Am J Cardiovasc Dis 2016;6(3):118-128
www.AJCD.us /ISSN:2160-200X/AJCD0036242

Original Article

Angiotensin type 2-receptor (AT_2R) activation induces hypotension in apolipoprotein E-deficient mice by activating peroxisome proliferator-activated receptor-γ

Ming Li[1,6*], Thor Tejada[1*], Jonathan P Lambert[3], Chad K Nicholson[3], Eiji Yahiro[1], Vats T Ambai[1], Syeda F Ali[1], Eddie W Bradley[4], Robert M Graham[2], Louis J Dell'Italia[4,5], John W Calvert[3], Nawazish Naqvi[1]

[1]Division of Cardiology, Department of Medicine, Emory University, Atlanta, Georgia, USA; [2]Victor Chang Cardiac Research Institute, Darlinghurst, NSW 2010, Australia; [3]Division of Cardiothoracic Surgery, Department of Surgery, Carlyle Fraser Heart Center, Emory University, Atlanta, Georgia, USA; [4]Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA; [5]VA Medical Center, Birmingham, Alabama, USA; [6]Cardiac Regeneration Research Institute, Wenzhou Medical University, Wenzhou 325035, China. [*]Equal contributors.

Received July 19, 2016; Accepted August 19, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: Angiotensin II (Ang II) modulates blood pressure and atherosclerosis development through its vascular type-1 (AT_1R) and type-2 (AT_2R) receptors, which have opposing effects. AT_2R activation produces hypotension, and is anti-atherogenic. Targeted overexpression of AT_2Rs in vascular smooth muscle cells (VSMCs) indicates that these effects are due to increased nitric oxide (NO) generation. However, the role of endogenous VSMC AT_2Rs in these events is unknown. Effect of 7-day low-dose Ang II-infusion (12 μg/kg/hr) on blood pressure was tested in 9-week-old apoE$^{(-/-)}$ mice fed a low or high cholesterol diet (LCD or HCD, respectively). Cardiac output was measured by echocardiography. Immunohistochemistry was performed to localize and quantify AT_2Rs and p-Ser^{1177}-endothelial nitric oxide synthase (eNOS) levels in the aortic arch. PD123319 and GW-9662 were used to selectively block the AT_2R and peroxisome proliferator-activated receptor-γ (PPAR-γ), respectively. Ang II infusion decreased blood pressure by 12 mmHg ($P < 0.001$) in LCD/apoE$^{(-/-)}$ mice without altering cardiac output; a response blocked by PD123319. Although, AT_2R stimulation neither activated eNOS (p-Ser^{1177}-eNOS) nor changed plasma NO metabolites, it caused an ~6-fold increase in VSMC PPAR-γ levels ($P < 0.001$) and the AT_2R-mediated hypotension was abolished by GW-9662. AT_2R-mediated hypotension was also inhibited by HCD, which selectively decreased VSMC AT_2R expression by ~6-fold ($P < 0.01$). These findings suggest a novel pathway for the Ang II/AT_2R-mediated hypotensive response that involves PPAR-γ, and is down regulated by a HCD.

Keywords: AT_2R, apoE$^{(-/-)}$, angiotensin II, blood pressure, eNOS, PPAR-γ

Introduction

Inhibition of the renin-angiotensin system is extensively used to lower blood pressure in hypertensive patients [1]. The main effector of this system is the peptide hormone angiotensin II (Ang II). Ang II exerts its effects on the vasculature through two distinct receptor subtypes: the Ang II type 1 (AT_1R) and type 2 (AT_2R) receptor [2]. Using subtype specific antagonists it has been established that the potent vasoconstrictor response produced by Ang II is mediated via AT_1R activation in vascular smooth muscle cells (VSMCs) [2].

AT_2Rs are abundantly expressed in fetal and newborn rat tissues, but in adults its expression is restricted to a few organs including the ovary, brain, and adrenal gland [3-5]. In blood vessels from adult rats, AT_2R is expressed in the thoracic aorta, mesenteric arteries, and coronary arteries [6]. The pressor response to low-dose, but not high-dose, Ang II is enhanced in $AT_2R^{(-/-)}$ mice [7] and Ang II produces a small vasodilator response in AT_1R antagonist-treated mice [8]. But, the expression of the AT_2R relative to the AT_1R in the vasculature of wild type mice is not sufficiently high for Ang II to produce a vasodilator response at any Ang II dose [7].

In the aortic arch of the apoE$^{(-/-)}$ mouse, AT_2R expression is prominently observed on macrophages and VSMCs in intimal lesions [9] and atherosclerotic lesion area and macrophage

infiltration is greater in $apoE^{(-/-)}/AT_2R^{(-/-)}$ mice versus $apoE^{(-/-)}$ controls maintained on a high cholesterol diet (HCD) for 16 weeks; a difference not seen after only 10 weeks of a HCD. The late effect is consistent with the site of expression of this receptor in advanced lesions. However, the lack of an early effect is unexpected because: 1) apoE-deficiency, *per se*, increases the expression of aortic arch AT_2R mRNA levels before the onset of lesion development [9]; and [2] Takata et al. [10] recently showed that, in $apoE^{(-/-)}$/α-smooth muscle actin (α-SMA): AT_2R transgenic mice, low-dose Ang II elicits an anti-atherogenic response after just 8-weeks of a HCD. The beneficial effect of AT_2R activation in transgenic mice was proposed to be due to activation of the endothelial kinin/NO axis in the aortic arch. Specifically, it was proposed that AT_2R activates kininogenase in VSMCs, which by increasing bradykinin production activates endothelial cell (EC) NO-dependent vasodilation and attenuates VCAM-1 expression and monocyte/macrophage accumulation; both of these events are important at the earliest stages of atherosclerosis development. While these findings show the early beneficial effects of forced AT_2R expression in VSMCs it does not explain why endogenous AT_2R expression in $apoE^{(-/-)}$ aortae does not slow disease progression. We hypothesized that either the AT_2R is not normally expressed in aortic arch VSMCs of young preatherosclerotic $apoE^{(-/-)}$ mice or that a HCD, which is normally used to accelerate lesion development, down regulates aortic arch VSMC AT_2Rs and consequently its modulation of NO generation. We tested this hypothesis in young preatherosclerotic $apoE^{(-/-)}$ mice given either a low cholesterol diet (LCD) or a HCD for 1 week.

Materials and methods

Ethics statement

Animals were handled according to University of Alabama (Animal Project Number: 07090-7642) or Emory University (DAR-2000436-010514-1) Institutional Animal Care and Use Committee guidelines.

