SUPPLEMENTAL MATERIAL

Angiotensin-II-dependent Hypertension Requires Cyclooxygenase 1-derived Prostaglandin E_2 and EP_1 Receptor Signaling in the Subfornical Organ of the Brain

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Short Title: EP_1R in the SFO and Hypertension

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Detailed Methods

Animals. All procedures were approved by the Animal Care and Use Committee at Cornell University. Studies were conducted in adult (8-10 wks old) male COX-1 null, COX-2 null, and EP_1R null mice which were obtained from in-house colonies[1-3]. Mice were congenic with the C57Bl/6 strain and age-matched C57Bl/6 mice (Harlan Laboratories) were used as wild-type (WT) controls. Mice were fed standard chow and water *ad libitum*. Care of the mice met or exceeded the standards set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, USDA regulations, and the AVMA Panel on Euthanasia.

Pharmacological agents. The COX-1 inhibitor SC-560 ($C_{17}H_{12}ClF_3N_2O$), the COX-2 inhibitor NS-398 ($C_{13}H_{18}N_2O_5S$) and the EP_1R inhibitor SC-51089 ($C_{22}H_{19}ClN_4O_3 \bullet HCL$) (Enzo Life Sciences, Plymouth Meeting, PA) were dissolved in dimethylsulfoxide (DMSO) and diluted with saline to the desired concentration. The final DMSO concentration was <0.2%, which does not affect physiological parameters tested. The Ang-II type 1 recptor (AT_1R) antagonist lorsartan potassium ($C_{22}H_{22}ClKN_6O$) (Sigma-Aldrich, St. Louis, MO) and PGE_2 ($C_{20}H_{32}O_5$) (Cayman Chemical, Ann Arbor, MI) were diluted in sterile saline. Specificity of SC-51089, SC-560 and NS-398 at similar doses used herein has been confirmed previously[1].

Blood pressure studies. Mice were anesthetized (ketamine, 150 mg/kg + xylazine, 15 mg/kg, ip) and instrumented with radiotelemetry probes (TA11PA-C10, Data Sciences International, Arden Hills, MN) as previously described[4, 5]. Briefly, the catheter of the telemeter was implanted in the thoracic aorta via the left common carotid artery, and the body of the probe was placed in a subcutaneous pocket created in the right flank. The wound was closed and sutured, and body temperature was maintained at 37°C using a heating pad until sternal recumbency was recovered. Mice remained undisturbed in their home cages for 7 days to achieve full recovery of normal circadian rhythm and cardiovascular parameters[4] before baseline BP recording over 3-4 days. Mice were then implanted subcutaneously with 14-day osmotic minipumps (ALZET®; Durect Corporation, Cupertino, CA) loaded with the slow-pressor dose of Ang-II (600ng/kg/min) as described[5]. BP was recorded daily for 2 hours (10am-12pm) for 3 weeks to monitor the effects of Ang-II during the entire infusion period as well as several days after infusion. It should be noted that although 14-day pumps were used, the actual calculated infusion times for these pumps ranges from 16 to 19 days (0.22-0.25μl/hr, fill volume of 100 ± 6μl).

In studies using intracerebroventricular (i.c.v.) infusion of SC-51089, mice were instrumented with i.c.v. cannulae (brain coordinates relative to bregma: 0.3mm caudal, 1.00 from midline, 3.3mm ventral)[5] during the same surgical session as radiotelemeter implantation. After 7 days of recovery and 3 days of baseline recording, two osmotic minipumps were implanted subcutaneously. One contained the 14-day slow-pressor dose of Ang-II (600ng/kg/min) as described above. The other one was loaded with SC-51089 (144μg/day, 14 days) and was connected to i.c.v. cannulae using

MicroRenathane® tubing (Braintree Scientific, Braintree, MA). BP monitoring was carried out as described above.

Power spectral analysis. Power spectral analysis of arterial pressure variability was performed as described[6-8]. Briefly, spectral power of mean arterial pressure (MAP) in the frequency domain was determined using custom-written functions in HemoLab Analyzer and Batch Processor software (version 9.3, provided by Dr. Harald Stauss, University of Iowa, Iowa City, IA). An average spectrum using 4096 point Fast Fourier Transforms (FFT) with 50% overlap was computed for a compact spectrum display[6]. Spectra were divided into the following frequency ranges: low frequency (LF: 0.4-1.0 Hz) and high frequency (HF: 1.0-3 Hz)[8]. Data were expressed as LF/HF in relation to baseline.

Measurement of dipsogenic responses. WT mice were instrumented with i.c.v. cannulae as described above and allowed 7 days recovery. Mice were administered either vehicle, SC-51089 (10μg/kg), SC-560 (10mg/kg) or NS-398 (10mg/kg) by introperitoneal injection (200 nl) 30 minutes prior to i.c.v. bolus administration of Ang-II (200ng, 200nl). Water drinking responses were measured over 1 hour as described[9, 10].

Quantitative real-time PCR detection of prostanoid-related transcripts. WT mice were decapitated and brains were removed and immediately placed on dry ice. The SFO was isolated by micropunch (0.75mm, Stoelting Co., Wood Dale, IL) as described[10]. Two SFO samples were used per biological sample. Total RNA was isolated by Trizol® (Invitrogen, Carlsbad, CA) extraction and reverse transcribed using random hexamer primers. Template samples (25 ng) were subjected in triplicate to real-time qPCR (ABI 7500FAST system) using Power SYBR Green (Applied Biosystems, Foster City, CA) as described[10]. All primers were derived from *Mus Musculus* gene (National Center for Biotechonology Information GenBank) and are shown in Table S1. Serial dilution was performed for each set of primers to determine qPCR amplification efficiency before the experimental run. A dissociation protocol (60-95°C melt) was performed at the end of each run to verify that only one amplicon was formed during the process of amplification. No RT and no template controls were performed during each run to ensure no contamination was present. β-actin was used as a normalizer gene in all experiments. Relative fold-change was calculated using the comparative $\Delta\Delta$Ct method as described[10].

PGE_2 assay. WT mice were implanted with osmotic minipumps loaded with the 14-day slow-pressor dose of Ang-II (600ng/kg/min, see above) or saline. Mice were decapitated at 3, 7 or 14 days after start of infusions and brains were removed and immediately flash frozen in liquid nitrogen for 10 sec. Micropunches of SFO, paraventricular nuclei (PVN), somatosensory cortex (CTX) and cerebellum (CBM) were collected from 2 mice per biological sample and weighed. Samples were then homogenized and prostanoids extracted as previously described[1, 11]. PGE_2 concentration was determined using an enzyme immunoassay kit (Cayman Chemical)[1, 11].

ROS detection. ROS production was assessed in dissociated SFO cells and in SFO-containing tissue using dyhidroethidium (DHE) as an indicator. For *in vitro* ROS detection in SFO cells[12], WT, COX-1-null, COX-2-null or EP_1R-null mice were sacrificed using CO_2, and the brains were removed and quickly transferred to a chamber containing ice-cold sucrose artificial cerebrospinal fluid (s-aCSF) composed of (in mM): 26 $NaHCO_3$, 1 NaH_2PO_4, 3 KCl, 5 $MgSO_4$, 0.5 $CaCl_2$, 10 glucose, and 248 sucrose, oxygenated with 95% O_2 and 5% CO_2, pH 7.35. Coronal slices (300μm) were then obtained using a Vibratome (Leica) and stored in a chamber filled with oxygenated lactic acid (l)-aCSF composed of (in mM): 124 NaCl, 26 $NaHCO_3$, 5 KCl, 1 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 4.5 lactic acid, pH 7.35. The SFO region was dissected and transferred to an oxygenated (l)-aCSF buffer containing 0.02% pronase and 0.02% thermolysin, and then incubated at 35^0C for 1.5 hrs. Isolated SFO cells were then transferred to a glass-bottom Petri dish and perfused with the oxygenated (l)-aCSF and incubated with 2μM DHE (Molecular Probes) for 30 minutes in the dark and were continuously perfused with 2μM DHE containing (l)-aCSF. Following 100-150 ms exposure to mercury light, time-resolved fluorescence was measured every 30s after addition of vehicle, Ang-II (100nM) or PGE_2 (100nM) using IPLab (Scanalytics Inc.). Recordings were initiated after a stable baseline was achieved. In a subset of *in vitro* studies, SC-51089 (10μM) or losartan (3μM) (in oxygenated (l)-aCSF) were applied 30 min prior to Ang-II or PGE_2.

ROS production in SFO tissue was assessed by DHE microfluorography as described[5]. Brains were removed on day 16 of Ang-II or vehicle infusions (peak of hypertension), flash frozen and coronal sections (20μM) were taken onto chilled microscope slides. Sections were then thawed at room temperature, rehydrated with phosphate-buffered saline (PBS), and incubated for 5 min in the dark with DHE (1μM) followed by 2 min wash with PBS. DHE fluorescence was visualized by confocal microscopy (Zeiss LSM 510 or Leica SP5). Detector and laser settings were kept constant across all samples within individual experiments, and control and experimental samples were always processed in parallel. Fluorescence intensity was quantified using ImageJ software and normalized to fluorescence levels observed in control samples as described[5].

