

RESEARCH PAPER

Angiotensin II stimulation alters vasomotor response to adenosine in mouse mesenteric artery: role for A₁ and A_{2B} adenosine receptors

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BACKGROUND AND PURPOSE

Stimulation of the A₁ adenosine receptor and angiotensin II receptor type-1 (AT₁ receptor) causes vasoconstriction through activation of cytochrome P450 4A (CYP4A) and ERK1/2. Thus, we hypothesized that acute angiotensin II activation alters the vasomotor response induced by the non-selective adenosine receptor agonist, NECA, in mouse mesenteric arteries (MAs).

EXPERIMENTAL APPROACH

We used a Danish Myo Technology wire myograph to measure muscle tension in isolated MAs from wild type (WT), A₁ receptor and A_{2B} receptor knockout (KO) mice. Western blots were performed to determine the expression of AT₁ receptors and CYP4A.

KEY RESULTS

Acute exposure (15 min) to angiotensin II attenuated the NECA-dependent vasodilatation and enhanced vasoconstriction. This vasoconstrictor effect of angiotensin II in NECA-treated MAs was abolished in A₁ receptor KO mice and in WT mice treated with the A₁ receptor antagonist DPCPX, CYP4A inhibitor HET0016 and ERK1/2 inhibitor PD98059. In MAs from A_{2B} receptor KO mice, the vasoconstrictor effect of angiotensin II on the NECA-induced response was shown to be dependent on A₁ receptors. Furthermore, in A_{2B} receptor KO mice, the expression of AT₁ receptors and CYP4A was increased and the angiotensin II-induced vasoconstriction enhanced. In addition, inhibition of K_{ATP} channels with glibenclamide significantly reduced NECA-induced vasodilatation in WT mice.

CONCLUSIONS AND IMPLICATIONS

Acute angiotensin II stimulation enhanced A₁ receptor-dependent vasoconstriction and inhibited A_{2B} receptor-dependent vasodilatation, leading to a net vasoconstriction and altered vasomotor response to NECA in MAs. This interaction may be important in the regulation of BP.

Abbreviations

CYP, cytochrome P450; KO, knock out; MAs, mesenteric arteries; NECA, 5'-(N-ethylcarboxamido) adenosine; PE, phenylephrine; WT, wild type, 20-HETE, 20-hydroxyeicosatetraenoic acid, DPCPX, 1,3-Dipropyl-8-cyclopentylxanthine

Tables of Links

| TARGETS | |
|---------------------------------|----------------------------|
| GPCRs^a | Enzymes^c |
| A ₁ receptor | CYP4A |
| A _{2B} receptor | ERK1 |
| AT ₁ receptor | ERK2 |
| Ion channels^b | PKC α |
| K _{ATP} channels | |

| LIGANDS | |
|------------------|--------------------|
| 20-HETE | L-NAME |
| Adenosine | Losartan |
| Angiotensin II | NECA |
| Arachidonic acid | PD98059 |
| DPCPX | Phenylephrine (PE) |
| Glibenclamide | |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b,c}Alexander *et al.*, 2013a,b,c).

Introduction

Adenosine is a metabolite of adenine nucleotide degradation generated in both extracellular and intracellular compartments in response to various physiological and pathological stimuli. Once released, adenosine regulates vascular tone by activating four subtypes of adenosine receptors. These adenosine receptors are GPCRs, namely, A₁, A_{2A}, A_{2B} and A₃. A₁ and A₃ receptor activation causes vasoconstriction, while stimulation of A_{2A} and A_{2B} receptors produces vasodilatation (Ohisalo, 1987; Mubagwa *et al.*, 1996; Olanrewaju and Mustafa, 2000; Olanrewaju *et al.*, 2000; Mubagwa and Flameng, 2001; Jacobson and Gao, 2006; Hein *et al.*, 2013). In mouse aorta, A₁ receptor-dependent smooth muscle contraction involves cytochrome P450 epoxygenase type 4A (CYP4A), PKC α and MAPKs, mainly p44/42 MAPK (ERK1/2) (Ansari *et al.*, 2009; Sanjani *et al.*, 2011; Kunduri *et al.*, 2013a; Kunduri *et al.*, 2013b). Moreover, A₁ receptor-mediated vasoconstriction occurs through the activation of PKC α , leading to ERK1/2 phosphorylation in coronary artery smooth muscle cells (Tawfik *et al.*, 2005; Ansari *et al.*, 2009). In contrast, A_{2A} receptor-dependent vasodilatation involves the activation of K_{ATP} channels (Ponnoth *et al.*, 2012b; Sharifi-Sanjani *et al.*, 2013; Zhou *et al.*, 2013). The mechanism involved in the vasodilatation mediated through A_{2B} receptors is less well established and may possibly involve the activation of K_{ATP} channels (Berwick *et al.*, 2010).

Interestingly, stimulation of angiotensin II receptor type 1 (AT₁ receptor) activates PLC, generates 20-hydroxyeicosatetraenoic acid (20-HETE) metabolite from the degradation of arachidonic acid by CYP4A, causing ERK1/2-dependent vasoconstriction and the development of hypertension (McGiff and Laniado-Schwartzman, 1988; Harder *et al.*, 1994; Haller *et al.*, 1996; McGiff *et al.*, 1996; Muthalif *et al.*, 1998; 2000; Zaman *et al.*, 2002). Notably, studies have revealed that inhibiting A₁ receptors attenuates hypertension in spontaneously hypertensive rats and Dahl salt-sensitive rats (Uehara *et al.*, 1995; Kost *et al.*, 2000). Moreover, in chronic angiotensin II- and L-NAME-induced hypertension in mice, genetic deletion of A₁ receptors attenuates arteriolar vasoconstriction and hypertension (Gao *et al.*, 2011; Lee *et al.*, 2012). Recently, increased

adenosine production and activation of A_{2B} receptors in kidneys of mice were implicated in chronic angiotensin II-dependent hypertension (Zhang *et al.*, 2013). Previously, we demonstrated that endothelium-dependent contractile responses to adenosine analogues in spontaneously hypertensive rat (SHR) aorta are mediated by A₁ receptors and vasodilatation is mediated by A₂ receptors (Fahim and Mustafa, 2001). Moreover, in SHR aorta, endothelium-dependent adenosine receptor-mediated vasodilatation was attenuated (Fahim *et al.*, 2001). Thus, a complex relationship exists between adenosine and angiotensin II signalling with important implications for chronic angiotensin II-dependent hypertension.