Animals and treatment

Nine-week-old male C57BL/6 and $apoE^{(-/-)}$ mice (Jackson Laboratory) were used. C57BL/6 mice were maintained on a standard chow diet. $ApoE^{(-/-)}$ mice were given a LCD (low fat rodent diet with 0% cholesterol, D12102C, Research Diet Inc.) or a HCD (high fat rodent diet with 11.25% cholesterol, D12108C, Research Diet Inc.) *ad libitum* for 1 week. $ApoE^{(-/-)}$ mouse weights were 26.1 ± 0.5 g (n = 11) and 25.7 ± 0.54 g (n = 11) after 1 week of a LCD or a HCD, respectively. Vehicle, Ang II (12 μg/kg/hr), Ang II + PD123319 (10 mg/kg/day), GWS9662 (2 mg/kg/day) or Ang II + GW-9662 were delivered via an osmotic minipump (Alzet, model 2002) placed in the peritoneal cavity for 7 days. These drugs were purchased from Sigma-Aldrich.

Blood pressure measurements, echocardiography and tissue collection

After induction of anesthesia with isofluorane (~1-2%) a 1.0 F high fidelity pressure transducer (Millar Instruments, Houston, TX) was passed via the right carotid artery into the left ventricle (LV) of the heart. Electrodes were attached to allow ECG and heart rate recordings. LV pressure, ECG and heart rate were monitored until stable recordings were obtained. The pressure transducer was then slowly withdrawn into the aorta for measurement of central arterial pressure as described [11]. Echocardiography was performed to measure cardiac output using a Vevo 2100 ultrasound system (VisualSonics) under ~1-2% isoflurane as described [12]. Blood (0.5-1 ml) was collected by heart puncture, under isoflurane anesthesia (3%), for lipid profiling. Subsequently, aortic arches were dissected and rinsed with ice-cold saline and then snap-frozen in OCT (OCT compound, Tissue-Tek).

Blood pressure in conscious mice

At 10 weeks of age a telemetry transmitter (PA-C10, Data Sciences International) was implanted into a carotid artery and 24-hr average MAP recorded. After baseline recordings, Ang II (12 μg/kg/hr) was delivered via an osmotic minipump (Alzet, model 2002) placed subcutaneously and 24-hr average MAP recorded daily over the following 7 days.

Immunohistochemistry

Mouse ascending aorta cryosections (5 μm) were used for quantitative immunohistochem-

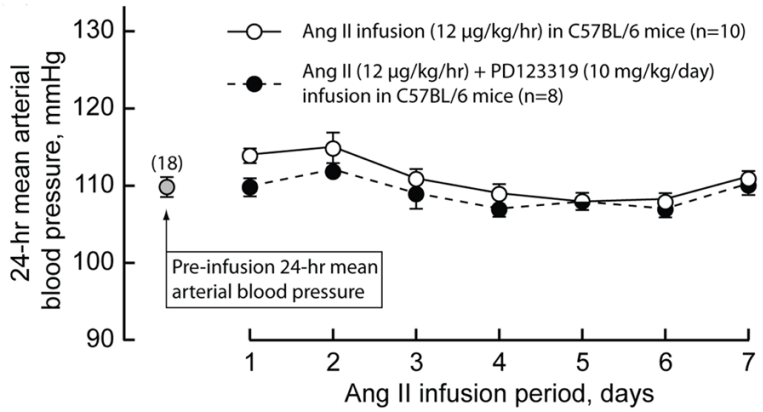

Figure 1. Determination of a subpressor dose of AII in C57BL/6 mice. Several doses of AII were studied to identify one that was just below the threshold for influencing blood pressure in conscious mice C57BL/6 mice. 12 μg/kg/hr AII given over a 1-week period did not significantly alter 24-hr MAP in conscious mice. This subpressor dose was used in subsequent experiments to activate AT_2Rs in vivo.

istry using antibodies against CD31 [an endothelial cell (EC) marker (1:20, ab-958)], α-smooth muscle actin (αSMA) [a VSMC marker (1:100, ab-8207)], CD68 [monocyte/macrophage cell marker, 1:100, Abcam), AT_2R (1:100, ab-19134), phospho-Ser^{1177}-endothelial nitric oxide synthase (eNOS) (p-Ser^{1177}eNOS; 1:100, sc-12972, Santa Cruz), and PPARγ (1:50, ab-19481). Immunohistochemistry was performed as described [12]. NIS-Elements AR3.0 program (Nikon) was used for quantitative fluorescence intensity (arbitrary units) analysis. Immunoreactivity (ir) in each tissue section was normalized relative to the total area measured for each section.

Quantitative real-time PCR

RNA extracted from snap frozen aortic tissue using a mirVana miRNA kit (Ambion) and real-time qRT-PCR performed as described [12]. The primer sets for AT_2R and GAPDH were as follows: AT_2R (forward: 5'-TCCCTGGCAAGCATCTTATGTAG-3'; reverse: 5'-GCGGTTTCC-AACAAAACAAT-3'); and GAPDH (forward: 5'-ATGGTGAAGGTCGGTGTG-3'; reverse: 5'-ACCAGTGGATGCAGGGAT-3').

Western blotting

Western blots were developed as described previously [13, 14]. The aortae were washed with 1XPBS and rapidly snap frozen in liquid nitrogen. The tissues were homogenized on ice in ice-cold RIPA lysis buffer (Cell Signaling) supplemented with PMSF (Sigma) in addition to Complete Mini protease inhibitors cocktail (Roche) and phosphatase inhibitors (Sigma). Lysates were centrifuged at 10,000 × g for 30 min at 4°C and the supernatants were collected. Protein concentrations were determined by a Bio-rad's Bradford protein assay. Equal amounts of protein (12 μg) were separated by SDS-PAGE and electroblotted onto PVDF membrane (Bio-rad). After pre-blocking with SuperBlock (Thermo-Pierce), membranes were incubated at 4°C overnight with polyclonal rabbit anti-eNOS, -p-Ser^{1177}-eNOS, or -GAPDH antibodies. The immunoblots were probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 hour at room temperature and developed with a SuperSignal West Dura Extended Duration chemiluminescence reagent kit (GE Healthcare) followed by X-ray film exposure. Densitometric analyses to measure relative intensity of each antibody specific signal were performed with Image J program from the National Institutes of Health.

Nitric oxide metabolite measurements

Nitrite and nitrate concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Nitrosothiol compounds (RXNO) were quantified using group specific reductive denitrosation by iodine-iodide with subsequent detection of the NO liberated by gas-phase chemiluminescence. All NO analysis procedures have been previously described in detail [15].

Data analysis

Values are mean ± S.E.M. Differences were assessed by ANOVA followed by Tukey's test for multiple comparisons, or by unpaired 2-tailed Student's t test. P values < 0.05 were considered significant.