Adenoviral-mediated reconstitution of EP_1R in EP_1R-null mice. A recombinant adenoviral vector encoding murine EP_1R tagged with HA on the N-terminus was engineered and then generated and characterized by the Iowa Gene Transfer Vector Core (IGTVC)[13]. An Ad vector encoding green fluorescent protein (AdGFP) was obtained from IGTVC and used as a control. Briefly, adenoviruses were based on the human Ad serotype 5, from which the E1a and E1b replication genes had been deleted[13]. The titer of both viruses was ~ $5x10^{10}$ pfu/mL. For $AdEP_1R$, HA (YPYDVPDYA) was N-terminally tagged to full length cDNA of *mus musculus* EP_1R gene (GenBank ID: NM013641). HA-EP_1R was under the control of the CMV promoter. In the same construct, a reporter gene GFP was driven off the RSV promoter (Fig S3A). To validate $AdEP_1R$ potency and stability, several experiments were performed. First, Neuro2A cells were infected with serial dilutions of $AdEP_1R$ (0-500 multiplicity of infection). 48 hrs after infection, cells were collected and qPCR analysis was performed as described above using a primer set spanning HA and EP_1R: Forward 5'-CCCATACGACGTACCAGATTACGCTAG-3'; Reverse 5'-GCAGCGCCAGCGCCAGCACGTTG-3' (Fig S3B). Second, for *in vivo* validation, WT mice underwent SFO-targeted injection of $AdEP_1R$ (500nl) as described[5, 10, 14] and HA-EP_1R expression was verified in regional micropunches and *in situ*. *In situ* expression of

$AdEP_1R$-induced HA-EP_1R in EP_1R null mice was examined using immunohistochemistry (Fig S3C). 9 days after SFO-targeted injection of titer-matched $AdEP_1R$ or AdGFP, mice were perfused with 37^{o}C saline followed by ice-cold 4% paraformaldehyde. Brains were removed and stored in 30% sucrose overnight before 20µm coronal sections were taken onto glass slides. Immunostaining was performed using a rabbit polyclonal HA antibody (2.5µg/ml, Abcam) followed by DyLight™549-conjugated AffiniPure goat anti-rabbit IgG (3µg/ml, Jackson ImmunoResearch Laboratories, Inc.). $AdEP_1R$ and AdGFP-treated samples were assayed on the same slides. Images were taken using a confocal microscope (Zeiss LSM 510). Transgene expression was limited to the SFO in all samples except in one animal in which a few cells in organum vasculosum of the lamina terminalis were transduced. In separate studies, brains were collected 9 or 28 days after SFO-targeted $AdEP_1R$ injection (a time-frame that covered the entire Ang-II infusion period in the following experiments). Micropunches from SFO, PVN, RVLM and CTX (0.75mm, Stoelting Co., Wood Dale, IL) were harvested and subjected to qPCR analysis using the HA-EP_1R primer set listed above (Fig S3D). β-actin was used as the normalizing gene. CTX samples from day 9 post-injection were used as the calibrator. Real-time qPCR was performed as described above. Finally, for BP and ROS studies, EP_1R-null mice underwent SFO-targeted injection of titer-matched AdGFP or $AdEP_1R$ (500nl). During the same surgical session, radiotelemeters were implanted as described above. Nine days later, osmotic minipumps loaded with the 14-day slow-pressor Ang-II dose were installed as described above. BP recording, spectral analysis and ROS measurements were performed as described above.

Data analysis. Data are expressed as mean±SEM. Comparisons between two groups were evaluated using the Student's *t* test. Multiple comparisons were evaluated by ANOVA followed by Dunnett's or Tukey's test. Differences were considered statistically significant at p<0.05.

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Table S1

qPCR Primer Sequences

Gene	Sequence	GenBank ID
EP_1R	F: TTTATTAGCCTTGGGCCTCGTGGA R: ATTGCACACTAATGCCGCAAGGAG	NM_013641
EP_2R	F: GATGAAGCAACCAGAGCAGAC R: CAGAGAGGACTCCCACATGAA	NM_008964
EP_3R	F: GGTCATCCTCGTGTACCTGTC R: GTCATGGTTAGCCCGAAGAA	NM_011196
EP_4R	F: GCCCTCTCCTGCCAATATAAC R: TTTCAACACTTTGGCCTGAAC	NM_008965
COX-1	F: CACTGGTGGATGCCTTCTCT R: TCTCGGGACTCCTTGATGAC	NM_008969
COX-2	F: TGGTGCCTGGTCTGATGATG R: GTGGTAACCGCTCAGGTGTTG	NM_011198
cPGES	F: TGCTGTACTGTCACAGGAAACCCT R: AAGTCACAACACTTGTCTGGCTGC	NM_019766
PGES1	F: TTTCTGCTCTGCAGCACACT R: CACATCTGGGTCACTCCTGTA	NM_022415
PGES2	F: GAGGACAAGGCCCAAACTTAC R: ATCCCTGTCAACAAGGGACTT	NM_133783
β-actin	F: CATCCTCTTCCTCCCTGGAGAAGA R: ACAGGATTCCATACCCAAGAAGGAAGG	NM_007393

Supplemental Figures

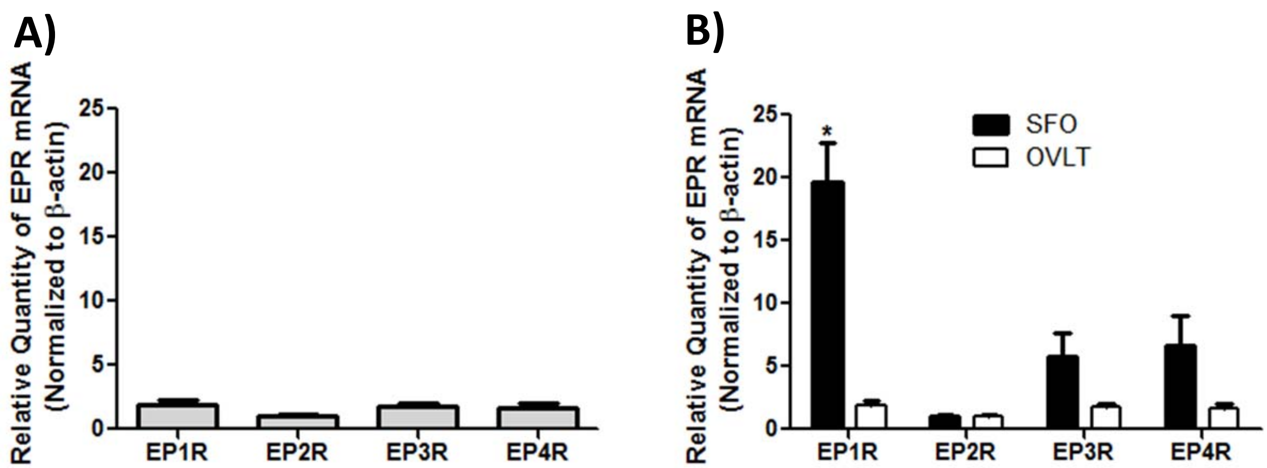

Figure S1. A) Basal mRNA levels of $EPR_{1\text{-}4}$ in adult WT organum vasculosum of the lamina terminalis (OVLT) tissue (n=3) as analyzed by quantitative real-time PCR. B) Comparsion of basal mRNA levels of $EPR_{1\text{-}4}$ in SFO vs OVLT. *p<0.05 vs. EP_1R in OVLT and $EP_{2\text{-}4}R$ in SFO and OVLT.