Previously, we have reported that all four adenosine receptors are present in mesenteric arteries (MAs) of mice and that the A₁ receptor mediates vasoconstriction, while the A_{2B} receptor mediates vasodilatation (Teng *et al.*, 2013). We hypothesized that acute exposure to angiotensin II alters adenosine receptor-dependent regulation of vascular tone in systemic arteries. In this study, we examined the effects of acute angiotensin II stimulation on the non-selective adenosine agonist, 5'-(N-ethylcarboxamido) adenosine (NECA)-induced concentration-response curves in isolated MAs using a Danish Myo Technology (DMT) wire myograph and identified a critical role for the A₁ and A_{2B} receptors in the response of MAs to NECA after acute angiotensin II-pretreatment.

Methods

Animals

All animal protocols were approved by the IACUC of West Virginia University Health Sciences Center. Male C57BL/6J wild type (WT), A₁ receptor knock out (KO) and A_{2B} receptor KO mice used for the experiments were raised as described previously (Teng *et al.*, 2013). Animals aged 14–18 weeks and body wt 25 \pm 3 g were used for isolation of MAs. Experiments were conducted in accordance with national legislation and with the Declaration of Helsinki regarding the use of experimental animals.

Muscle tension measurement

Muscle tension measurements were performed using a DMT myograph (Danish Myo Technology A/S, Aarhus, Denmark) as described previously (Teng *et al.*, 2013). Briefly, after anaesthesia with pentobarbital sodium (100 mg·kg⁻¹ i.p.), the mesentery with intestines from mice was excised and placed in cold oxygenated (5% CO₂ and 95% O₂) modified Krebs–Henseleit physiological salt solution (PSS, in mM: NaCl 120, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 1.8, MgSO₄ 1.2, glucose 15 and EDTA 0.05, pH 7.4). First-order MAs were carefully dissected from the mesentery under a microscope in ice-cold PSS. The MA rings (3–5 mm long, 75–150 μm diameter) were mounted on the DMT myograph in PSS continuously oxygenated at 37°C. The MAs were stretched to a preload of 300 mg and equilibrated for 90 min. During the equilibration period, MAs were tested for viability using KCl (50 mM). The presence of an intact endothelium was tested by assessing the response to ACh (1 μM) of MAs precontracted with phenylephrine (PE; 1 μM). NECA-induced responses (0.5, 5, 50 and 500 nM and 5 μM) were performed in MAs precontracted with PE (1 μM). The MAs were incubated with various pharmacological agents, and NECA-induced responses were obtained and normalized to % contraction obtained with PE (1 μM). The stable contraction to PE was attained in 10 min, and muscle tension measurements at this time were used to quantify data. In all of our muscle tension measurement experiments, the endothelia were intact. ACh-induced relaxations were -86.35 ± 3.211 , -81.48 ± 5.868 and -91.34 ± 4.421 (% contractions to PE) in MAs obtained from WT, A_{2B} and A₁ receptor KO mice, respectively. Similar levels of PE-induced stable contractions were observed whenever serial NECA-induced responses were performed.

The role of A₁ receptors was investigated by incubating MAs with the A₁ receptor antagonist DPCPX (1 μM) (Li *et al.*, 1998; Tawfik *et al.*, 2005; Ansari *et al.*, 2009; Pradhan *et al.*, 2014). Furthermore, A₁ receptor KO mice were used to determine the role of the A₁ receptor. To study the effect of CYP4A, MAs were pre-incubated with HET0016 (10 μM) (Ponnoth *et al.*, 2012a; Kunduri *et al.*, 2013a), an inhibitor of CYP4A and 20-HETE production. In addition, the role of ERK1/2 was determined by using the inhibitor PD98059 (1 μM) (Ponnoth *et al.*, 2012a; El-Awady *et al.*, 2013; Kunduri *et al.*, 2013b). In addition, the role of K_{ATP} channels in NECA-induced vasodilatation was investigated by pre-incubation of the MAs with glibenclamide (1 μM), a K_{ATP} channel blocker (Fujii *et al.*, 1999; Ponnoth *et al.*, 2012b).

Western blotting

Western blot analyses were performed as described previously (Ponnoth *et al.*, 2012a; Kunduri *et al.*, 2013a). Briefly, MAs isolated from WT and A_{2B} receptor KO mice were homogenized with 125 μL of RIPA lysis and extraction buffer (Peirce #89901 mixed with 1 mM of Na₃VO₄, 1 mg·mL⁻¹ of leupeptin and 1 mM NaF), vortexed and centrifuged for 10 min at 10 000 g at 4°C. Supernatants were stored at -80°C. Protein was measured by Lowry's method using Bio-Rad Laboratories protein assay concentrate (Hercules, CA, USA). The samples (2–5 μg of total protein) were loaded on slab gels (10% acrylamide; 1.5 mm thick), separated by SDS-PAGE, and transferred

to nitrocellulose membranes (Hybond-ECL). Membranes were blocked with 5% dried skimmed milk for 45 min and incubated overnight with primary antibodies. The 1:1000 dilutions were used for CYP4A and A₁ receptor antibodies. All membranes were stripped and probed for β-actin. Secondary antibodies were HRP conjugated. Membranes were developed using enhanced chemiluminescence (Amersham BioSciences, Amersham, UK) and X-ray film. Densitometry analysis was made using Multi Gauge V3.0 (Fuzifilm Medical Systems, CT, USA) software. The data obtained were normalized to actin and presented as the normalized ratio (% change) of target protein to WT protein expression.

Statistical analysis

Data are presented as mean ± SEM % contraction of MAs observed with 1 μM PE. The data from the concentration response curves were analysed between groups at the same concentrations. The comparison between two groups was made using Student's *t*-test. Experiments comprising three or more groups were compared using one-way ANOVA followed by Tukey's test to compare the significance between various groups. *P* < 0.05 was considered statistically significant. For all data, two or more MA rings from one animal were averaged together and presented as one 'n'; MAs from a minimum of six animals were tested and analysed in each group of experiments.