Results

Comparative studies between wild type and AT_2R deficient mice have revealed that as compared to wild type mice an enhanced pressor

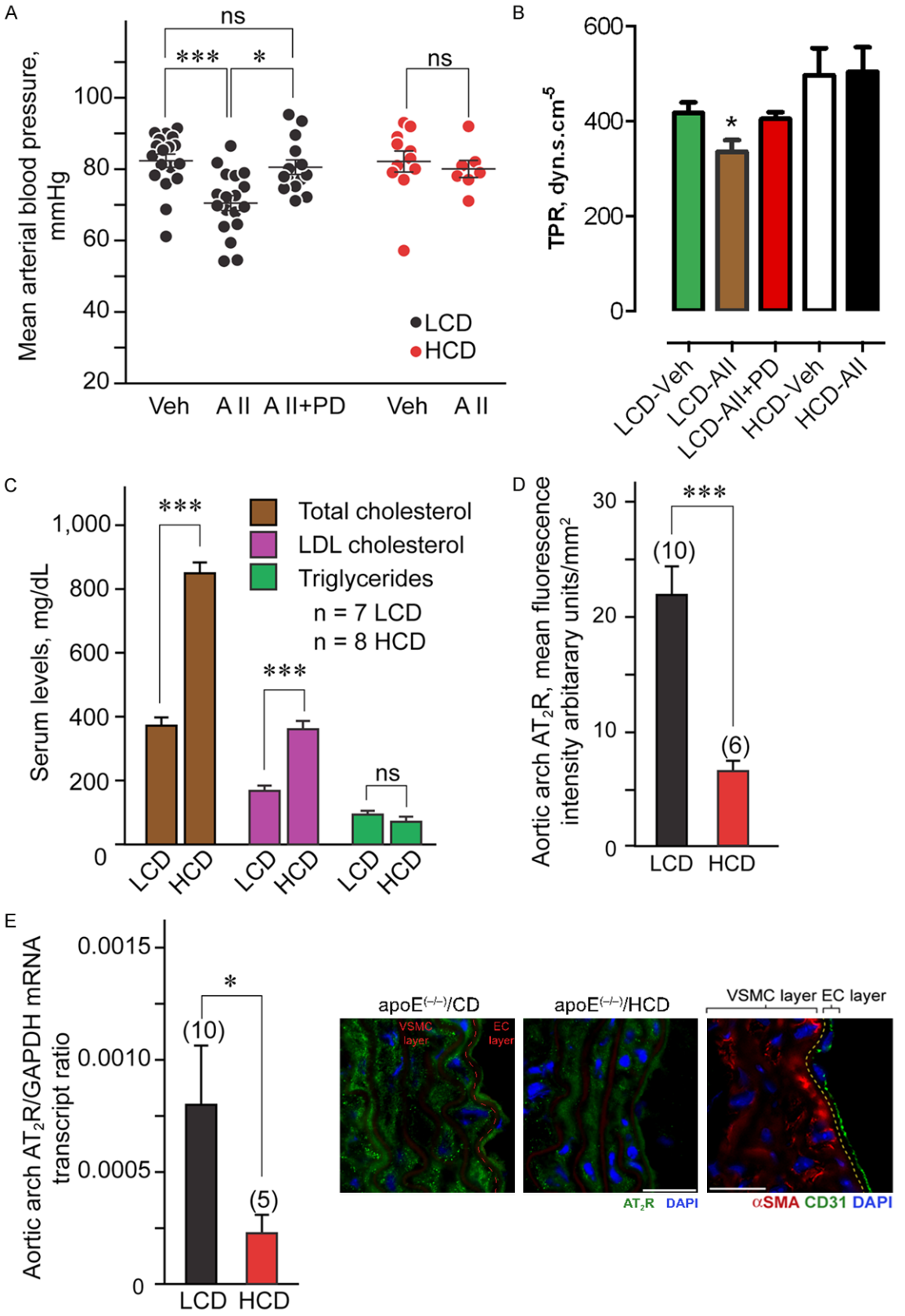
A
ns
***
*
ns
Mean arterial blood pressure, mmHg
100
80
60
40
20
LCD
HCD
Veh
A II
A II+PD
Veh
A II
B
TPR, dyn.s.cm-5
600
400
200
0
*
LCD-Veh
LCD-AII
LCD-AII+PD
HCD-Veh
HCD-AII
C
***
Total cholesterol
LDL cholesterol
Triglycerides
n = 7 LCD
n = 8 HCD
***
ns
Serum levels, mg/dL
1,000
800
600
400
200
0
LCD
HCD
LCD
HCD
LCD
HCD
D
***
Aortic arch AT2R, mean fluorescence intensity arbitrary units/mm2
30
20
10
0
(10)
(6)
LCD
HCD
E
*
Aortic arch AT2R/GAPDH mRNA transcript ratio
0.0015
0.0010
0.0005
0
(10)
(5)
LCD
HCD
apoE(−/−)/CD
apoE(−/−)/HCD
VSMC layer EC layer
VSMC layer
EC layer
AT2R DAPI
αSMA CD31 DAPI

Figure 2. Effect of high cholesterol diet (HCD) on the vasodilator response to low-dose Ang II infusion and AT_2R expression in young $apoE^{(-/-)}$ mice. (A) A 7-day low-dose (12 μg/kg/day) Ang II infusion in 9-week-old low cholesterol (LCD)/$apoE^{(-/-)}$ mice decreased MAP. The AT2R antagonist PD123319 blocked this low-dose Ang II effect. In HCD/$apoE^{(-/-)}$ mice, low-dose AII was unable to produce a vasodilator effect. (B) We also measured total peripheral resistance (tpr) using the formula: Total peripheral resistance (tpr) = (80 x MAP)/Cardiac Output. This study revealed that low dose Ang II infusion significantly reduced tpr in 9-week-old $apoE^{(-/-)}$ mice, fed a LCD for 1 week (C). A pressor response (that is, a subpressor dose) in C56BL/6 mice (their genetic controls). This dose was empirically determined to be 12 μg/kg/day (low-dose Ang II) (**Figure 1**). We compared effects of the vehicle or an AT2R antagonist (PD123319) on responses produced by 7-day Ang II infusion (12 μg/kg/hr) in $apoE^{(-/-)}$ mice. This duration/dose of Ang II was not sufficient to influence MAP in conscious mice (**Figure 1**). Effect of a 1-week HCD or LCD in 9-week-old LCD/$apoE^{(-/-)}$ mice on serum total cholesterol, LDL cholesterol, and triglycerides levels. Relative to the LCD, HCD increased serum total cholesterol, LDL cholesterol without significantly increasing triglyceride levels. In these mice, HCD also significantly decreased aortic arch AT2R protein expression (C) and mRNA expression (D). This effect of the HCD on AT2R expression was consistent with the loss of vasodilator response to low-dose Ang II seen in (A). Values are mean ± S.E.M. Numbers in parenthesis represent the number of independent replications. Statistical analyses performed by ANOVA (Tukey) or by Student's *t* test. *P < 0.05; ***P < 0.001; n.s., not significant.