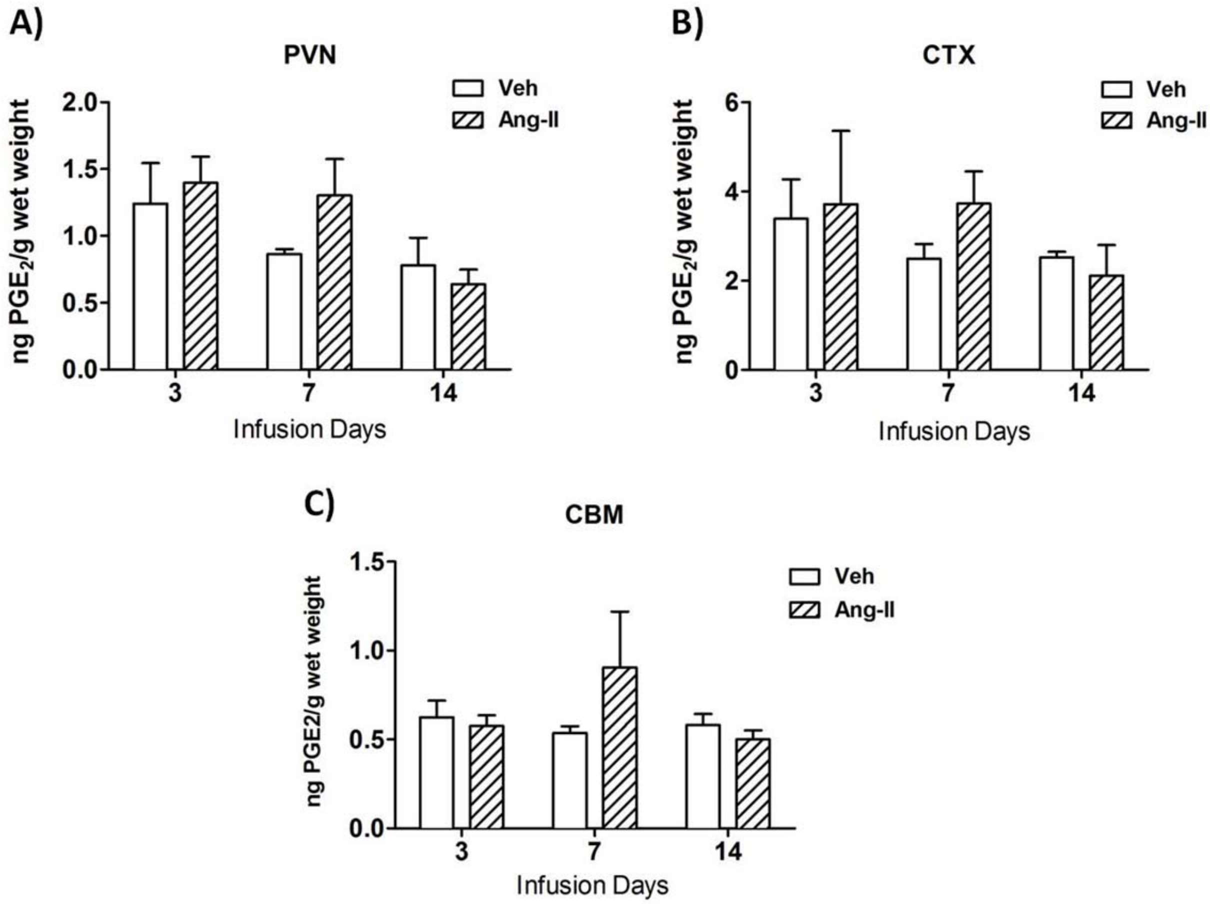

Figure S2. PGE_2 levels in WT mouse brain regions during slow-pressor Ang-II infusion. PGE_2 levels measured by ELISA in micropunches of PVN (A), CTX (B) and CBM (C) at 3, 7 and 14 days of slow-pressor Ang-II (n=3) or vehicle infusions (n=3). $p>0.05$ vs vehicle at all time-points in all regions. Two brains were pooled per biological sample for all regions.

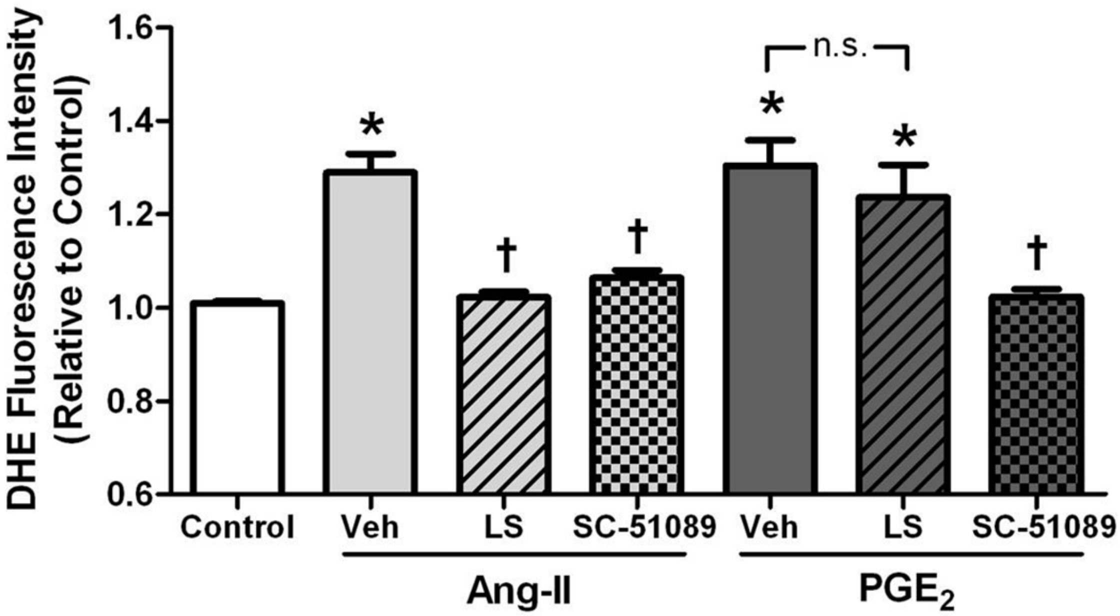

Figure S3. Ang-II- and PGE_2-induced increases in ROS formation in cells dissociated from the SFO of WT mice. Histogram showing the effects of Ang-II or PGE_2 on ROS formation as measured by DHE fluorescence intensity in cells dissociated from SFO of WT mice (n=9-24) before and after vehicle (Veh), losartan (LS) or SC-51089. All data are expressed as a ratio of DHE fluorescence relative to baseline (control). *$p<0.05$ vs. control; †$p<0.05$ vs. vehicle; n.s., not significant.

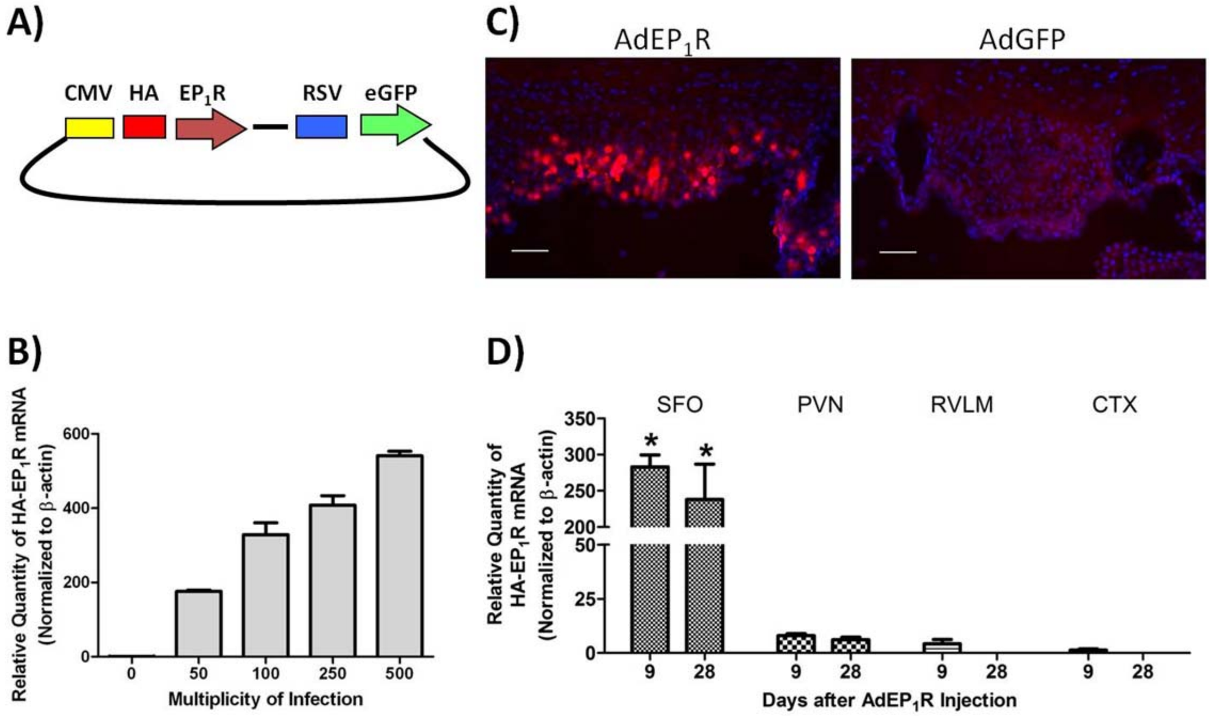

Figure S4. Ad-mediated EP_1R transgene expression in the SFO *in vitro* and *in vivo*. A) Schematic of $AdEP_1R$ vector containing HA-tagged full-length murine EP_1R gene driven off CMV and enhanced green fluorescent protein (eGFP) driven off RSV. B) qPCR data using a primer set spanning the HA tag and EP_1R gene revealed concentration-dependent (0-500 multiplicity of infection) effects of $AdEP_1R$ on HA-EP_1R transcript levels in Neuro2A cells. C) Representative immunohistochemistry of SFO in $EP_1R^{-/-}$ mice with SFO-targeted injections of $AdEP_1R$ (n=3) or AdGFP (n=3) using an antibody targeting the HA tag. D) Real-time RT-PCR data showing HA-EP_1R mRNA levels in the SFO, PVN, RVLM and CTX of WT mice at day 9 (n=3) and 28 (n=3) after SFO-targeted $AdEP_1R$ injection. *$p<0.05$ vs. day 9 and 28 PVN, RVLM, CTX. Two brains were pooled per biological sample for each region.