Materials

NECA, phenylephrine (PE), ACh, angiotensin II, DPCPX, PD98059 and all other chemicals and buffers were purchased from Sigma-Aldrich (St. Louis, MO, USA). HET0016 and glibenclamide were purchased from Cayman Chemical (Ann Arbor, MI, USA). Antibodies A₁ receptor (product # sc-1173), CYP4A (sc-271983) and actin (sc-47778) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Results

Acute angiotensin II stimulation causes NECA-induced vasoconstriction in MAs

With an aim to investigate whether angiotensin II stimulation alters adenosine-induced vascular responses, we first assessed angiotensin II-induced vasoconstriction. We observed that angiotensin II concentration-dependently (1, 10 and 100 nM) produced vasoconstriction (Figure 1A and B). As shown in Figure 1A, angiotensin II-induced vasoconstriction rapidly waned. Next, we determined the effect of acute angiotensin II stimulation on NECA-induced vascular responses. We used the non-selective adenosine agonist NECA to determine the effect on all adenosine receptors. First, we measured NECA-induced responses without angiotensin II pretreatment (Control, Figure 2A) and then exposed the MAs to angiotensin II (10 nM) and performed NECA-induced responses in the same tissues (Figure 2B). Angiotensin II pretreatment inhibited the vasodilatation produced by high concentrations of NECA and facilitated a vasoconstrictor response to low doses of NECA that was not observed in control tissues that were not pretreated with

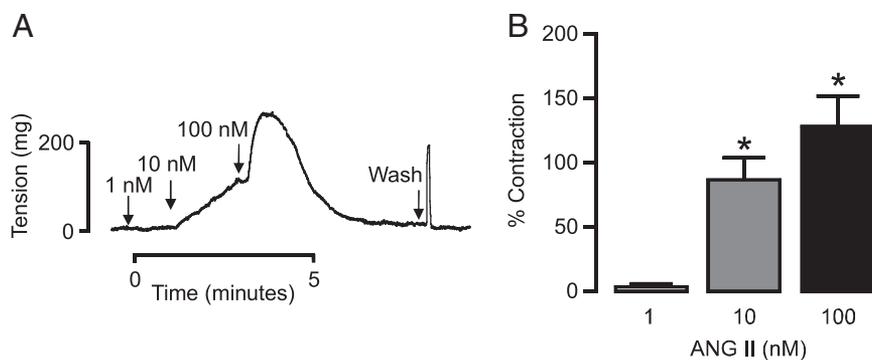


Figure 1

Angiotensin (ANG) II induces concentration-dependent vasoconstrictions in MAs. (A) Representative trace showing angiotensin II concentration-dependent (1, 10 and 100 nM) vasoconstriction. (B) Bar graph summarizes ANG II-induced concentration-dependent muscle tension generated in MAs from WT mice ($n = 6$). * $P < 0.05$ compared with vasoconstriction observed at 1 nM angiotensin II.

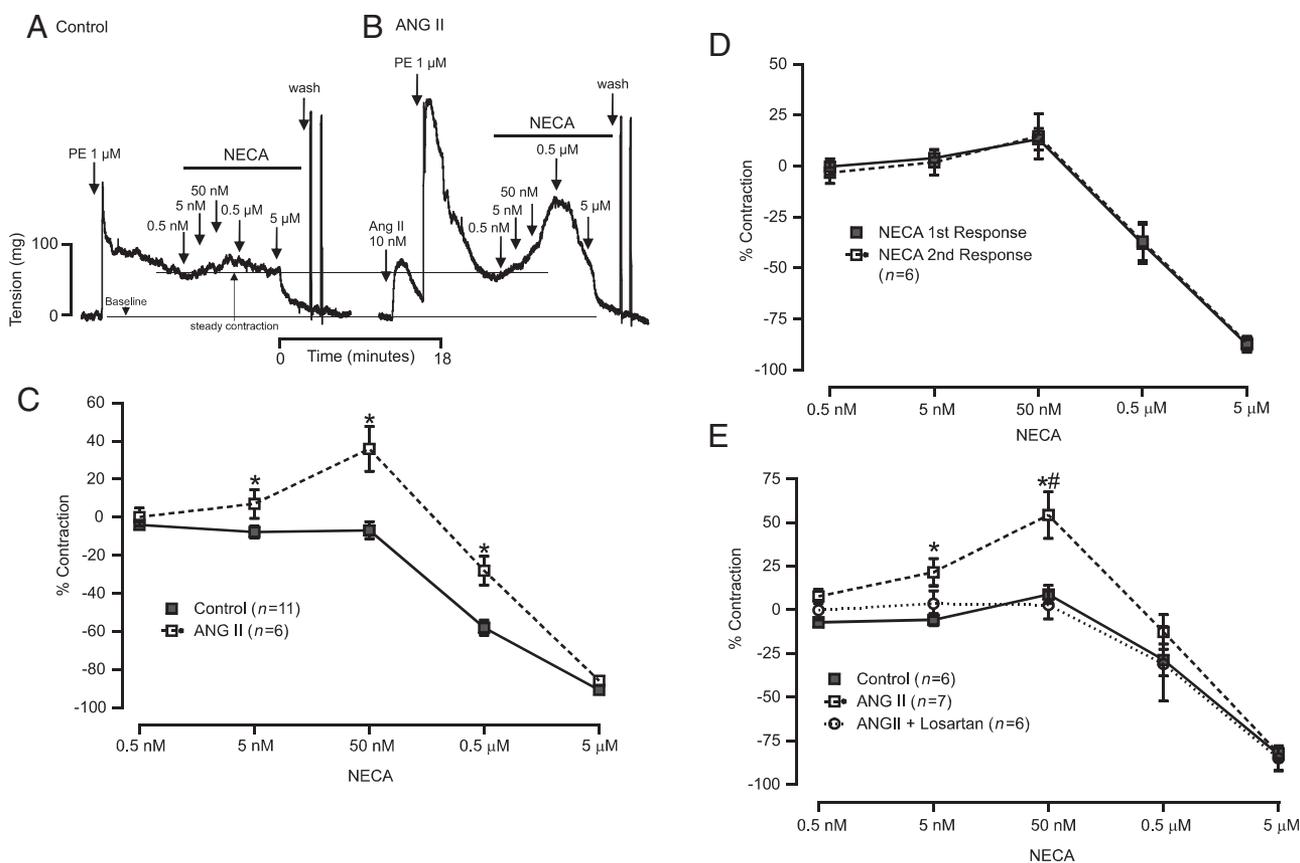


Figure 2

Acute angiotensin (ANG) II stimulation alters NECA-induced responses in MAs from WT mice. (A) Control tracing. (B) NECA-induced responses after ANG II (10 nM) stimulation (bottom line represents baseline, and upper line shows stable contraction to PE). (C) Summarizes quantification of NECA-induced responses in control and ANG II-stimulated MAs. (D) Graph shows that responses of MAs to a series of increasing concentrations of NECA were comparable. (E) Effect of losartan (1 μM, 10 min) on NECA-induced altered responses after ANG II stimulation. The graph shows that losartan pre-treatment completely blocked altered responses to NECA after ANG II stimulation. Asterisk (*) indicates $p < 0.05$ compared with control, and number sign (#) denotes $p < 0.05$ compared with losartan-treated response.

angiotensin II (Figure 2C). To make sure that the altered responses to NECA were indeed due to angiotensin II stimulation, we performed serial NECA concentration-response experiments.