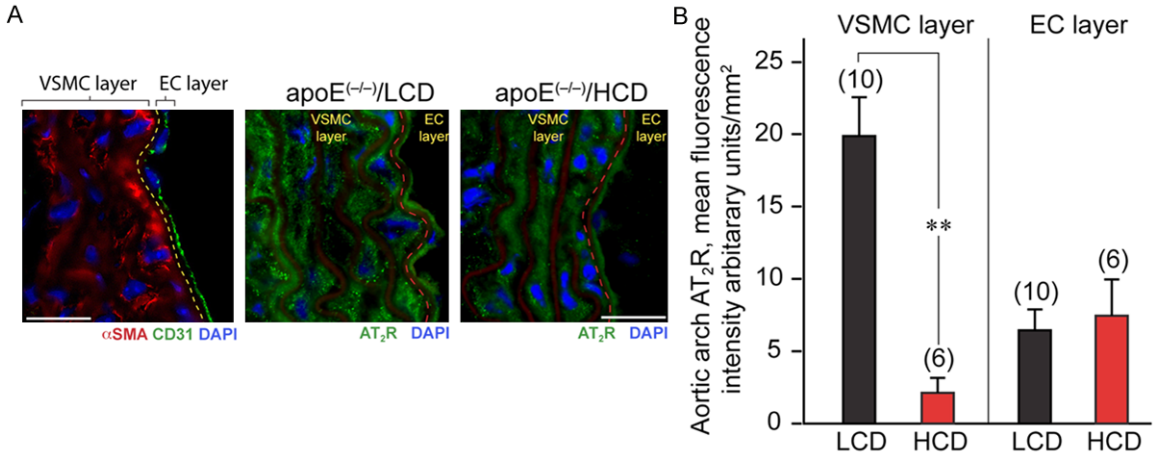

Figure 3. HCD down regulates VSMC AT_2R expression in the aortic arch of $apoE^{(-/-)}$ mice. A. In the *left panel* is a representative section stained with anti-aSMA (red) and anti-CD31 (green) to demarcate the VSMC and EC layers, respectively. The dotted lines (red or yellow) identify the border between the EC and VSMC layers. The *middle* and *right panels* show AT_2R immunoreactivity (ir) (green) and nuclei labeled with DAPI (blue) in representative sections from $apoE^{(-/-)}$ aortae fed LCD or HCD, respectively. B. Quantification of AT_2R-ir in the aortic arch showing that HCD markedly down regulates VSMC, but not EC, AT_2R-ir in aortae of $apoE^{(-/-)}$ mice. Bars denote 20 μm. Values are mean ± S.E.M. Numbers in parenthesis represent the number of independent replications. Statistical differences of continuous variables were determined by Student's *t* test. **P < 0.01.

response to Ang II in AT_2R-deficient mice is observed only at very low doses of the hormone [7]. Because the Kd of the interaction between Ang II and these receptor subtypes is similar [3], these blood pressure findings may be interpreted to suggest that the maximal AT_2R-mediated effect is obtained without full receptor occupancy. We reasoned, therefore, that potential AT_2R-mediated vascular responses may become manifest in $apoE^{(-/-)}$ mice at a dose of Ang II that just fails to elicit a pressor response (that is, a subpressor dose) in C56BL/6 mice (their genetic controls). This dose was empirically determined to be 12 μg/kg/day (low-dose Ang II) (**Figure 1**). We compared effects of the vehicle or an AT_2R antagonist (PD123319) on responses produced by 7-day Ang II infusion (12 μg/kg/hr) in $apoE^{(-/-)}$ mice. This duration/dose of Ang II was not sufficient to influence MAP in conscious (**Figure 1**). In 9-week-old $apoE^{(-/-)}$ mice, fed a LCD for 1 week, a 7-day low-dose Ang II infusion produced a 12 mmHg decrease in MAP (P < 0.001) that was abolished by the selective AT_2R antagonist, PD123319 (**Figure 2A**). Echocardiographic and hemodynamic analyses in these mice indicated that this vasodepressor response was not associated with significant changes in heart rate (553 ± 21 beats/min in vehicle-infused mice, n = 7 versus 545 ± 15 beats/min in low-dose Ang II-infused mice, n = 8; P = 0.753) or cardiac output (15.5 ± 1.4 ml/min in vehicle-