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Angiotensin-II-dependent Hypertension Requires Cyclooxygenase 1-derived Prostaglandin E_2 and EP_1 Receptor Signaling in the Subfornical Organ of the Brain

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Abstract

Cyclooxygenase (COX)-derived prostanoids have long been implicated in blood pressure (BP) regulation. Recently prostaglandin E_2 (PGE_2) and its receptor EP_1R have emerged as key players in angiotensin II (Ang-II)-dependent hypertension (HTN) and related end-organ damage. However, the enzymatic source of $PGE_{2,}$ ie COX-1 or COX-2, and its site(s) of action are not known. The subfornical organ (SFO) is a key forebrain region that mediates systemic Ang-II-dependent HTN via reactive oxygen species (ROS). We tested the hypothesis that cross-talk between PGE_2/EP_1R and ROS signaling in the SFO is required for Ang-II HTN. Radiotelemetric assessment of BP revealed that HTN induced by infusion of systemic "slow-pressor" doses of Ang-II was abolished in mice with null mutations in EP_1R or COX-1 but not COX-2. Slow-pressor Ang-II-evoked HTN and ROS formation in the SFO were prevented when the EP_1R antagonist SC-51089 was infused directly into brains of wild-type mice, and Ang-II-induced ROS production was blunted in cells dissociated from SFO of $EP_1R^{-/-}$ and COX-$1^{-/-}$ but not COX-$2^{-/-}$ mice. In addition, slow-pressor Ang-II infusion caused a ~3-fold increase in PGE_2 levels in the SFO but not in other brain regions. Finally, genetic reconstitution of EP_1R selectively in the SFO of EP_1R-null mice was sufficient to rescue slow-pressor AngII-elicited HTN and ROS formation in the SFO of this model. Thus, COX-1-derived PGE_2 signaling through EP_1R in the SFO is required for the ROS-mediated HTN induced by systemic infusion of Ang-II, and suggests that EP_1R in the SFO may provide a novel target for antihypertensive therapy.

Key Words

Prostanoids, PGE_2, COX, reactive oxygen species, blood pressure, central nervous system

Introduction

Hypertension is a global health problem, afflicting nearly a third of the population and predisposing to serious diseases affecting the brain, heart and kidneys[1]. Cyclooxygenase (COX)-derived prostanoids, endogenous fatty acid metabolites known to play critical roles in a wide variety of biological processes, have long been implicated in blood pressure (BP) regulation[2]. Clinical use of prostanoid synthesis-inhibiting nonsteroidal anti-inflammatory drugs (NSAIDs) are associated with hypertension[3], suggesting that endogenous prostanoids generally reduce blood BP. However, recently a more complex picture has emerged in which specific components of the prostanoid system have divergent effects and can be pro-hypertensive. For example, the major prostanoid prostaglandin E_2 (PGE_2) and its receptor subtype 1 (EP_1R), one of four G-protein-coupled receptors ($EP_{1-4}R$) mediating the effects of PGE_2 [4], are now considered key players in hypertension and related end-organ damage[5-7]. In particular, recent studies utilizing mice with global targeted disruption of EP_1R revealed a critical role for this receptor subtype in systemic Ang-II-dependent hypertension[8]. However, the underlying mechanisms involved in this, including the enzymatic source of PGE_2, i.e. COX-1 or COX-2 and its site(s) of action at EP_1R, remain poorly defined.

There is abundant evidence that neurohumoral dysfunction is a key contributor to Ang-II-dependent hypertension[9]. In particular, regions devoid of a blood-brain-barrier can be activated by elevated levels of blood-borne Ang-II, triggering alterations in downstream signaling pathways and hypertension[10]. One of these regions, the subfornical organ

(SFO), is strongly implicated in sympathoexcitation and hypertension caused by elevated levels of circulating Ang-II, particularly the model involving chronic infusion of subpressor doses of Ang-II, ie “slow-pressor”, which is thought to recapitulate key features of human essential hypertension[11-13]. Reactive oxygen species (ROS) signaling in the SFO is clearly involved in this model[11, 14, 15]; however, the factors regulating ROS production evoked by Ang-II in this brain region and contributing to neural dysregulation and hypertension remain poorly understood.

Here we sought to determine if COX-derived PGE_2 and EP_1R signaling in the SFO provide an essential link between Ang-II, ROS and the central neural changes that give rise to slow-pressor Ang-II hypertension. Utilizing genetic and pharmacologic tools to selectively target distinct components of the PG system in mice, we provide evidence that COX-1-derived PGE_2 signaling through EP_1R in the SFO is required for the ROS-mediated hypertension induced by systemic infusion of Ang-II.

Methods

An expanded Materials and Methods section is available in the Online Supplement at http://hyper.ahajournal.org/.

Animals. Adult EP_1R-null, COX-1-null and COX-2-null mice (8-10 weeks old) were obtained from in-house colonies. Mice were congenic with the C57Bl/6 strain and age-matched C57Bl/6 mice were used as wild-type (WT) controls. All procedures were approved by the Animal Care and Use Committee at Cornell University. Care of the mice met or exceeded standards set forth by the NIH *Guide for the Care and Use of Laboratory Animals*, USDA regulations, and the AVMA Panel on Euthanasia.

Pharmacological agents. Inhibitors of EP_1R (SC-51089), COX-1 (SC-560), COX-2 (NS398) and Ang-II type 1 receptors (AT_1R, losartan) were utilized.

Blood pressure studies. Mice were anesthetized and instrumented with radiotelemetry devices as described[11]. After 7 days recovery, baseline BP measurements were taken over 3-4 days, after which mice were implanted subcutaneously with osmotic minipumps loaded with the slow-pressor dose of Ang-II (600ng/kg/min, 14 days) as described[11]. BP was recorded daily for 3 weeks to monitor the effects of Ang-II during the entire infusion period as well as several days post-infusion. In studies using intracerebroventricular (i.c.v) infusion of SC-51089, mice were instrumented with i.c.v. cannulae[11] during the same surgical session as radiotelemeter implantation. For these studies, at the time of Ang-II pump installation, a second 14-day osmotic minipump containing SC-51089 (144μg/day) was implanted and connected to the i.c.v. cannulae and BP monitoring was carried out as described above.

Meaurement of dipsogenic responses. WT mice were instrumented with i.c.v. cannulae and allowed 7 days recovery. Mice were administered either vehicle, SC-51089 (10μg/kg), SC-560 (10mg/kg) or NS-398 (10mg/kg) by i.p. injection (200 nl) 30 minutes prior to i.c.v. bolus administration of Ang-II (200ng, 200nl). Water drinking responses were measured over 1 hour as described previously[14, 16].

Quantitative real-time PCR detection of prostanoid-related transcripts. WT mice were decapitated and brains flash frozen. SFO tissue was collected by micropunch as described[14]. Total RNA was harvested and cDNA was generated using random hexamers. Templates (25ng) were subjected in triplicate to real-time RT-PCR using Power SYBR Green and specific primers for COX-1, COX-2, $EP_{1\text{-}4}R$ and PGE synthases as described[14]. β-actin was used for relative quantification by ΔΔCt method[14].

PGE_2 assay. WT mice were implanted with osmotic minipumps loaded with the 2 week slow-pressor dose of Ang-II (see above) or saline. Mice were euthanized at 3, 7 or 14 days after start of infusions and brains flash frozen. Micropunches of SFO, paraventricular nuclei (PVN), somatosensory cortex (CTX) and cerebellum (CBM) were collected from 2 mice per biological sample and weighed. Samples were processed and PGE_2 concentration was determined using an enzyme immunoassay kit as described[17].

ROS detection. ROS production was assessed in dissociated SFO cells and in SFO-containing tissue sections using dihydroethidium (DHE) as an indicator. For *in vitro* ROS detection, WT, COX-1-null, COX-2-null or EP_1R-null mice were sacrificed, brains removed and coronal slices containing the SFO obtained. SFO cells were dissociated, incubated with DHE and time-resolved fluorescence was measured every 30s before (vehicle) and after addition of Ang-II (100nM) as described[18]. Additional *in vitro* studies were performed the same way except pre-treatment with SC-51089 (10μM) or losartan (3 μM) were used, and PGE_2 (100nM) was also applied. For *in situ* ROS detection, brains were removed on day 16 of Ang-II or vehicle infusions, frozen sections were incubated with DHE and fluorescence was visualized and quantified as described[11]. Data are expressed as DHE fluorescence intensity relative to control samples.

Adenoviral-mediated reconstitution of EP_1R in EP_1R-null mice. A recombinant adenoviral vector encoding murine EP_1R tagged with HA on the N-terminus (AdEP_1R; Fig S4A) was engineered and then generated by the Iowa Gene Transfer Vector Core (IGTVC). AdEP_1R potency and stability was validated both *in vitro* and *in vivo* (Fig S4). An Ad vector encoding GFP (AdGFP) obtained from IGTVC was used as the control vector. EP_1R-null mice underwent SFO-targeted injection of AdEP_1R ($5x10^{10}$ pfu/mL, 500nl) or titer-matched AdGFP as described[11, 14]. During the same surgical session, radiotelemeters were implanted as described above. Nine days later, osmotic minipumps loaded with the 2 week slow-pressor Ang-II dose were implanted. BP recording and ROS measurements were performed as described above.

Data analysis. Data are expressed as mean ± SEM. Comparisons between two groups were evaluated using the Student's *t* test. Multiple comparisons were evaluated by ANOVA followed by Dunnett's or Tukey's test. Differences were considered statistically significant at $p<0.05$.

Results

Genetic disruption of EP_1R prevents hypertension during slow-pressor Ang-II infusion. EP_1R are implicated in the BP elevation induced by high doses of Ang-II[8]. Here, using EP_1R-null and WT mice, we determined whether EP_1R are involved in hypertension caused by chronic slow-pressor doses of Ang-II, a model thought to mimic human hypertension and in which there is a strong CNS component[9]. Baseline mean arterial pressure (MAP) was not different between the groups ($EP_1R^{-/-}$ 98±2, WT 97±3 mmHg; p>0.05). In accordance with previous studies[11, 19], Ang-II induced a gradual rise in MAP in WT mice that peaked at ~30mmHg above baseline following 2 weeks Ang-II infusion (Fig 1A). In contrast, this Ang-II-induced rise in BP was absent in EP_1R-null mice (Fig 1A).