Reproducible serial NECA-induced responses were achieved in MAs (Figure 2D). Losartan (1 μM, AT_1 receptor blocker) completely blocked the angiotensin II-induced vasoconstriction

in MAs ($0.49 \pm 0.34\%$ to PE). Furthermore, the vasoconstrictor response to NECA after angiotensin II stimulation was also blocked by losartan pretreatment (Figure 2E), suggesting that acute angiotensin II stimulation of AT_1 receptors altered the NECA-induced responses to produce vasoconstriction.

To determine the role of the endothelium, we examined the NECA-induced responses in endothelium-denuded MAs. In these vessels, the ACh-induced vascular relaxation was -6.53 ± 2.0 (% contraction to PE). In MAs not pretreated with angiotensin II, NECA-induced relaxation was partially attenuated by removal of the endothelium (Figure 3A). As shown in Figure 3B, partial attenuation of the NECA-induced vasodilatation was observed in angiotensin II treated MAs in endothelium-denuded MAs. In addition, NECA-induced vasoconstriction was absent in endothelium-denuded MA (Figure 3B) compared with MAs with intact endothelium (Figure 2C). These data suggest that NECA-induced vasodilatation is partially endothelium-dependent and that the

effects of angiotensin II on NECA-induced vasoconstrictions are largely endothelium-dependent.

A₁ receptors, CYP4A and ERK1/2 mediate the altered NECA-induced response following angiotensin II stimulation in MAs

After establishing that the vascular responses to NECA were changed following angiotensin II stimulation, we next wanted to determine the signalling mechanism of the angiotensin II-induced vasoconstriction. Thus, we performed muscle tension measurements to angiotensin II (10 nM) in MAs incubated (15 min) with various pharmacological inhibitors, namely, the A_1 receptor antagonist DPCPX (1 μ M), CYP4A inhibitor HET0016 (10 μ M) and ERK1/2 inhibitor PD98059 (1 μ M).

We found that DPCPX (Figure 4A) and HET0016 (Figure 4B) significantly reduced vasoconstrictor responses to angiotensin II. Pretreatment of MAs with the A_1 receptor antagonist DPCPX (1 μ M) or CYP4A inhibitor HET0016 (10 μ M) for 15 min completely abolished the vasoconstriction observed to NECA after angiotensin II stimulation (Figure 4C). We also observed that pretreatment with the ERK1/2 inhibitor PD98059 (1 μ M) significantly inhibited angiotensin II-induced vasoconstriction (Figure 5A) and NECA-induced contractions in angiotensin II-treated MAs

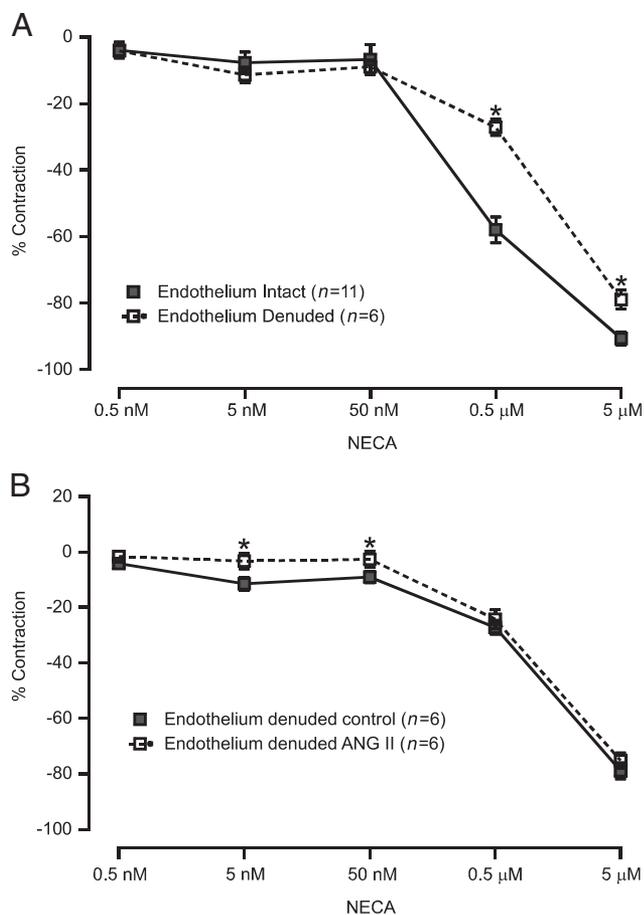


Figure 3

Endothelium plays an important role in vasodilatation to NECA. After endothelium denudation, we performed NECA responses in MAs. (A) Graph shows that NECA-induced vascular responses were partially dependent on endothelium. (B) Denuded MAs were exposed to angiotensin (ANG) II (10 nM), and serial NECA-induced responses were performed. The graph shows that the contraction to NECA was absent, and a significant inhibition of relaxation was observed after ANG II stimulation. * $P < 0.05$ compared with control.

