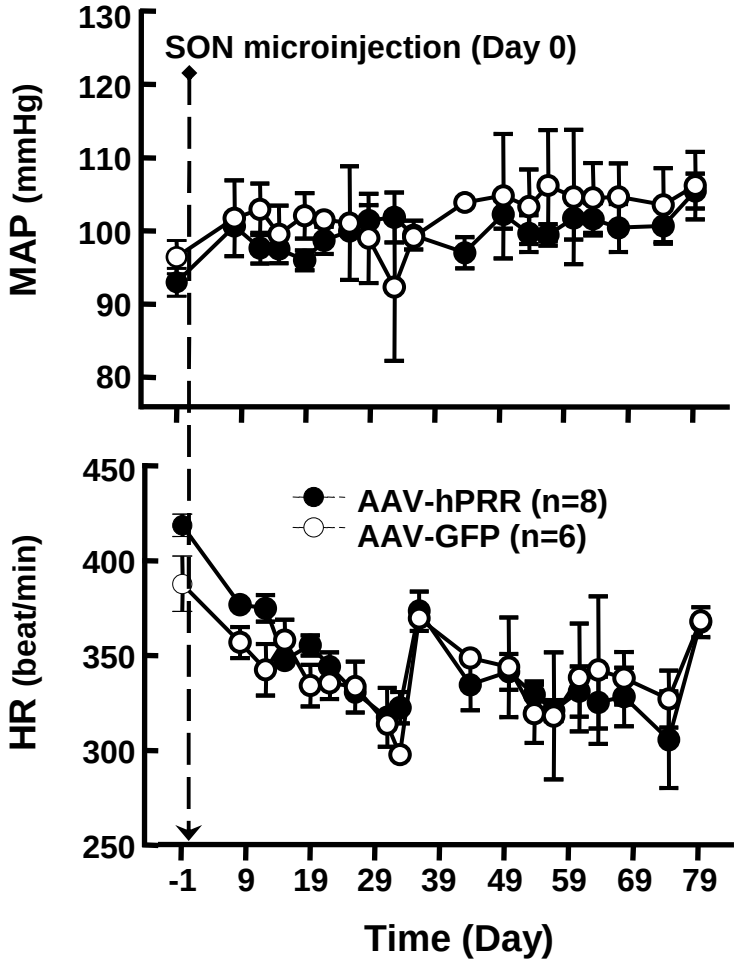

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