Ang-II slow-pressor hypertension is ameliorated in COX-1-null but not COX-2-null mice. Data in Figure 1A suggest that PGE_2 signaling is needed for slow-pressor Ang-II hypertension. Since PGE_2 is a major reaction product of both COX isozymes[20], we next sought to determine the enzymatic source of PGE_2 involved in the slow-pressor effects of Ang-II using mice with null mutations in either COX-1 or COX-2. Baseline MAP did not differ between the groups ($COX\text{-}1^{-/-}$ 93±2, $COX\text{-}2^{-/-}$ 96±7, WT 98±3 mmHg; p>0.05). Similar to WT mice in Figure 1A, Ang-II induced the classic slow rise in MAP which peaked during the second week of infusion (Fig 1B). This response was abolished in COX-1-null mice, whereas it remained intact in COX-2-null mice (Fig 1B).

EP_1R and COX-1 in the CNS are implicated in Ang-II-induced cardiovascular and dipsogenic effects. Data in Figure 1 suggest that COX-1-derived PGE_2 and EP_1R are involved in the rise in BP during slow-pressor Ang-II infusion, but the use of global knockouts prevents us from pinpointing the site(s) of these effects. To test the hypothesis that slow-pressor Ang-II hypertension is caused by a PGE_2/EP_1R mechanism operating in the CNS, we utilized three separate approaches. First, given abundant evidence that slow-pressor Ang-II hypertension is mediated via CNS-driven increases in sympathetic activity[9], power spectral analysis was used to assess slow-pressor Ang-II-induced sympathetic responses in EP_1R-null vs WT mice. Increased low frequency (LF)/high frequency (HF) oscillations of arterial pressure reflect increased sympathetic activity[21]. Consistent with previous findings, Ang-II infusions caused a doubling of the LF/HF ratio in WT mice by the end of the 2 week infusion period (pumps empty completely on day 16) (Fig 2A) when the hypertensive response is maximal (see Fig 1 and 2B). In contrast, the LF/HF ratio was unchanged in EP_1R-/- mice over the course of Ang-II infusions (Fig 2A). Second, using chronic infusion of the EP_1R antagonist SC51089 into brains (i.c.v.) of WT mice, data shown in Figure 2B demonstrate that the slow-pressor Ang-II-induced rise in MAP observed in i.c.v. vehicle-treated controls was prevented by blockade of EP_1R in the CNS. It should be noted that i.c.v. infusions at this volume do not escape into the peripheral circulation[11]. Third, given the well established role of the CNS in mediating Ang-II effects on dipsogenesis[16, 22], we employed the classic assay of bolus injection of Ang-II in the brain (i.c.v.) coupled with measurement of drinking responses in WT mice pre-treated (i.p.) with either SC51089 or the selective inhibitors of COX-1 (SC-560) or COX-2 (NS-398). As seen in Figure

2C, i.c.v. Ang-II elicited the well-established dipsogenic response in vehicle-treated mice. This response was intact in mice treated with the COX-2 inhibitor, whereas it was markedly attenuated in mice treated with either the COX-1 inhibitor or the EP_1R antagonist.

PGE_2 synthetic enzymes and receptors are expressed in the SFO under basal conditions, and PGE_2 production is augmented in this brain region during slow-pressor Ang-II infusion. Data in Figures 1 and 2 suggest that COX-1-derived PGE_2 acting at EP_1R in the CNS is important in slow-pressor Ang-II hypertension. Since the SFO is a key region of the CNS mediating this form of hypertension[11, 12], next we examined the capacity of the SFO for PGE_2 formation and signaling. Real-time qPCR was performed to determine basal mRNA levels of COX isozymes, PGE synthases and EP receptor subtypes in SFO tissue harvested from adult WT mice. First, COX-1 was expressed at ~7-fold higher levels in SFO than COX-2 (Fig 3A). Second, of the PGE synthases that convert PGH_2 to PGE_2, ie PGES-1, PGES-2 or cytosolic PGES (c-PGES), the latter was expressed at much higher levels in the SFO than either of the other two isoforms (Fig 3B). Third, of the four receptor subtypes mediating PGE_2 effects, mRNA levels of EP_1R were more than 10-fold higher than EP_2R, EP_3R or EP_4R in SFO tissue (Fig 3C). Importantly, all four of the EPR subtypes were expressed at very low levels in organum vaculosum of the lamina terminalis (Fig S1). Finally, we sought to directly evaluate the effects of slow-pressor Ang-II infusions on PGE_2 formation in the SFO. As seen in Figure 3D, Ang-II caused a significant increase in PGE_2 levels in the SFO as early as day 3 of the infusion compared to saline controls. This was sustained through 7 days of the infusion, but by day 14, PGE_2 levels were not different from controls. PGE_2 levels were also measured in PVN, CTX and CBM of WT mice infused with slow-pressor doses of Ang-II or vehicle, but no significant Ang-II-induced changes in PGE_2 levels were observed in any of these regions compared to controls (Fig S2).

PGE_2/EP_1R signaling is coupled to Ang-II-induced ROS accumulation in the SFO. Slow-pressor Ang-II infusion causes increased ROS formation in the SFO[11, 15]. Thus, several experiments were performed to test the hypothesis that PGE_2/EP_1R are required for ROS formation in the SFO in response to Ang-II. First, ROS production was assessed *in vitro* in single cells dissociated from the SFO of adult WT and null mice. Consistent with earlier reports[14, 16], Ang-II caused a significant increase in DHE signal in WT SFO cells compared to control (Fig 4A). This response was absent in SFO cells dissociated from either $EP_1R^{-/-}$ or COX-$1^{-/-}$ mice, whereas it was intact in COX-$2^{-/-}$ SFO cells (Fig 4A). Pharmacological studies using the EP_1R antagonist SC-51089 in SFO cells dissociated from WT mice confirmed the results observed in the $EP_1R^{-/-}$ mice (Fig S3), and further verified earlier data[16] that Ang-II-induced increases in ROS formation are sensitive to losartan (Fig S3). Interestingly, PGE_2 elicited increases in DHE intensity to a similar extent as Ang-II, and while this response was inhibited by SC-51089, it was unaffected by losartan (Fig S3). Next, *in situ* DHE microfluorography was used to assess Ang-II-induced ROS production in the SFO of WT mice receiving systemic slow-pressor Ang-II infusions concomitant with i.c.v. infusions of either SC-51089 or vehicle. Consistent with earlier reports[11], DHE fluorescence intensity was ~2.5-fold higher in the

SFO of mice receiving Ang-II (day 16) compared to untreated mice (Fig 4B). This response was abolished in mice receiving i.c.v. infusions of SC-51089 (Fig 4B).

Virally-mediated reconstitution of EP_1R selectively in the SFO rescues slow-pressor Ang-II hypertension and ROS formation in EP_1R-null mice. Our data thus far show that global knockout of EP_1R prevents slow-pressor AngII-induced hypertension, and the CNS, particularly the SFO, may be involved. To directly test the hypothesis that SFO-selective expression of EP_1R is sufficient to induce gradual hypertension elicited by slow-pressor doses of Ang-II, we utilized a genetic rescue approach to reconstitute EP_1R selectively in the SFO with $AdEP_1R$ in EP_1R-null mice. First, the potency and stability of $AdEP_1R$ were evaluated. $AdEP_1R$ increased exogenous EP_1R mRNA levels as measured by qPCR in Neuro2A cells in a concentration-dependent manner (Fig S4B). Furthermore, *in vivo* targeting of the virus to the SFO induced highly robust HA-EP_1R levels in the SFO as revealed by immunohistochemistry (Fig S4C). This was confirmed at the mRNA level, with qPCR showing highly abundant expression of the HA-EP_1R transgene in SFO and barely detectable levels in PVN, rostral ventrolateral medulla and CTX (Fig S4D). Transgene expression was also stable over time as indicated by similar high levels of HA-EP_1R mRNA in SFO at 9 and 28 days post-transduction (Fig S4D). Having verified the potency and stability of the virus, $EP_1R^{-/-}$ mice underwent SFO-targeted injections of $AdEP_1R$ or control vector AdGFP. Prior to initiating slow-pressor Ang-II infusions 9 days after viral transduction, baseline MAP was not different in the two groups (AdGFP 97±5 mmHg, $AdEP_1R$ 105±2; $p>0.05$). In control AdGFP-treated $EP_1R^{-/-}$ mice, there was no change in MAP at any time throughout the Ang-II infusion (Fig 5A), verifying data in Figure 1 showing that slow-pressor Ang-II hypertension is abolished in $EP_1R^{-/-}$ mice. In contrast, the classic gradual rise in MAP was restored in $AdEP_1R$-treated $EP_1R^{-/-}$ mice (Fig 5A). This was accompanied by LF/HF ratios that were similar to those observed in WT mice at the end of the 2 week infusion period (1.72±0.6, $p>0.05$). To determine whether this was accompanied by restoration of Ang-II-induced ROS accumulation in the SFO, a separate cohort of $EP_1R^{-/-}$ mice with SFO-targeted $AdEP_1R$ or AdGFP were subjected to DHE studies as described above. In AdGFP-treated $EP_1R^{-/-}$ mice, DHE fluorescence intensity in the SFO did not change from baseline, confirming in this null strain findings in Figure 4B obtained using the EP_1R antagonist. In contrast, ROS levels in the SFO of $AdEP_1R$-treated $EP_1R^{-/-}$ mice were re-established to that of Ang-II-treated WT mice (see Fig 4B), with a ~3-fold increase in DHE intensity compared to controls (Fig 5B).