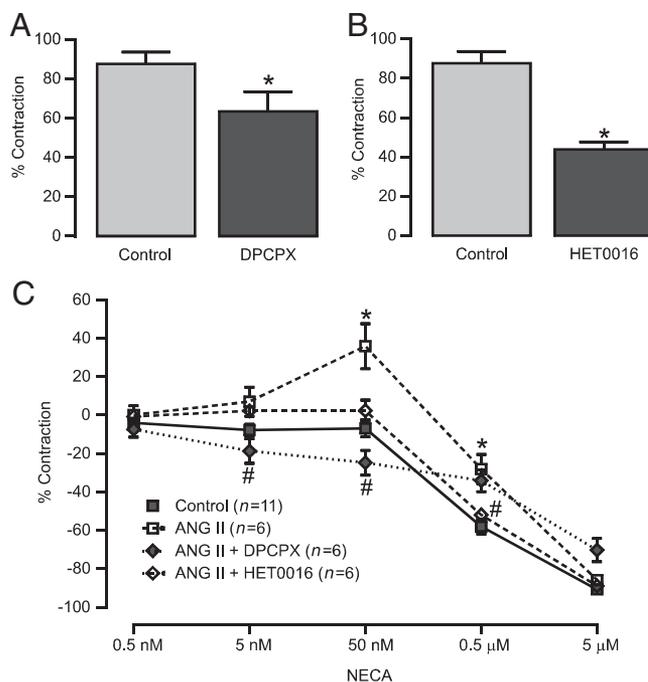


Figure 4

Acute angiotensin (ANG) II stimulation alters the A_1 receptor- and CYP4A-dependent NECA-induced responses in MAs from WT mice. Pre-incubation of MAs with the A_1 receptor antagonist DPCPX 1 μ M (Control $n=10$, DPCPX $n=6$) (A) and CYP4A inhibitor HET0016 10 μ M (Control $n=7$, HET0016 $n=7$) (B) significantly inhibited ANG II-induced (10 nM) vasoconstrictions. (C) ANG II-induced altered responses to NECA were blocked by DPCPX and HET0016. * $P < 0.05$ compared with control, and # $P < 0.05$ compared with ANG II-stimulated response.

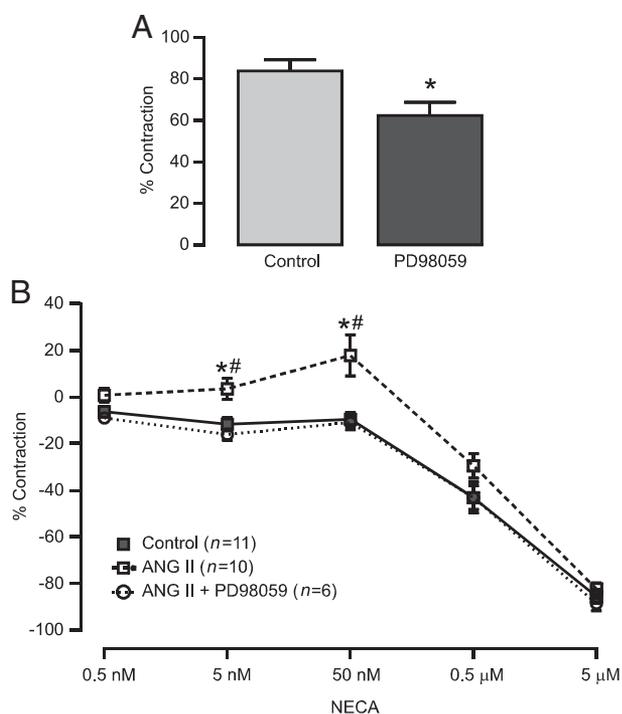


Figure 5

ERK1/2 inhibition reduces angiotensin (ANG) II-induced vasoconstriction and altered responses to NECA in MAs from WT mice. (A) PD98059 (1 μ M), an ERK1/2 inhibitor, significantly reduced ANG II (10 nM)-induced vasoconstrictions (control $n=10$, PD98059 $n=6$; * $P < 0.05$ compared with control). (B) Altered response to NECA after ANG II stimulation was also blocked by PD98059. * $P < 0.05$ compared with control NECA response, and # $P < 0.05$ compared with response after PD98059 treatment.

(Figure 5B). Taken together, these data show that the enhanced vasoconstriction to NECA after angiotensin II treatment occurs through activation of the A_1 receptor and involves signalling through CYP4A and ERK1/2.

A_{1A} and A_{2B} receptors regulate angiotensin II- and NECA-induced vasoconstriction in MAs

To determine the role of the A_{2B} receptor and substantiate the role of the A_1 receptor in angiotensin II-induced vasoconstriction and the altered responses to NECA, we performed muscle tension measurements in MAs obtained from A_{2B} receptor KO mice. We observed that angiotensin II-induced vasoconstriction in MAs from A_{2B} KO mice was significantly higher than that from WT mice (Figure 6A). Furthermore, DPCPX (1 μ M) and HET0016 (10 μ M) (Figure 6B and C, respectively) significantly reduced angiotensin II-induced vasoconstriction. Western blot analysis of MAs revealed that the expression of AT_1 receptors and CYP4A was higher in A_{2B} receptor KO mice compared with WT mice (Figure 7A and B). In contrast to observations in WT mice, NECA alone (without angiotensin II pretreatment) induced a vasoconstrictor response in A_{2B} receptor KO mice, suggesting un-opposed A_1 receptor activation. Similar to WT mice, acute angiotensin II stimulation potentiated NECA-induced vasoconstriction

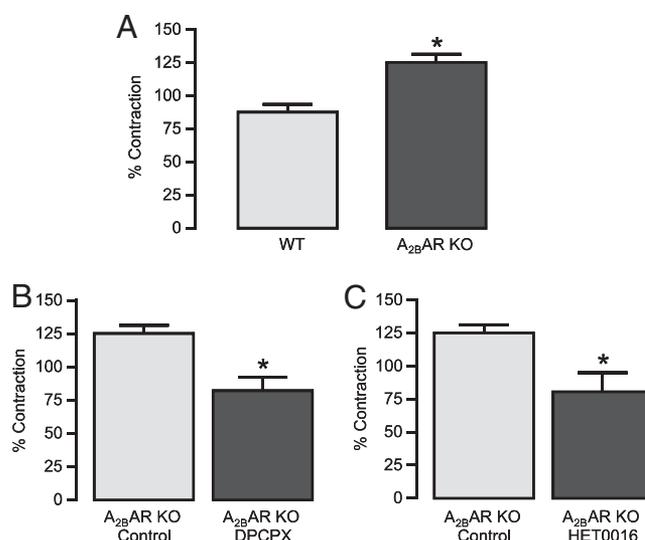


Figure 6

Angiotensin (ANG) II-induced vasoconstrictions in MAs obtained from A_{2B} receptor (A_{2B} AR) KO mice. (A) Significantly higher vasoconstrictions to ANG II were observed in MAs from A_{2B} receptor KO mice (WT $n=10$, A_{2B} AR KO $n=6$); (B) DPCPX (1 μ M) significantly reduced ANG II-induced vasoconstrictions (control $n=6$, DPCPX $n=6$), and (C) HET0016 (10 μ M) significantly attenuated ANG II-induced vasoconstrictions (control $n=6$, HET0016 $n=8$). * $P < 0.05$ compared with respective controls.

(Figure 8). Pre-incubation of A_{2B} receptor KO MAs with DPCPX (1 μ M, 15 min) completely abolished NECA-induced vasoconstriction (Figure 8). The CYP4A inhibitor HET0016 (10 μ M) partially but not significantly attenuated the enhanced vasoconstriction to NECA following angiotensin II stimulation (Figure 8). These data suggest that A_{2B} receptor expression limits NECA-induced contraction by (1) reducing AT_1 receptor and CYP4A expression and by (2) providing an opposing vasodilator signalling pathway in vascular smooth muscle.