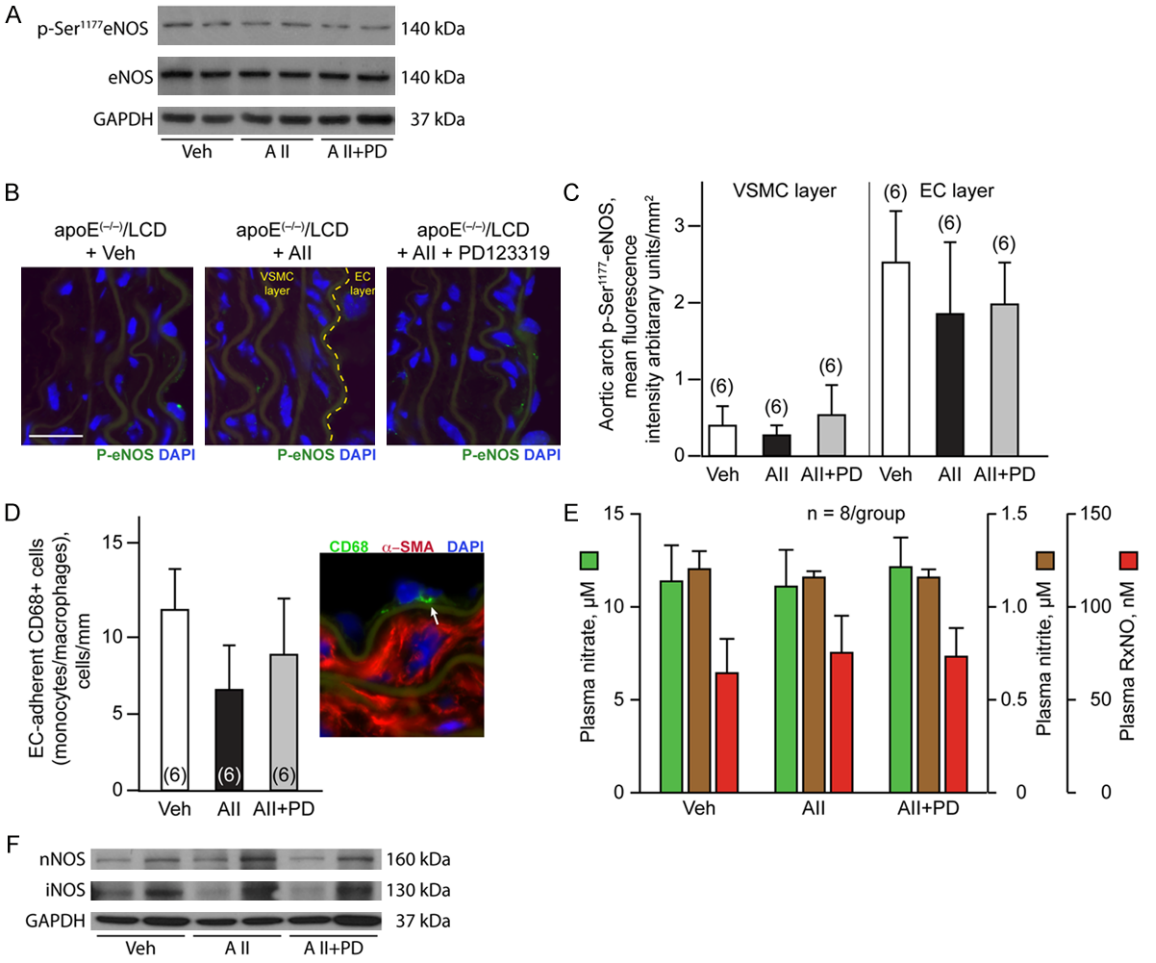

Figure 4. Ang II/AT$_2$R activation does not activate aortic arch eNOS or increase plasma NO metabolites in LCD/apoE$^{(-/-)}$ mice. (A) Aortic arch homogenates from LCD/apoE$^{(-/-)}$ mice infused with vehicle (Veh), Ang II (AII), or Ang II plus PD123319 (AII + PD) were analyzed by Western blotting using anti-p-Ser^{1177}eNOS, anti-eNOS, and anti-GAPDH antibodies. Representative data of 3 separate experiments (3 aortae/pool) are shown. GAPDH-corrected intensities revealed no significant differences in activated (p-Ser^{1177}eNOS) or total eNOS (not shown). (B) In the *left panel* is a representative aortic arch section stained with anti-p-Ser^{1177}eNOS (green) and the nuclei labeled with DAPI (blue) from an apoE$^{(-/-)}$ mouse fed a LCD and infused with vehicle for 7 days. In the *middle panel* is a similarly stained representative section from an apoE$^{(-/-)}$ mouse fed a LCD and infused with low-dose Ang II for 7 days. Note no change in the p-Ser^{1177}eNOS-ir in cells from the VSMC or EC layers. The dotted yellow line shows the demarcation between ECs and VSMCs. In the *right panel* is a similarly stained representative section from an apoE$^{(-/-)}$ mouse fed a LCD and infused with low-dose Ang II + PD123319 for 7 days. Note that this treatment had no effect on p-Ser^{1177}eNOS-ir levels. Bar denotes 20 μm. (C) Quantification of p-Ser^{1177}eNOS-ir in the aortic arch showing that neither Ang II-infusion nor its antagonism by PD123319 affected activated eNOS levels in the VSMC or EC layers. (D) CD68-positive cells attached to EC layer were quantified using immunohistochemistry. Inset shows a representative section from a control aorta (vehicle-treated) stained with anti-α-SMA to detect VSMCs (red) and anti-CD68-positive cells to detect adherent monocytes/macrophages (green). Nuclei labeled with DAPI (blue). No significant differences were found between vehicle, Ang II, or Ang II plus PD123319 treatment groups. (E) Plasma NO metabolites (nitrate, nitrite and RxNO) or the expression of nNOS and iNOS (F) in LCD/apoE$^{(-/-)}$ mice are not significantly influenced by low-dose Ang II- or Ang II + PD123318-infusions. Values are mean ± S.E.M. Numbers in parenthesis represent the number of independent replications. Statistical analyses performed by ANOVA (Tukey) test. ***P < 0.001.

infused mice, n = 7 versus 16.7 ± 1.0 ml/min in low-dose Ang II-infused mice, n = 8; P = 0.501), suggesting that Ang II/AT$_2$R activation decreases total peripheral resistance (tpr) in LCD/apoE$^{(-/-)}$ mice. Thus we measured tpr using the formula: Total peripheral resistance (tpr) = (80 x MAP)/Cardiac Output. This study revealed that low dose Ang II infusion significantly reduced tpr in 9-week-old apoE$^{(-/-)}$ mice, fed a LCD for 1 week (**Figure 2B**).

