NADPH oxidase pathway is involved in aortic contraction induced by A3 adenosine receptor in mice


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Running Title Page:
Adenosine A3 receptors and NADPH oxidase

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List of Non-Standard Abbreviations: Adenosine receptors (ARs), NADPH oxidases (Nox), reactive oxygen species (ROS), A3 adenosine receptors (A3AR), A3 adenosine receptors knockout (A3KO), wild type (WT), superoxide dismutase-polyethylene glycol (PEG-SOD), catalase-polyethylene glycol (PEG-Catalase), Krebs-Henseleit buffer (KH buffer), concentration-response curves (CRCs), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 2',7'-dichlorofluorescin (DCF), 3-Propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS1523), 2-Chloro-N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (CI-IBMECA), diphenyleneiodonium chloride (DPI), dimethyl sulfoxide (DMSO).

Recommended section assignment: Cardiovascular Pharmacology
Abstract

The NADPH oxidase (Nox) subunits 1, 2 (gp91 phox) and 4 are the major sources for reactive oxygen species (ROS) in vascular tissues. In conditions such as ischemia-reperfusion and hypoxia, both ROS and adenosine are released suggesting a possible interaction. Our aim in this study was to examine the A3 adenosine receptors (A3AR)-induced vascular effects and its relation to ROS and Nox1, 2 and 4 using aortic tissues from wild type (WT) and A3AR knockout (A3KO) mice. The selective A3AR agonist Cl-IBMECA (10^{-10} - 10^{-5} M) induced contraction of the aorta from WT but not from A3KO mice, and this contraction was inhibited by the Nox inhibitor apocynin (10^{-5} M) and the ROS scavengers superoxide dismutase-polyethylene glycol and catalase-polyethylene glycol PEG-(SOD+Catalase) (100 U/ml each). Cl-IBMECA-induced contraction was not affected by mast cell degranulator compound 48/80 (100 µg/ml) or stabilizer cromolyn sodium (10^{-4} M). In addition, Cl-IBMECA (10^{-7} M) increased intracellular ROS generation by 35±14% in WT but not in A3KO aorta and this increase was inhibited by apocynin (10^{-5} M), DPI (10^{-5} M) and the A3AR antagonist MRS1523 (10^{-5} M). Furthermore, Cl-IBMECA selectively increased the protein expression of Nox2 subunit by 150±15% in WT but not in A3KO mice without affecting either Nox1 or 4, and this increase was inhibited by apocynin. The mRNA of Nox2 was unchanged by Cl-IBMECA in either WT or A3KO aortas. In conclusion, A3AR enhances ROS generation, possibly through activating Nox2, with subsequent contraction of the mouse aorta.
Introduction

Adenosine is an autacoid that plays an important role in the regulation of cardiovascular functions. The cardiovascular effects of adenosine are mediated by activation of four well known cell surface receptors (A₁, A₂A, A₂B, A₃) (Mustafa, et al., 2009; Tabrizchi and Bedi, 2001). The role of adenosine receptors (ARs) in vascular contraction and relaxation has been studied in several species, with A₂AAR and A₂BAR showing vasorelaxant effects, while A₁AR showing vasoconstricting effects (Ansari, et al., 2007a; Ansari, et al., 2009; Tabrizchi and Bedi, 2001). However, the physiological role of A₃AR in vascular responses is not fully characterized, although its role in myocardial ischemia and reperfusion injury has been demonstrated (Maddock, et al., 2003; Zatta, et al., 2006). We previously demonstrated that activation of A₃AR leads to endothelium-dependant aortic contraction through cyclooxygenase-1 (COX-1) using A₃AR knockout (A₃KO) mice (Ansari, et al., 2007b). A₃AR has also been shown to inhibit or negatively modulate coronary flow in isolated mouse heart (Talukder, et al., 2002), causes vasoconstriction in hamster arterioles (Shepherd, et al., 1996) and reverses vascular hyporeactivity after hemorrhagic shock in rats (Zhou, et al., 2010).