Angiotensin II stimulation inhibits A_{2B} receptor-dependent vasodilatation in MAs

To determine whether angiotensin II stimulation inhibits NECA-induced vasodilatation, we examined MAs obtained from A_1 receptor KO mice. As shown in Figure 9A, acute angiotensin II stimulation significantly reduced NECA-induced vasodilatation in MAs from A_1 receptor KO mice. Notably, the PE-induced vasoconstriction observed in control and angiotensin II-stimulated MAs were 181.5 ± 26.39 and 212.3 ± 28.72 (mg tension). One potential mechanism for these effects of angiotensin II is through the inhibition of K_{ATP} channels. To test this hypothesis, we examined NECA-induced responses in MAs pre-incubated with the K_{ATP} channel blocker glibenclamide (1 μ M). As shown in Figure 9B, glibenclamide significantly attenuated NECA-induced vasodilatation in MAs from WT mice.

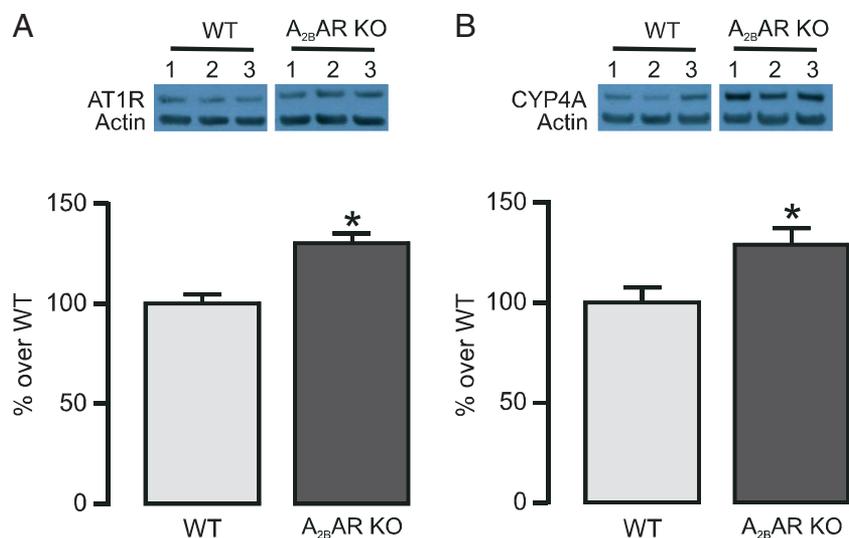


Figure 7

Western blot analysis of AT₁ receptors (AT1R) and CYP4A in MAs from WT and A_{2B} receptor (A_{2B}AR) KO mice. Top panel represents the expression of AT₁ receptors (A) and CYP4A (B) in MAs from three mice. Bottom panel summarizes the quantification of data obtained from six mice in each group. * $P < 0.05$ compared with WT.

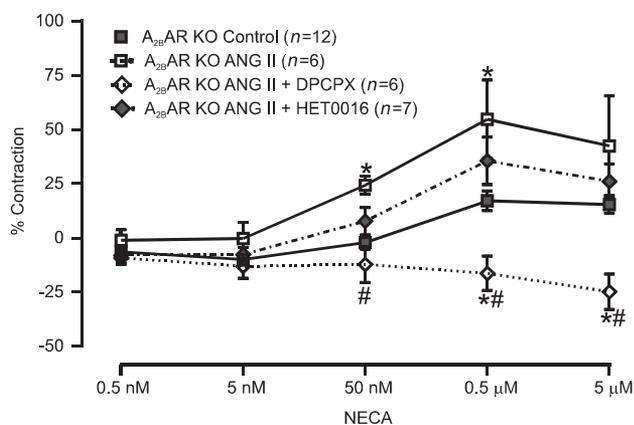


Figure 8

NECA-induced responses were altered by angiotensin (ANG) II stimulation in MAs from A_{2B} receptor KO mice. ANG II stimulation altered NECA-induced responses in MAs. These altered responses were completely abolished when MAs were pretreated (15 min) with DPCPX (1 μM) and partly reduced by HET0016 (10 μM). * $P < 0.05$ compared with control NECA response. # $P < 0.05$ compared with ANG II-stimulated responses.

Discussion

In the present study, we found that acute angiotensin II stimulation significantly altered adenosine receptor-dependent vascular tone in MAs. Angiotensin II pretreatment of MAs resulted in the response to NECA being vasoconstriction. This vasoconstrictor response was A₁ receptor-dependent and mediated by CYP4A and ERK1/2. Angiotensin II stimulation also inhibited the A_{2B} receptor-dependent vasodilatation. These data suggest an intricate relationship between

adenosine receptors and angiotensin II-induced vascular tone regulation in MAs.

Angiotensin II is a potent vasoconstrictor; we observed concentration-dependent vasoconstriction in mouse isolated MAs (Figure 1). Angiotensin II receptors are rapidly desensitized, and the muscle tension generated diminishes in whole animals, isolated vessels and in cultured cells (Abdellatif *et al.*, 1991; Sasamura *et al.*, 1994; Paul *et al.*, 2006). Similarly, angiotensin II-induced vasoconstriction rapidly diminished in MAs (Figure 1A). Evidently, angiotensin II activates CYP4A-dependent metabolism of arachidonic acid into 20-HETE, which constricts blood vessels. In addition, CYP4A contributes to the development of angiotensin II-dependent hypertension (McGiff and Laniado-Schwartzman, 1988; Harder *et al.*, 1994; Haller *et al.*, 1996; McGiff *et al.*, 1996; Muthalif *et al.*, 1998; 2000; Zaman *et al.*, 2002). PLC, 20-HETE and ERK1/2 pathways are involved in the regulation of vascular tone by adenosine through the activation of A₁ receptors (Rogel *et al.*, 2005; Jacobson and Gao, 2006; Ponnath *et al.*, 2012a; Kunduri *et al.*, 2013a; Kunduri *et al.*, 2013b). Thus, we believe that acute angiotensin II stimulation may alter adenosine receptor-dependent signalling, establishing an important role for adenosine in the regulation of vascular tone in MAs. We found that stimulation of MAs with angiotensin II uncovered a NECA-induced vasoconstriction and inhibited NECA-induced vasodilatation (Figure 2A, B and C). After angiotensin II stimulation, at low concentrations, NECA produced significant vasoconstriction that was not observed in NECA-treated MAs that were not treated with angiotensin II. We found that altered responses to NECA after angiotensin II stimulation were due to the stimulation of AT₁ receptors (Figure 2E). Importantly, responses to a series of increasing concentrations of NECA were identical (Figure 2D), suggesting that there is no desensitization of the NECA response (Figure 2D).