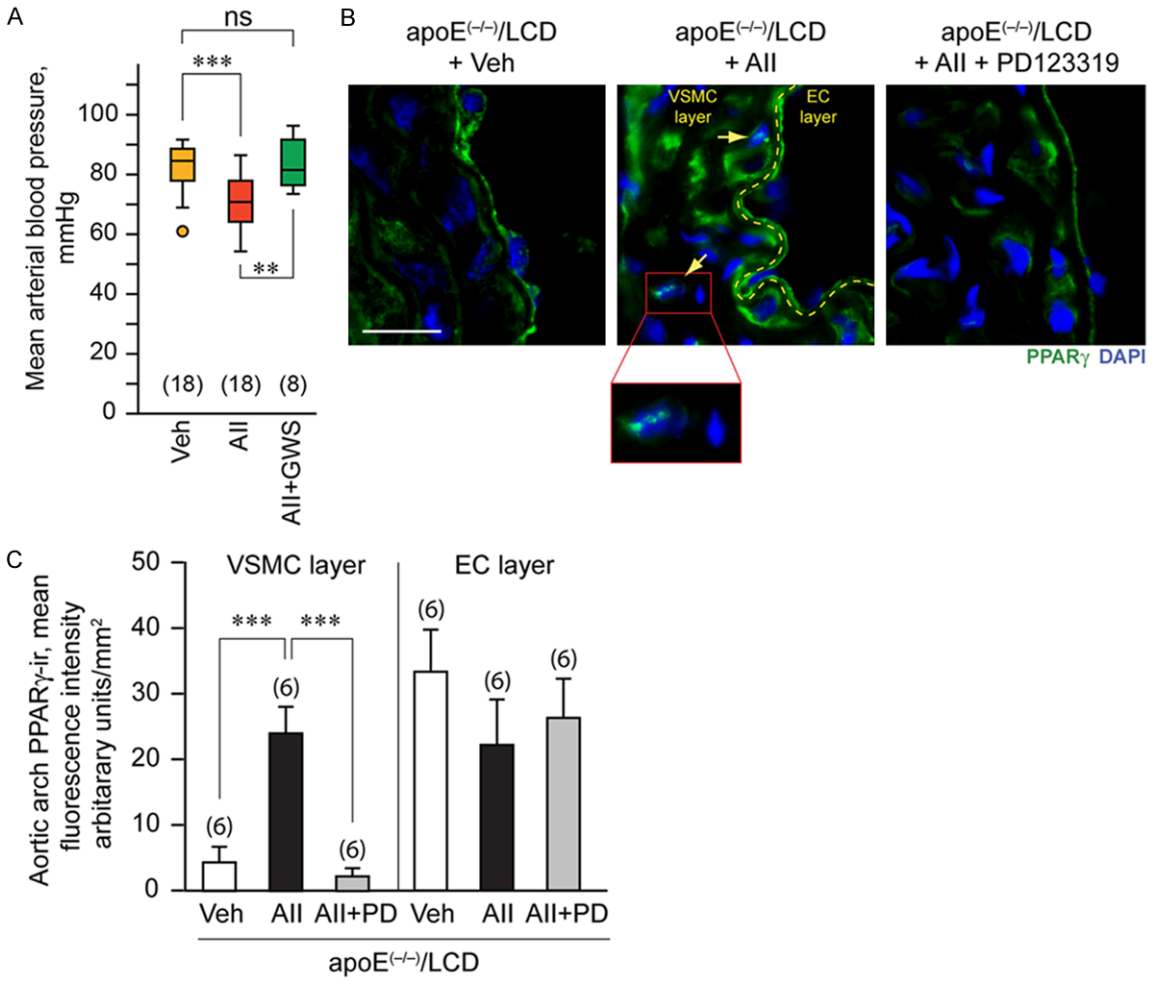

Figure 5. Low-dose Ang II-dependent vasodilator response involves PPARγ activation. A. The AT_2R-dependent vasodepressor response to low-dose Ang II (AII) is not observed in LCD/apoE$^{(-/-)}$ mice pretreated with GWS9662 (GWS), a selective PPARγ antagonist. This indicates that the vasodilator response to Ang II requires PPARγ. Mean arterial blood pressure is shown in a box-and-whiskers plot, with a value lower than the first percentile shown as a circle. Statistical analyses performed by ANOVA (Tukey). ns, not significantly different. Data for Veh and AII is from (**Figure 2A**). B. In the *left panel* is a representative aortic arch section stained with anti-PPARγ (green) and the nuclei labeled with DAPI (blue) from an apoE$^{(-/-)}$ mouse fed a LCD and infused with vehicle for 7 days. In the *middle panel* is a similarly stained representative section from an apoE$^{(-/-)}$ mouse fed a LCD and infused with low-dose Ang II for 7 days. Note the increase in PPARγ immunoreactivity (ir) in cells from the VSMC layer. The dotted yellow line shows the demarcation between ECs and VSMCs. The arrow shows nuclear localization of PPARγ. This nuclear localization is shown in greater magnification in the inset. In the *right panel* is a similarly stained representative section from an apoE$^{(-/-)}$ mouse fed a LCD and infused with low-dose Ang II + PD123319 for 7 days. Note the increase in PPARγ immunoreactivity in VSMCs is markedly inhibited by AT_2R antagonism with PD123319. Bar denotes 20 μm. C. Quantification of PPARγ-ir in the aortic arch showing that low dose Ang II-infusion markedly up regulates PPARγ-ir (by ~6-fold) in VSMCs, but not in ECs, of LCD/apoE$^{(-/-)}$ mice and that this effect is inhibited by PD123319. Values are mean ± S.E.M. Numbers in parenthesis represent the number of independent replications. Statistical analyses performed by ANOVA (Tukey) test. ***P < 0.001.

Next we examined the effect of a 1-week HCD in 9-week-old apoE$^{(-/-)}$ mice on aortic arch AT_2R mRNA and AT_2R expression. We selected a commercial AT_2R antibody that specifically recognizes a single band by Western blot analysis and selectively identifies (by immunohistochemistry) AT_2R expression in cell layers that are known to expresses this receptor subtype [16]. A 1-week HCD in apoE$^{(-/-)}$ mice increased serum total cholesterol by 2.3-fold ($P < 0.001$) and LDL cholesterol by 2.2-fold ($P < 0.001$), without significantly changing triglycer-

ide levels (**Figure 2C**). Quantitative immunohistochemistry indicated that a HCD produced a 72% decrease in aortic arch AT_2R expression ($P < 0.001$) (**Figure 2D**), which was associated with a 65% decrease in aortic arch *agtr2* gene expression ($P < 0.05$) (**Figure 2E**). In these HCD/apoE$^{(-/-)}$ mice, MAPs were not different between vehicle- and low-dose Ang II-infused mouse groups (**Figure 2A**). These findings indicate that short-term increases in total serum cholesterol and LDL cholesterol are sufficient to decrease the AT_2R-mediated blood pressure effects in apoE$^{(-/-)}$ mice, which are likely due to decreased vascular AT_2R expression. (**Figure 3A** and **3B**), show that VSMC AT_2Rs, but not EC AT_2Rs, were selectively down regulated (by ~6-fold; $P < 0.01$) by the HCD indicating a cell layer specific effect in the aortic arch of apoE$^{(-/-)}$ mice.

To evaluate an Ang II/AT_2R-mediated activation of NO, we examined PD213319-inhibitable eNOS activation in the aortic arch in response to low-dose Ang II. The activity of eNOS is regulated by phosphorylation at multiple sites. The two most thoroughly studied sites are the activation site Ser^{1177} and the inhibitory site Thr^{495} [17]. Low-dose Ang II did not significantly change total eNOS or p-Ser^{1177}-eNOS in the aortic arch (**Figure 4A**). To determine if these Western blot analyses mask a selective increase in p-Ser^{1177}-eNOS in the EC layer we performed quantitative immunohistochemistry. This also failed to show an effect of low-dose Ang II on EC p-Ser^{1177}-eNOS levels (**Figure 4B**, **4C**). NO generation, secondary to Ang II/AT_2R activation has been reported to decrease monocyte attachment in the aortic arch of apoE$^{(-/-)}$/α-SMA-AT_2R transgenic mice [10]. We evaluated this by immunohistochemistry using an antibody to CD68, a marker for the various cells of the macrophage lineage, including monocytes. In 10-week-old apoE$^{(-/-)}$ mice, monocyte/macrophages begin to adhere to aortic arch ECs. We found that low-dose Ang II did not significantly influence this attachment in LCD/apoE$^{(-/-)}$ mice (**Figure 4D**). These findings do not support a role for Ang II/AT_2R-mediated NO production in the aortic arch of LCD/apoE$^{(-/-)}$ mice.