Discussion

Brain Ang-II, prostanoids and ROS have each been proposed as important mediators of BP regulation and hypertension[2, 9, 23]. Here we provide evidence that these factors are mechanistically linked in the pathogenesis of slow-pressor Ang-II-elicited hypertension. We show that elevations in BP during slow-pressor Ang-II infusions are abolished in mice with global null mutations of EP_1R or COX-1 but not COX-2. Pharmacologic inhibition of EP_1R selectively in the CNS prevents slow-pressor Ang-II hypertension, and central Ang-II-driven sympathetic and dipsogenic responses are also mediated by brain EP_1R. Markedly elevated levels of COX-1, cPGES and EP_1R are observed in the SFO relative to other PGE_2 synthetic enzymes and receptors, making this forebrain structure an ideal platform for COX1-derived PGE_2 signaling through EP_1R. Indeed, slow-pressor Ang-II infusions induce early robust PGE_2 production in the SFO. Both *in vitro* and *in vivo* inhibition of EP_1R prevents Ang-II-induced ROS accumulation in the SFO, a response that is known to have a causative role in slow-pressor Ang-II hypertension[11, 15]. Finally, virally-mediated reconstitution of EP_1R selectively in the SFO of EP_1R-null mice restores hypertension and SFO ROS formation in response to slow-pressor Ang-II infusions. This provides the first evidence that COX-1-derived PGE_2/EP_1R signaling in the SFO is required for the ROS-mediated hypertension elicited in the slow-pressor Ang-II model.

The significance of these findings lies in the complex picture that has recently emerged concerning specific components of the prostanoid system and their divergent effects on BP. COX-inhibiting NSAIDs are among the most widely prescribed classes of therapeutic agents, many of the effects of which are mediated by their actions in the CNS[2]. Their general association with hypertension in humans has suggested that endogenous prostanoids lower BP[2, 3]. However, the ubiquitous tissue distribution and biological complexity of the prostanoid system[2], coupled with recent evidence that certain components are pro-hypertensive[6-8, 24] underscores the importance of understanding how prostanoids influence BP regulation, particularly as newer agents with a higher selectivity of action within the prostanoid system are being developed. Our data bolster the emerging concept that endogenous PGE_2-mediated EP_1R activation contributes to Ang-II-dependent hypertension and related end-organ damage[5, 8]. Although the study by Guan et al. established that the pressor effects of high-dose systemic Ang-II are blunted in $EP_1R^{-/-}$ mice[8], the enzymatic source of PGE_2 and the tissue site(s) of action remained poorly defined. Here, utilizing tissue-specific reconstitution of EP_1R in EP_1R-null mice, our data now point to a key role for PGE_2/EP_1R signaling in the CNS, particularly the SFO, in mediating systemic Ang-II-dependent hypertension. Whereas these studies do not rule out the possibility that other EPR subtypes and/or other tissues sites are involved in this model of Ang-II hypertension, the complete restoration of the slow-pressor response, coupled with the markedly higher levels of EP_1R expression in SFO compared to the other subtypes strongly supports this concept. This is important information in considering EP_1R as a novel target for treatment of hypertension.

Another major finding of the present study is that we identified COX-1 in the SFO as the sole source of the PGE_2 required for Ang-II slow-pressor hypertension. Although it is well established that Ang-II stimulates PGE_2 synthesis[25], the enzymatic source of PGE_2 in Ang-II-evoked hypertension has remained poorly defined. Our data showing an absence of slow-pressor Ang-II-evoked hypertension in COX-1-null mice but an intact response in COX-$2^{-/-}$ mice implicates COX-1 as the source of PGE_2 and mediator of hypertension in this model. The relative roles of COX-1 versus COX-2-derived products in Ang-II-dependent responses have been controversial, but our findings are consistent with previous studies showing that pharmacological blockade or genetic deletion of COX-1, but not COX-2, reduced the acute pressor effects of Ang-II in mice[24]. In addition, Capone et al. recently showed that selective pharmacological inhibition of COX-1, but not COX-2, prevented the cerebrovascular effects of Ang-II, and that COX-1 is the major source of PGE_2 in the somatosensory cortex[5]. Our evidence that COX-1 is expressed at much higher levels than COX-2 in the SFO, and that the COX-1-coupled PGE synthase cPGES[26] is the predominant isoform in the SFO suggests COX-1 as the source of increased PGE_2 levels in this brain region during slow-pressor Ang-II infusion. The importance of COX-1 is further suggested by our *in vitro* data demonstrating that Ang-II-evoked ROS formation in dissociated SFO cells is prevented by inhibition of COX-1 but not COX-2. Thus, our data suggest that at least in the SFO, COX-1 predominates under basal conditions. Further studies will be required to define the cell-type localization of these enzymes in the SFO before and after Ang-II infusion to better understand the cellular mechanisms involved.

It is notable that systemic Ang-II induced increases in PGE_2 production in the SFO but not in other brain regions including the PVN, cortex and cerebellum, despite these regions being enriched in PGE_2 synthesis enzymes and receptors[27, 28]. This suggests that the links between Ang-II and PGE_2 are specific rather than due to generalized CNS activation in this model, and is also consistent with the fact that the SFO lacks a blood-brain-barrier and can be accessed by circulating Ang-II via AT_1 binding[9]. Indeed Ang-II has been shown to elicit PGE_2 synthesis in cultured CNS cells via stimulation of AT_1 receptors[29, 30], and interestingly, autoradiographic studies have demonstrated intense PGE_2 binding in the anteroventral region of the third ventricle (AV3V), a region that encompasses the SFO[31]. It is also important to note that the Ang-II-induced increase in PGE_2 production in the SFO occurred early in the infusion period (3 and 7 days), prior to a significant rise in sympathetic outflow and BP. Furthermore, PGE_2 levels had returned to baseline by 14 days, a time when hypertension is at its peak. This suggests that PGE_2 in the SFO *per se* is not directly causing neural changes leading to hypertension in this model, but rather serves as a critical signaling intermediate that then triggers downstream pathways involved in central Ang-II-mediated hypertension. Indeed, essential hypertension has a slow and insidious onset, and its underlying pathophysiological mechanisms precede the elevation in BP[32]. Increasingly, adaptive changes in CNS neurons are considered highly relevant to hypertension[9, 33]. Determining whether early induction of PGE_2 in the SFO by slow-pressor Ang-II is involved in such changes in CNS circuitries involved in the delayed sympathetic activation and hypertension in this model will require further investigation.

We and others have demonstrated that Ang-II induces ROS formation in the SFO via the activation of NADPH oxidase, and this is a key signaling event in the hypertensive and dipsogenic actions of Ang-II in the brain[11, 14-16, 34]. A key finding of the present study demonstrates that COX-1-derived PGE_2 and EP_1R are required for this Ang-II-evoked ROS formation in the SFO *in vitro* and *in vivo*. This is consistent with recent studies demonstrating a similar mechanism in Ang-II-mediated ROS formation in cerebral blood vessels, which likely involves NADPH oxidase[5]. Given that Ang-II-induced NADPH oxidase activation in neurons is Ca^{2+}-dependent[18], along with evidence that activation of EP_1R results in IP3-mediated release of intracellular Ca^{2+}[35] and reduced Ca^{2+} efflux through the Na/Ca^{2+} exchanger[17], it is reasonable to speculate that NADPH oxidase mediates PGE_2/EP_1R-mediated ROS formation in the SFO through Ca^{2+} signaling. Further studies will be required to elucidate the detailed cellular mechanisms linking Ang-II, prostanoids, ROS and neuronal signaling in the SFO. For example, it will be important to define the relationship between early pre-hypertensive induction of PGE_2 in the SFO by slow-pressor Ang-II and ROS accumulation in this region during the later hypertensive phase in the model.

Perspectives. COX-derived prostanoid signaling has long been implicated in the pathogenesis of Ang-II-dependent hypertension, and this study provides evidence for the first time that a mechanism involving increased COX-1-dependent PGE_2 formation and EP_1R signaling in the SFO region of the forebrain is a key underlying mechanism. Determining how these various players are spatially and functionally linked in the SFO to provide the substrate for adaptive neural changes that lead to gradually developing Ang-II hypertension is a critical next step. However, in the meantime, this is important information as new therapeutic agents targeting the prostanoid system are being developed.

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Disclosures

None.

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Figure Legends

Figure 1. Slow-pressor Ang-II hypertension is prevented in mice with null mutations in EP_1R or COX-1 but not COX-2. A) Summary of MAP before, during and after 2 wk slow-pressor Ang-II infusions in WT (n=5) and $EP_1R^{-/-}$ (n=7) mice. B) MAP throughout slow-pressor Ang-II in WT (n=7), COX-$2^{-/-}$ (n=4) and COX-1-/- (n=5) mice. *p<0.05 vs. baseline (WT in panels A and B; COX-2 in panel B); †p<0.05 vs. WT or COX-2-/-. Arrow indicates start of Ang-II infusions.