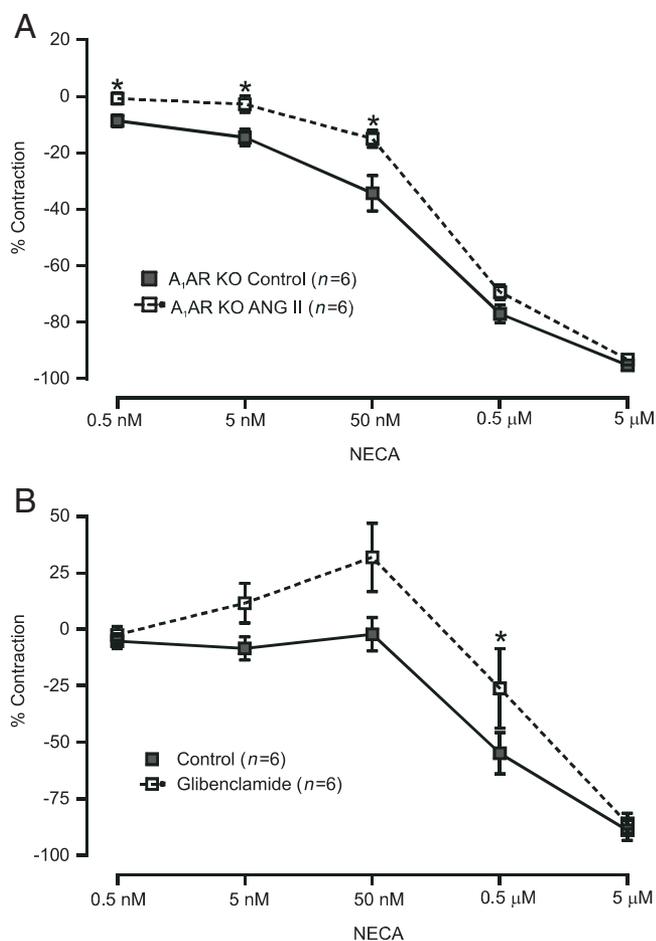


Figure 9

Acute angiotensin (ANG) II stimulation inhibits NECA-induced vasodilation in MAs. (A) We found that after ANG II stimulation, NECA-induced vasodilator responses were partly but significantly inhibited in MAs obtained from A₁ receptor KO mice. (B) In MAs from WT mice, the K_{ATP} channel blocker, glibenclamide (1 μM), significantly reduced NECA-induced vasodilation. **P* < 0.05 compared with control.

Adenosine receptors are present on endothelium as well as smooth muscle cells of arteries (Ohisalo, 1987; Mubagwa *et al.*, 1996; Mubagwa and Flameng, 2001; Jacobson and Gao, 2006; Hein *et al.*, 2013). Thus, we determined the role of the endothelium in NECA-induced vascular responses and subsequent effects of acute angiotensin II stimulation. We found that NECA-induced vasodilation was partially endothelium-dependent (Figure 3A). Furthermore, in the absence of endothelium, angiotensin II stimulation inhibited NECA-induced vasodilation (Figure 3B). In addition, no vasoconstriction to NECA was noted in endothelium-denuded vessels after angiotensin II stimulation (Figures 2C and 3B). Previously, in mouse aorta, we demonstrated that the endothelium mediates A₃ receptor-dependent vasoconstriction through the generation of TXB₂ by COX-1 (Ansari *et al.*, 2007). Similarly, no vasoconstriction to adenosine receptor stimulation was observed in endothelium-denuded mouse aorta (Nayeem *et al.*, 2008; Ponnoth *et al.*, 2012b). Our data suggest that the endothelium plays a role in A₁

receptor-dependent vasoconstriction in MAs, which involves the activation of CYP4A possibly through the generation of 20-HETE (Figures 3B and 4C). Overall, acute angiotensin II stimulation of AT₁ receptors alters the adenosine receptor-dependent regulation of vascular tone in MAs.

In the quest to identify the mechanism for the altered responses to NECA induced by angiotensin II, we first investigated the mechanism of angiotensin II-induced vasoconstriction in MAs. We found that angiotensin II-induced vasoconstriction in MAs involves the A₁ receptor (Figure 4A) and CYP4A (Figure 4B). Angiotensin II-induced vasoconstriction has been shown to be mediated through CYP4A activation, leading to 20-HETE production, resulting in MAPK activation (McGiff and Laniado-Schwartzman, 1988; Harder *et al.*, 1994; Haller *et al.*, 1996; McGiff *et al.*, 1996; Muthalif *et al.*, 1998; 2000; Zaman *et al.*, 2002). Our data are in agreement with these findings. However, inhibition of angiotensin II-induced vasoconstriction by an A₁ receptor antagonist was intriguing and needs further investigation. Possibly, inhibition of A₁ receptors by DPCPX, in part, blocks further downstream activation of CYP4A, PLC and ERK1/2, which mediates angiotensin II-induced vasoconstriction. Another possibility is that DPCPX may directly inhibit angiotensin II receptors. Notably, our data revealed that enhanced vasoconstriction to NECA after angiotensin II pretreatment was A₁ receptor-dependent (Figure 4C). Furthermore, CYP4A plays an important role in the enhanced vasoconstriction to NECA observed in MAs after angiotensin II pretreatment (Figure 4C). In addition, our data revealed that angiotensin II-induced vasoconstriction and altered responses to NECA in MAs were ERK1/2 dependent (Figures 5A and B). Evidently, the constrictor effect of A₁ receptors was enhanced in the presence of angiotensin II, as mentioned previously (Lai *et al.*, 2006). Moreover, in chronic angiotensin II and L-NAME hypertension mouse models, genetic deletion of A₁ receptors has been shown to attenuate arteriolar and BP responses (Gao *et al.*, 2011; Lee *et al.*, 2012). Previously, we demonstrated that CYP4A and ERK1/2 are involved in the A₁ receptor-dependent contractile response in mouse aorta (Ponnoth *et al.*, 2012a; Kunduri *et al.*, 2013b; Kunduri *et al.*, 2013a). In agreement, our data revealed that enhanced vasoconstriction to NECA in MAs following angiotensin II stimulation was completely dependent on the A₁ receptor and mediated through CYP4A and ERK1/2 (Figures 4 and 5).