We next considered the possibility that the vasodilator effect of low-dose Ang II may be due to local NO production in resistance vessels that is not reflected by Ang II/AT_2R activation in the aortic arch. To test this we evaluated NO metabolites nitrate, nitrite and nitroso (RXNO)-species in plasma. We found that plasma nitrate, nitrite and RXNO levels were not significantly changed by low-dose Ang II or by an infusion of low-dose Ang II + PD123319 (**Figure 4E**). Furthermore, the expression of nNOS or iNOS was no significantly influenced by low-dose Ang II or by an infusion of low-dose Ang II + PD123319 (**Figure 4F**). Thus, our findings failed to support a role for Ang II/AT_2R in activating eNOS in the aortic arch, despite a prominent Ang II/AT_2R-mediated vasodilator response in 10-week-old LCD/apoE$^{(-/-)}$ mice (**Figure 2A**).

Ang II/AT_2R increases peroxisome proliferator-activated receptor γ (PPARγ) in PC12W rat pheochromocytoma cells [18]. Because PPARγ suppression in VSMCs causes hypertension [19], we investigated the possibility that low-dose Ang II causes a hypotensive response in LCD/apoE$^{(-/-)}$ mice by increasing PPARγ levels. **Figures 2A** and **5A** show that GWS9662, a PPARγ antagonist, inhibited the hypotensive response to low-dose Ang II in LCD/apoE$^{(-/-)}$ mice. Moreover, low-dose Ang II selectively increased PPARγ expression in the VSMC layer by ~6-fold ($P < 0.001$); an effect that was blocked by PD123319 (**Figure 5B**, **5C**). Together, these findings suggest a role for Ang II/AT_2R-mediated increase in vascular PPARγ in mediating a hypotensive response.

Discussion

Monocyte adhesion to the endothelium is an early inflammatory response that characterizes the initiation of atherosclerosis development in apoE$^{(-/-)}$ mice. This process, which begins at 8- to 10 week of age in apoE$^{(-/-)}$ mice, is accelerated by diets that are rich in cholesterol [20]. Endothelium-derived NO inhibits adhesion molecule expression [21]. Transgenic overexpression of AT_2Rs in VSMCs activates kinin-dependent NO generation in apoE$^{(-/-)}$ ECs and reduces monocyte adhesion to vascular ECs [10]. Thus, apoE deficiency, which tonically increases AT_2R mRNA expression in the aortic arch [9], is expected to increase AT_2R-dependent NO generation and suppress monocyte adhesion. Detrimental effects of AT_2R deficiency, however, are not apparent in the aortic arch of 14-week-old HCD/apoE$^{(-/-)}$ mice where foam cell lesions are abundant [9]. Here we show that

short-term (7-day) administration of a HCD to 9-week-old apoE$^{(-/-)}$ mice markedly decreases AT_2R mRNA and protein levels in the aortic arch; this expression was selectively decreased in VSMCs. The cholesterol rich diet also suppressed the Ang II/AT_2R-dependent vasodilator effect. This down regulation of VSMC AT_2Rs could explain why beneficial effects of AT_2R-activation are not observed early in the apoE$^{(-/-)}$ mouse model of atherosclerosis that requires a HCD to accelerate the disease process [9]. However, a beneficial effect of vascular AT_2R is possible when its expression is artificially driven in VSMCs by an α-SMA promoter [10].

It is believed that a prominent effect of VSMC AT_2R activation is kinin production and its paracrine activation of EC eNOS, NO generation, and vasodilatation [8]. We also show a prominent AT_2R-dependent vasodilator response to low-dose Ang II infusion in LCD/apoE$^{(-/-)}$ mice. This response was observed in the absence of an AT_1R antagonism, which normally is required to unmask the AT_2R effect in wild type and HCD/apoE$^{(-/-)}$ mice [8]. However, by contrast to the findings of Tsutsumi et al. [8], levels of total eNOS and active eNOS (p-Ser^{1177}-eNOS) were not significantly changed during low-dose Ang II infusion. This despite abundant AT_2R expression in the aortic arch VSMC layer of these LCD/apoE$^{(-/-)}$ mice. We considered the possibility that the vasodilator effect of low-dose Ang II might be due to local NO production in resistance vessels. We previously showed that NO synthesized within a specified location (cardiomyocytes) is transported in the blood as a bioactive NO species (nitrate, nitrite and nitroso (RXNO)-species) [22]. We evaluated NO metabolites in plasma to test whether Ang II/AT_2R-activation increases NO generation in other tissues of LCD/apoE$^{(-/-)}$ mice. However, low-dose Ang II was unable to significantly increase plasma nitrate, nitrite and RXNO levels. These findings are not consistent with Ang II/AT_2R-dependent eNOS activation as a mechanism for the vasodilator effect of low-dose Ang II in LCD/apoE$^{(-/-)}$ mice. By contrast, we found that a PPARγ antagonist inhibits the Ang II/AT_2R-dependent vasodilator response. A finding supported by a prominent increase in aortic arch VSMC PPARγ levels that are inhibited by an AT_2R antagonist. This novel effect of the AT_2R in VSMCs is also supported by the report that transgenic overexpression of a dominant negative-PPARγ mutant in VSMCs produces hypertension [19]. While our studies do not negate the extensive data that supports a role for AT_2R-dependent eNOS activation in the vasculature via increased kinin production, it does suggest a novel mechanism, involving PPARγ, for the Ang II/AT_2R-dependent vasodilator effect. The prominent down regulation of aortic arch AT_2R by a HCD would suggest that the beneficial effects of vascular AT_2R may not become apparent at an early stage of atherosclerosis development in experimental models that combine apoE deficiency with a HCD. This, as well as the substantial protective effects of VSMC PPARγ in lesion development [23] leads us to speculate that VSMC AT_2R activation may substantially delay the onset of atherosclerosis development in apoE$^{(-/-)}$ mice maintained on a LCD. If substantiated, AT_2R agonist therapy may reduce atherosclerotic lesion development and, thus, augment the clinically efficacious effects of dietary fat restriction. However, effective translation of this potentially beneficial effect of AT_2R activation may best be achieved by initiating AT_2R therapy after an initial period of fat restriction, since a high fat diet suppresses AT_2R expression, which would mask beneficial effects of AT_2R agonist therapy on atherosclerotic lesion development.