Figure 2. EP_1R and COX-1 in the CNS are involved in Ang-II-induced cardiovascular and dipsogenic effects. A) Power spectral analysis of arterial pressure variability at several time-points before, during and after slow-pressor Ang-II infusion in WT (n=5) and $EP_1R^{-/-}$ mice (n=7). Data are presented as the LF/HF ratio relative to day 0. *p<0.05 vs. day 0; †p<0.05 vs. WT at day 16. B) Summary of MAP in WT mice with chronic i.c.v. infusion of the EP_1R antagonist SC-51089 (n=7) or saline (n=6) at the same time as systemic slow-pressor Ang-II infusions. *p<0.05 vs. baseline; †p<0.05 vs. saline. Arrow indicates start of i.c.v. and Ang-II infusions. C) Drinking responses elicited by bolus i.c.v. injection of Ang-II in WT mice treated 30 min earlier with i.p. injections of vehicle (Veh, n=11), EP_1R antagonist SC51089 (n=11), COX-1 inhibitor SC560 (n=4) or COX-2 inhibitor NS398 (n=4). Data are expressed as the total time drinking (seconds) for 30 min after i.c.v. injection of Ang-II. †p<0.05 vs. vehicle; n.s., not significant.

Figure 3. PGE_2 synthetic enzyme and receptors are expressed at high levels in the SFO under basal conditions, and PGE_2 levels are increased in the SFO early after slow-pressor Ang-II infusion. A-C) Basal mRNA levels of EPR_{1-4}, COX isozymes and

PGE synthases in adult WT SFO tissue (n=3) as analyzed by quantitative real-time PCR. †p<0.05 vs. $EP_{2\text{-}4}R$ (A), vs. COX-2 (B), vs. PGES1 and PGES2. D) PGE_2 levels measured by ELISA in micropunches of SFO from WT mice at day 3, 7 and 14 of slow-pressor Ang-II (n=3) or saline infusion (n=3). *p<0.05 vs. saline. In all assays, two brains were pooled for per biological sample.

Figure 4. PGE_2/EP_1R signaling is required for Ang-II-induced ROS formation in the SFO. A) Summary of the effects of Ang-II vs. vehicle (Veh) on ROS production as measured by DHE fluorescence intensity in cells dissociated from SFO of adult WT (n=24), $EP_1R^{-/-}$ (n=9), $COX1^{-/-}$ (n=9) and $COX2^{-/-}$ (n=9) mice. *p<0.05 vs. Veh; n.s., not significant. B) Left: Representative confocal images showing DHE fluorescence in SFO tissue at day 16 of slow-pressor Ang-II infusion in mice treated concomitantly with either i.c.v. vehicle or SC-51089. Mice left untreated served as controls. Right: Summary of DHE fluorescence intensity in SFO tissue of mice with no treatment (n=7) or at day 16 of slow-pressor Ang-II infusions treated concomitantly with either i.c.v. vehicle (Veh, n=6) or SC-51089 (n=7). †p<0.05 vs.untreated; #p<0.05 vs. Ang-II + i.c.v. Veh. Scale bar: 50 µm.

Figure 5. Adenoviral-mediated reconstitution of EP_1R selectively in the SFO rescues slow-pressor Ang-II hypertension and ROS formation in $EP_1R^{-/-}$ mice. A) Summary of MAP before, during and after slow-pressor Ang-II infusion in $EP_1R^{-/-}$ mice with SFO-targeted AdGFP (n=6) or $AdEP_1R$ (n=6). *p<0.05 vs. baseline; †p<0.05 vs. $AdEP_1R$. B) Left: Representative confocal images showing DHE fluorescence in the SFO at the end of the slow-pressor Ang-II infusion period in $EP_1R^{-/-}$ mice with SFO-targeted AdGFP or $AdEP_1R$. Right: Summary of DHE fluorescence intensity in the SFO

of $EP_1R^{-/-}$ mice with SFO-targeted AdGRP (n=3) or $AdEP_1R$ (n=4) at the end of the slow-pressor Ang-II infusion period. †p<0.05 vs. AdGFP. Scale bar: 50μm.

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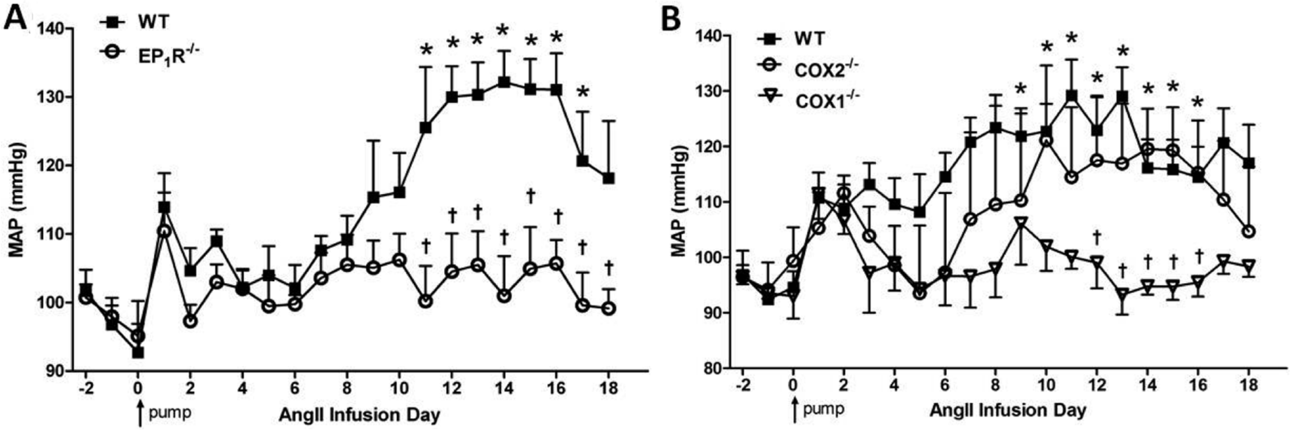

A
WT
EP1R-/-
MAP (mmHg)
140
130
120
110
100
90
-2
0
2
4
6
8
10
12
14
16
18
pump
AngII Infusion Day
B
WT
COX2-/-
COX1-/-
MAP (mmHg)
140
130
120
110
100
90
80
-2
0
2
4
6
8
10
12
14
16
18
pump
AngII Infusion Day

Type of file: figure
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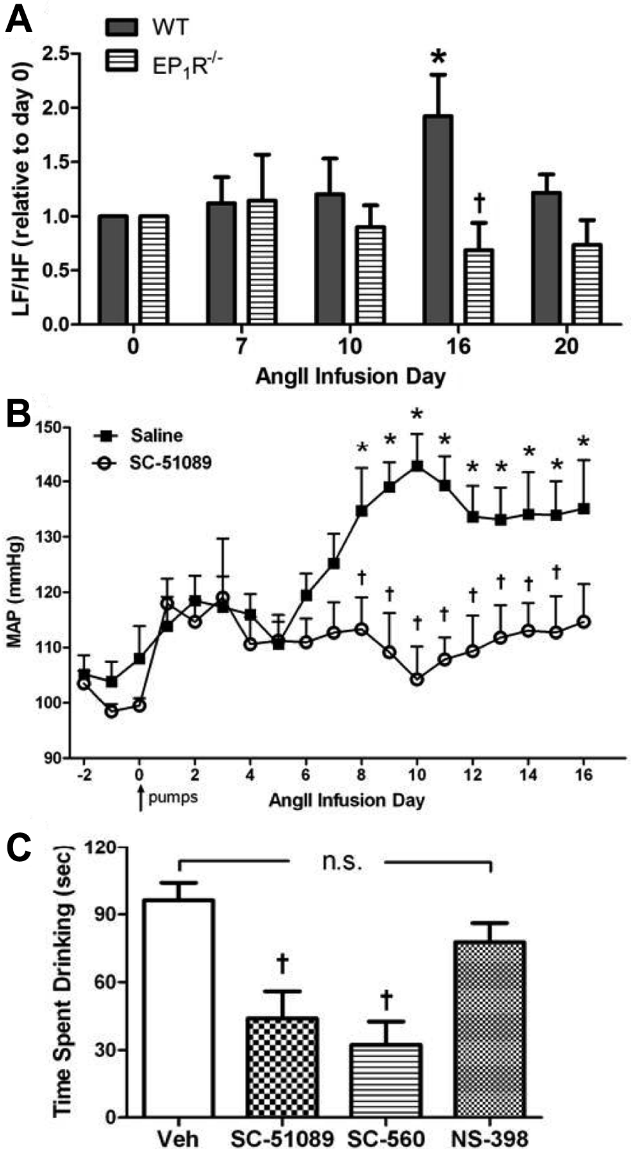
A
WT
EP1R-/-
LF/HF (relative to day 0)
2.5
2.0
1.5
1.0
0.5
0.0
0
7
10
16
20
AngII Infusion Day
B
Saline
SC-51089
MAP (mmHg)
150
140
130
120
110
100
90
-2
0
2
4
6
8
10
12
14
16
pumps
AngII Infusion Day
C
Time Spent Drinking (sec)
120
90
60
30
0
n.s.
Veh
SC-51089
SC-560
NS-398