We further investigated the role of A₁ARs in altered NECA-induced responses using isolated MAs from A_{2B}AR KO mice. Previously, we demonstrated that in MAs, the A₁ receptor regulates vasoconstriction, while the A_{2B} receptor regulates vasodilation (Teng *et al.*, 2013). In addition, the expression of A₁ receptors was higher in MAs obtained from A_{2B} receptor KO mice (Teng *et al.*, 2013). We believe that the absence of A_{2B} receptors may potentiate the contractile responses to NECA or angiotensin II. As expected, we found that angiotensin II-induced vasoconstriction was higher in MAs obtained from A_{2B} receptor KO mice (Figure 6A), possibly due to enhanced signalling through AT₁ receptors and CYP4A, as protein expression of these proteins was greater in MAs from A_{2B} receptor KO mice (Figure 7A and B). Angiotensin II-induced vasoconstriction was reduced by A₁ receptor and CYP4A inhibitors (Figure 6B and C). Thus, our data suggest an intricate regulation of angiotensin II-induced contraction through A_{2B}

receptors. As the A_{2B} receptor mediates vasodilatation in MAs (Teng *et al.*, 2013), we found no vasodilatation to NECA in MAs obtained from A_{2B} receptor KO mice (Figure 8). Furthermore, the vasoconstriction to NECA was potentiated after stimulation of MAs with angiotensin II (Figure 8). Importantly, the angiotensin II-induced increased vasoconstrictor responses to NECA were abolished by the A_1 receptor antagonist DPCPX and partially reduced by CYP4A inhibition (Figure 8). The elevated expression of CYP4A (Figure 7B) may account for the lack of inhibition of NECA-induced vasoconstriction after angiotensin II stimulation by HET0016. Notably, our data suggest the possibility that an absence of A_{2B} receptors could also potentiate the vasoconstrictor response to angiotensin II stimulation in MAs.

Recently, enhanced A_{2B} receptor-dependent production of endothelin-1 in the kidneys was implicated in angiotensin II-induced hypertension in mice, while no change in endothelin receptors was reported (Zhang *et al.*, 2013). In addition, angiotensin II led to enhanced production of adenosine in the kidneys whereas the blood levels of adenosine were unchanged (Zhang *et al.*, 2013). However, no vascular reactivity data were recorded in this study. In the scenario of unchanged levels of adenosine in the blood, altered responses to angiotensin II-induced stimulation of adenosine receptors may contribute to the regulation of BP in angiotensin II-induced hypertension. An absence of A_{2B} receptors enhances angiotensin II-induced vasoconstriction in MAs (Figure 6A), whereas it suppresses the production of endothelin-1, a potent vasoconstrictor, from kidneys in angiotensin II-induced hypertension in mice (Zhang *et al.*, 2013). This complicated relationship between adenosine receptors and angiotensin II signalling warrants further detailed investigations, as it is involved in the regulation of BP in chronic hypertension models.

After establishing the role of the A_1 receptor in vasoconstriction, we further determined the role of A_{2B} receptors in the vasodilator response to NECA in MAs pretreated with angiotensin II. To address this, we performed muscle tension studies in MAs obtained from A_1 receptor KO mice. According to our previous findings, A_{2B} receptor expression was higher in MAs obtained from A_1 receptor KO mice. In addition, the vasodilator response to NECA was increased in MAs from A_1 receptor KO mice (Teng *et al.*, 2013). Similarly, an increased vasodilator response to NECA was observed in the present study (data not shown). Furthermore, acute angiotensin II stimulation significantly inhibited this NECA-induced vasodilatation in MAs obtained from A_1 receptor KO mice (Figure 9A). MAs lack functional A_{2A} receptors, which have previously been shown to mediate vasodilatation (Teng *et al.*, 2013). Previously, we demonstrated that A_{2A} receptor-dependent vasodilatation of aorta occurred through the activation of K_{ATP} channels (Ponnoth *et al.*, 2012b). In addition, K_{ATP} channels are involved in A_{2A} receptor-dependent vasodilatation in coronary reactive hyperaemia (Sharifi-Sanjani *et al.*, 2013; Zhou *et al.*, 2013). Interestingly, angiotensin II inhibits K_{ATP} channels of rat mesenteric arterial smooth muscle cells (Hayabuchi *et al.*, 2001) and aortic smooth muscle cells (Sampson *et al.*, 2007). Also, A_{2B} receptor-dependent activation of K_{ATP} channels has been implicated in the regulation of coronary flow (Berwick *et al.*, 2010). Similarly, we found that inhibition of K_{ATP} channels

produces vasoconstriction to NECA in MA from WT mice (Figure 9B). Probably, inhibition of K_{ATP} channels by angiotensin II may contribute to the inhibition of NECA-induced dilation, enhancing the vasoconstrictor response after angiotensin II stimulation in MA.

In conclusion, our data reveal that acute angiotensin II stimulation potentiates endothelium- and A_1 receptor-dependent vasoconstriction mediated by CYP4A and ERK1/2 in MAs. Angiotensin II also inhibited the A_{2B} receptor-dependent vasodilatation, thus augmenting the vasoconstrictor response to NECA. In addition, the expression of AT_1 receptor and CYP4A was increased and the contraction to angiotensin II enhanced in MAs from A_{2B} receptor KO mice. Thus, a complex interaction exists between adenosine receptors and angiotensin II signalling pathways, which contribute to an altered vascular tone in MAs, and this interaction may be important in the regulation of BP. Findings from the current study suggest that adenosine may regulate the angiotensin II-mediated altered vascular tone and potentially contribute to BP regulation in angiotensin II-dependent hypertension. Further studies are needed to investigate the role of A_1 and A_{2B} receptors in the regulation of vascular tone contributing to high BP in murine models of chronic angiotensin II-induced hypertension so as to substantiate the involvement of adenosine receptors in angiotensin II-dependent hypertension. In addition, determining the various factors involved and delineating their mechanisms will provide novel therapeutic targets for the control of BP in angiotensin II-induced hypertension.

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Author contributions

V.R.Y., M.A.N. and S.J.M. conceived and designed the research; S.L.T. provided the KO mice; V.R.Y. performed the experiments and analysed the data; V.R.Y. drafted the manuscript; V.R.Y., M.A.N., S.J.M. and S.L.T. revised and edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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