Conclusions

Activation of PPARγ decreases blood pressure and attenuates the development of atherosclerotic lesions. Here we show that the vascular AT_2R regulates PPARγ in the arthrosclerosis-prone vasculature of the apoE$^{(-/-)}$ mouse and that dyslipidemia suppresses VSMC AT_2R expression, a vascular protective mechanism.

Acknowledgements

We thank Dr. Ahsan Husain for helpful discussion and guidance in preparation of this manuscript. This work was funded by grants from the Department of Medicine, Emory University; Carlyle Fraser Heart Center, Emory University Hospital Midtown; NIH (NIH 1RO1HL127726-01A1; HL079040; HL127726; 5R01HL098481-05); American Heart Association (13SDG16460006); and National Health and Medical Research Council of Australia (573732) and the public welfare of science and technology grants in 2014, the city bureau of finance in

science and technology of Wenzhou, China (Y20140671).

Disclosure of conflict of interest

None.

Authors' contribution

M.L., and N.N. designed research; M.L., T.T., J.P.L., C.K.N., E.Y., V.T.A., S.F.A., E.W.B., J.W.C., and N.N. performed research; L.J.D. helped in data analysis and interpretation; R.M.G., and N.N. wrote the paper.

Address correspondence to: Dr. Nawazish Naqvi, Cardiology, Emory University, 101 Woodruff Circle NE, Atlanta, GA 30322, USA. Tel: 404-712-6309; E-mail: nnaqvi@emory.edu

References

[1] Zaman MA, Oparil S, Calhoun DA. Drugs targeting the renin-angiotensin-aldosterone system. Nat Rev Drug Discov 2002; 1: 621-636.

[2] de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev 2000; 52: 415-472.

[3] Pucell AG, Hodges JC, Sen I, Bumpus FM, Husain A. Biochemical properties of the ovarian granulosa cell type 2-angiotensin II receptor. Endocrinology 1991; 128: 1947-1959.

[4] Tsutsumi K, Strömberg C, Viswanathan M, Saavedra JM. Angiotensin-II receptor subtypes in fetal tissue of the rat: autoradiography, guanine nucleotide sensitivity, and association with phosphoinositide hydrolysis. Endocrinology 1991; 129: 1075-1082.

[5] Chang RS, Lotti VJ. Angiotensin receptor subtypes in rat, rabbit and monkey tissues: relative distribution and species dependency. Life Sci 1991; 49: 1485-1490.

[6] Viswanathan M, Tsutsumi K, Correa FM, Saavedra JM. Changes in expression of angiotensin receptor subtypes in the rat aorta during development. Biochem Biophys Res Commun 1991; 179: 1361-1367.

[7] Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. Nature 1995; 377: 744-747.

[8] Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K, Miwa T, Kawada N, Mori Y, Shibasaki Y, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Takahashi H, Iwasaka T. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. J Clin Invest 1999; 104: 925-935.

[9] Sales VL, Sukhova GK, Lopez-Ilasaca MA, Libby P, Dzau VJ, Pratt RE. Angiotensin type 2 receptor is expressed in murine atherosclerotic lesions and modulates lesion evolution. Circulation 2005; 112: 3328-3336.

[10] Takata H, Yamada H, Kawahito H, Kishida S, Irie D, Kato T, Wakana N, Miyagawa S, Fukui K, Matsubara H. Vascular angiotensin II type 2 receptor attenuates atherosclerosis via a kinin/NO-dependent mechanism. J Renin Angiotensin Aldosterone Syst 2015; 16: 311-20.

[11] Li M, Liu K, Michalicek J, Angus JA, Hunt JE, Dell'Italia LJ, Feneley MP, Graham RM, Husain A. Involvement of chymase-mediated angiotensin II generation in blood pressure regulation. J Clin Invest 2004; 114: 112-120.

[12] Li M, Naqvi N, Yahiro E, Liu K, Powell PC, Bradley WE, Martin DI, Graham RM, Dell'Italia LJ, Husain A. c-kit is required for cardiomyocyte terminal differentiation. Circ Res 2008; 102: 677-685.

[13] Kondo K, Bhushan S, King AL, Prabhu SD, Hamid T, Koenig S, Murohara T, Predmore BL, Gojon G Sr, Gojon G Jr, Wang R, Karusula N, Nicholson CK, Calvert JW, Lefer DJ. H_2S protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. Circulation 2013; 127: 1116-1127.

[14] Nicholson CK, Lambert JP, Chow CW, Lefer DJ, Calvert JW, Chronic exercise downregulates myocardial myoglobin and attenuates nitrite reductase capacity during ischemia-reperfusion. J Mol Cell Cardiol 2013; 64: 1-10.

[15] Calvert JW, Condit ME, Aragón JP, Nicholson CK, Moody BF, Hood RL, Sindler AL, Gundewar S, Seals DR, Barouch LA, Lefer DJ. Exercise protects against myocardial ischemia-reperfusion injury via stimulation of β(3)-adrenergic receptors and increased nitric oxide signaling: role of nitrite and nitrosothiols. Circ Res 2011; 108: 1448-1458.

[16] Portela VM, Gonçalves PB, Veiga AM, Nicola E, Buratini J Jr, Price CA. Regulation of angiotensin type 2 receptor in bovine granulosa cells. Endocrinology 2008; 149: 5004-5011.

[17] Fleming I, Fisslthaler B, Dimmeler S, Kemp BE, Busse R. Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. Circ Res 2001; 88: E68-E75.

[18] Zhao Y, Foryst-Ludwig A, Bruemmer D, Culman J, Bader M, Unger T, Kintscher U. Angiotensin II induces peroxisome proliferator-activated receptor gamma in PC12W cells via angiotensin type 2 receptor activation. J Neurochem 2005; 94: 1395-1401.

[19] Halabi CM, Beyer AM, de Lange WJ, Keen HL, Baumbach GL, Faraci FM, Sigmund CD. Interference with PPAR gamma function in smooth muscle causes vascular dysfunction and hypertension. Cell Metab 2008; 7: 215-226.
[20] Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb 1994; 14: 133-140.
[21] Khan BV, Harrison DG, Olbrych MT, Alexander RW, Medford RM. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. Proc Natl Acad Sci U S A 1996; 93: 9114-9119.
[22] Elrod JW, Calvert JW, Gundewar S, Bryan NS, Lefer DJ. Nitric oxide promotes distant organ protection: evidence for an endocrine role of nitric oxide. Proc Natl Acad Sci U S A 2008; 105: 11430-11435.
[23] Halabi CM, Sigmund CD. Peroxisome proliferator-activated receptor-gamma and its agonists in hypertension and atherosclerosis: mechanisms and clinical implications. Am J Cardiovasc Drugs 2005; 5: 389-398.