Type of file: figure
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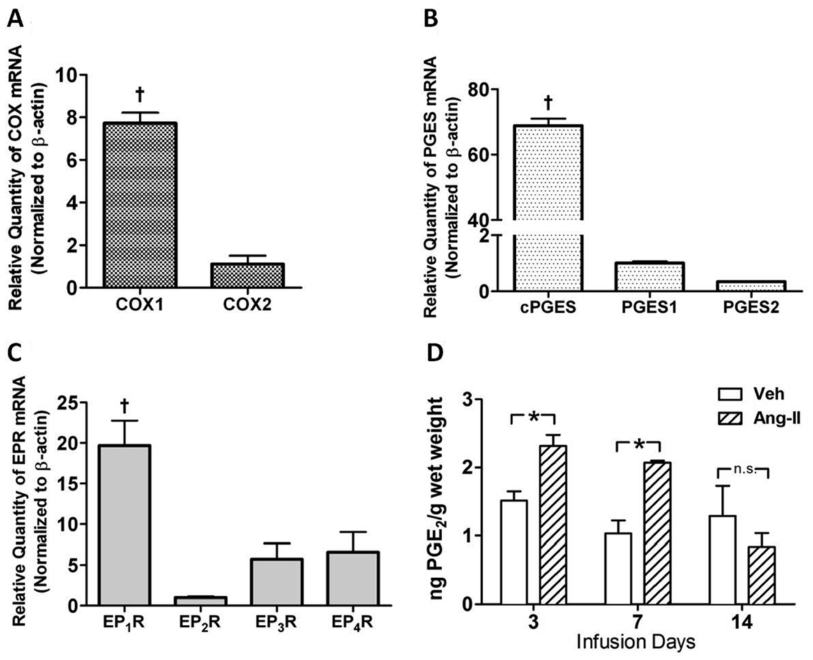
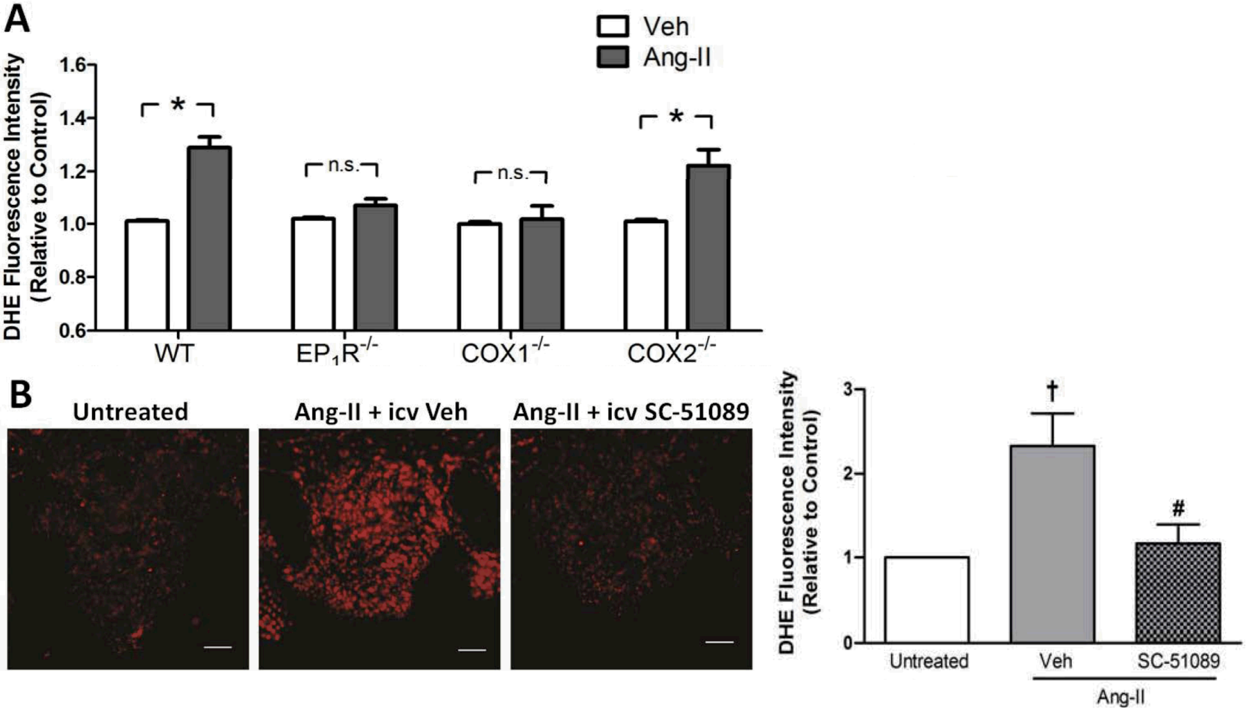


A
Relative Quantity of COX mRNA (Normalized to β-actin)
0
2
4
6
8
10
†
COX1
COX2
B
Relative Quantity of PGES mRNA (Normalized to β-actin)
0
2
40
60
80
†
cPGES
PGES1
PGES2
C
Relative Quantity of EPR mRNA (Normalized to β-actin)
0
5
10
15
20
25
†
EP1R
EP2R
EP3R
EP4R
D
ng PGE2/g wet weight
0
1
2
3
Veh
Ang-II
*
*
n.s.
3
7
14
Infusion Days

Type of file: figure
Label: 4
Filename: HYP200904Fig4.tif


A
Veh
Ang-II
DHE Fluorescence Intensity (Relative to Control)
1.6
1.4
1.2
1.0
0.8
0.6
*
n.s.
n.s.
*
WT
EP1R-/-
COX1-/-
COX2-/-
B
Untreated
Ang-II + icv Veh
Ang-II + icv SC-51089
DHE Fluorescence Intensity (Relative to Control)
3
2
1
0
†
#
Untreated
Veh
SC-51089
Ang-II

Type of file: figure
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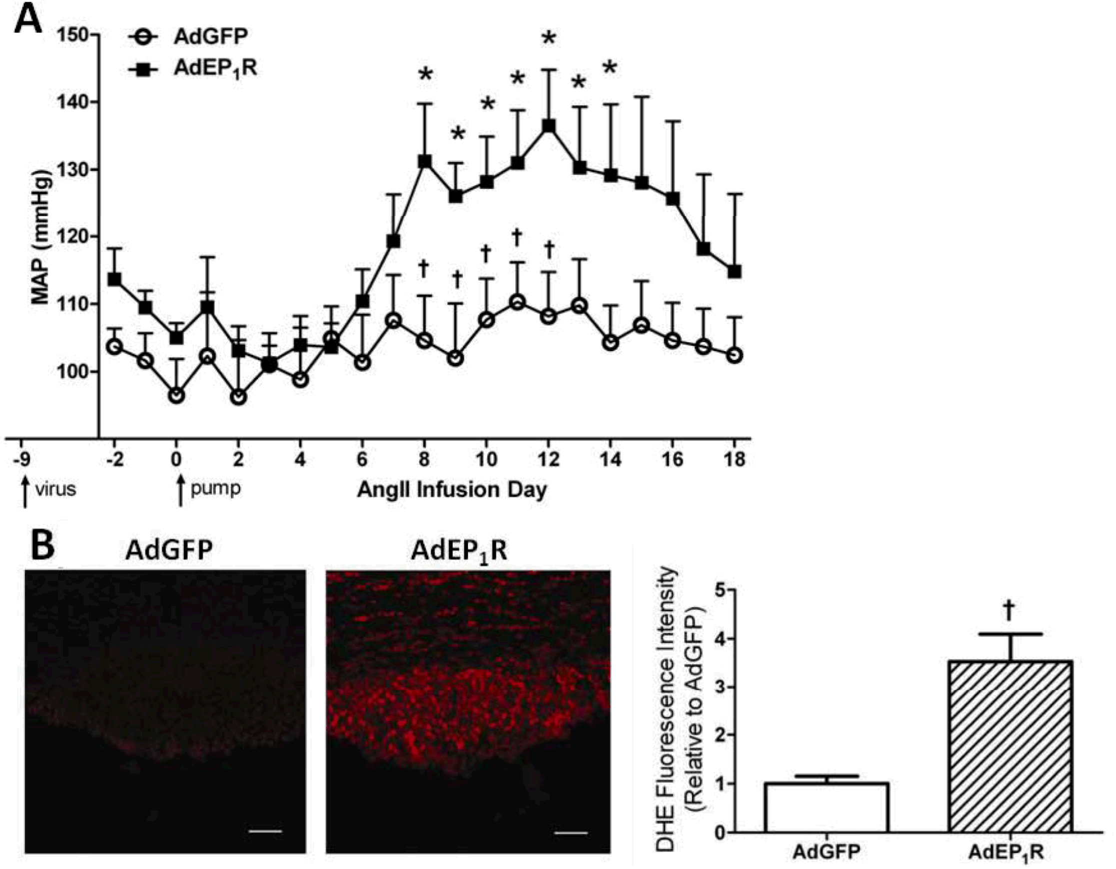
Filename: HYP200904Fig5.tif



A
AdGFP
AdEP1R
MAP (mmHg)
150
140
130
120
110
100
-9
-2
0
2
4
6
8
10
12
14
16
18
virus
pump
AngII Infusion Day
B
AdGFP
AdEP1R
DHE Fluorescence Intensity (Relative to AdGFP)
5
4
3
2
1
0
AdGFP
AdEP1R