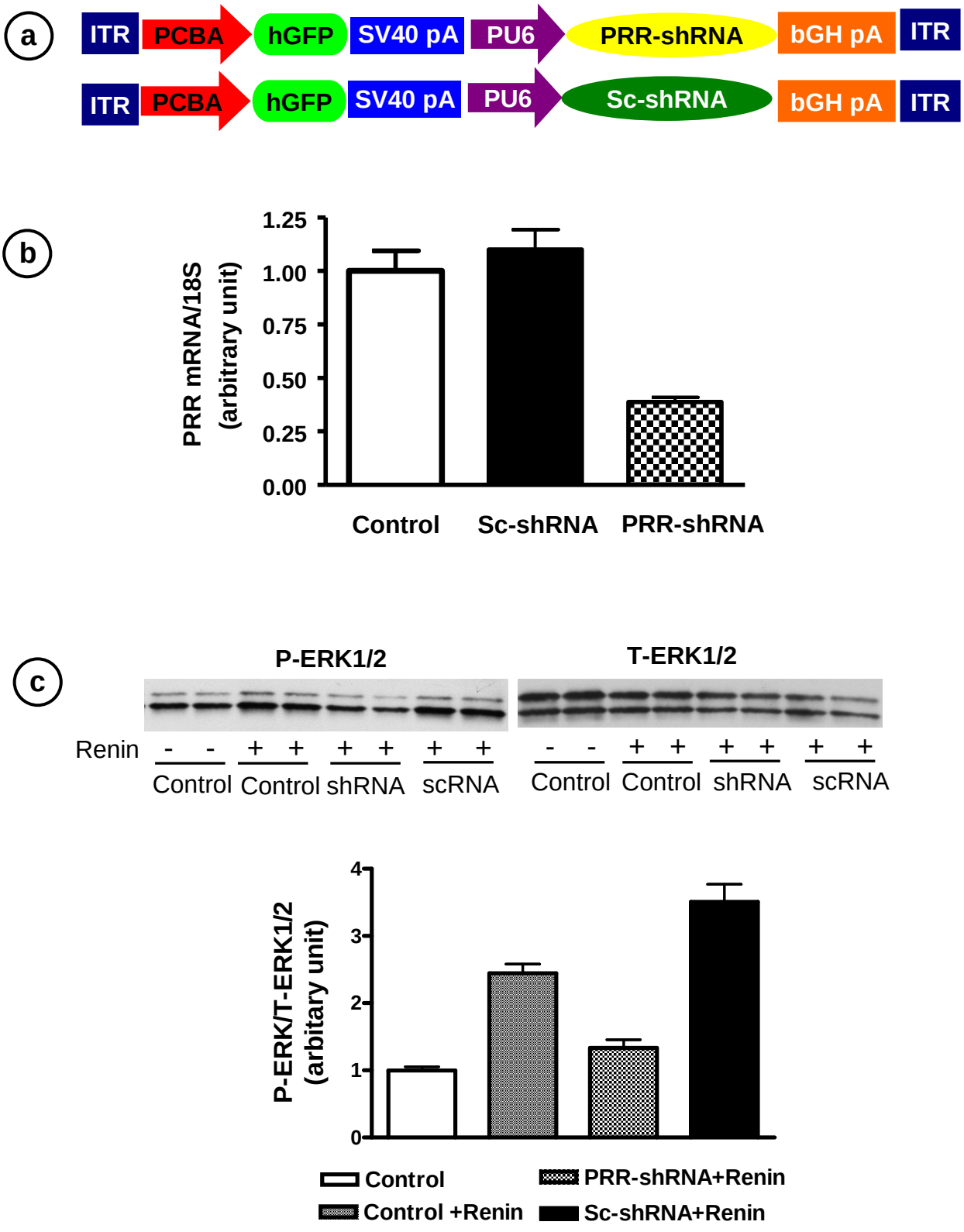
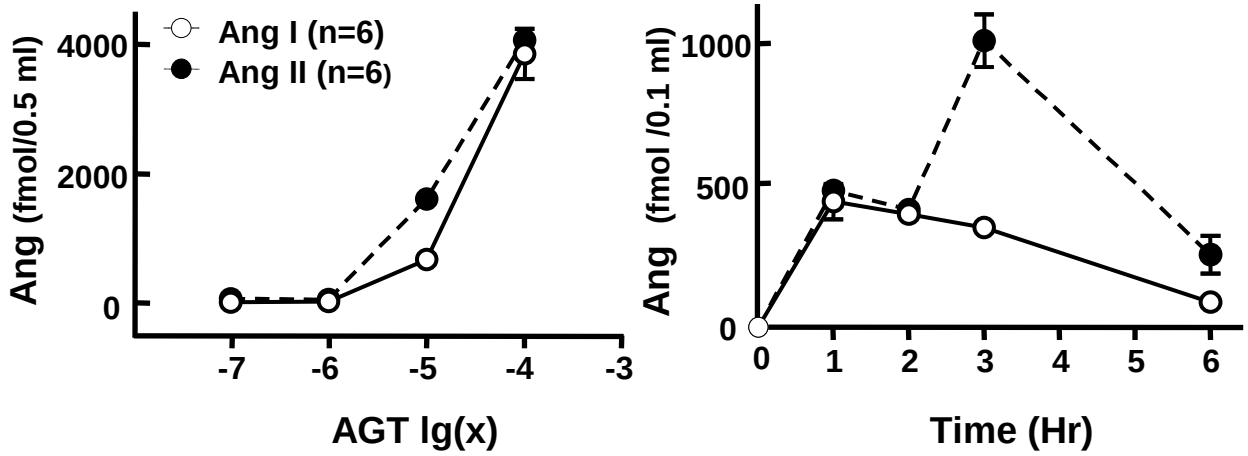


Online Figure V



Online Figure VI