NADPH oxidases (Nox) are the major source of ROS in the vasculature that play both physiological and pathophysiological roles in the control of vascular tone (Carlstrom, et al., 2009). The family of NADPH oxidases consists of seven members, Nox1–Nox5 and Doux1 and Doux2 (Schroder, 2010). Among these, Nox1, Nox2 and Nox4 are of relevance in the cardiovascular system. Nox5 is not expressed in rodents due to gene deletion (Kawahara, et al., 2007). Endothelial cells express Nox2 and Nox4 whereas VSMC express Nox1 and Nox4 (Cheng, et al., 2001; Gorlach, et al.,...
2000; Sorescu, et al., 2004). In conditions such as ischemia-reperfusion and hypoxia, both ROS and adenosine are released suggesting a possible interaction between them (Gebremedhin, et al., 2010; Zatta, et al., 2006). It is becoming increasingly clear that adenosine may exhibit some of its actions through modulating Nox activity (Carlstrom, et al., 2009; Jajoo, et al., 2009; Nadeem, et al., 2009; Ribe, et al., 2008).

The question arises whether A3AR-induced contraction in mouse aorta is mediated through ROS from Nox. Our data indicate that A3AR activation using Cl-IBMECA leads to aortic contraction in wild type (WT) but not in A3AR knockout (A3KO) mice. In addition, Cl-IBMECA induced intracellular ROS generation through selective activation of Nox2 subunit in WT but not in A3KO mice without affecting Nox1 or 4. Nox inhibitor apocynin inhibited A3AR-induced aortic contraction, ROS generation and Nox2 activation in the aorta of WT mice but not in A3KO mice.
Methods

All of the experimental protocols were performed according to the guidelines and approval of the Animal Care and Use Committee at West Virginia University. A3KO (male/female) mice were generated, as previously described (Salvatore, et al., 2000) and backcrossed 12 generations to the C57 BL/6 background. The corresponding WT (C57 BL/6) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). A3KO and their corresponding WT mice (10-12 weeks old) were used in this study.

Preparation of isolated mouse aorta

The mice were sacrificed by deep anesthesia with pentobarbital sodium (65 mg/kg ip) followed by thoracotomy. The aorta was gently removed and cleaned of fat and connective tissue. The aorta was cut transversely into four rings that measured 3–4 mm in length with extreme care to avoid damaging the endothelium. The rings were mounted vertically between two wire hooks and then suspended in 10 ml organ baths containing Krebs-Henseleit buffer (KH buffer, pH 7.4) of the following composition: 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 11 mM glucose, and 2.5 mM CaCl2. The KH buffer was maintained at 37°C with continuous bubbling of 95% O2 and 5% CO2. The aortic rings were equilibrated for 90 min with a resting force of 1 g, with changes of the bathing solution at 15-min interval. The changes in isometric tension were monitored continuously with a fixed range precision force transducer (TSD, 125 C, BIOPAC system, Santa Barbara, CA) connected to a differential amplifier (DA 100B, BIOPAC system). The data were recorded on a digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC system).
At the end of the equilibration period, aortic rings were contracted with 50 mM KCl to check their viability. The tissues were then contracted with phenylephrine (PE, 10^{-7} M) to produce consistent submaximal (~90%) response in our experiments. The aortic rings were then washed several times with KH buffer and allowed to equilibrate for 30 min before the experimental protocol began.

**Experimental protocol**

All experiments were performed in endothelium-intact aortic rings, since we previously shown that activation of A3AR leads to endothelium-dependant aortic contraction due to the higher expression of A3AR in endothelium compared to aortic smooth muscle in mouse (Ansari, et al., 2007b). The cumulative concentration-response curves (CRCs) for the selective A3AR agonist Cl-IBMECA (10^{-10}-10^{-5} M) were run in parallel in aortic rings from both WT and A3KO mice. Inhibitors and antagonist (apocynin, 10^{-5} M; DPI, 10^{-5} M; MRS 1523, 10^{-5} M; superoxide dismutase–polyethylene glycol (PEG-SOD) and catalase-polyethylene glycol (PEG-catalase), 100 U/ml each; cromolyn sodium, 10^{-4} M; compound 48/80, 100 µg/ml) were added 30 min prior to aortic contraction with PE and were present throughout the experiments.

**Reactive oxygen species (ROS) generation in mouse aorta**

For reactive oxygen species generation, mice aortic tissues were cut in to 3-4mm length and were first pre-incubated for 75 min at 37°C in 1 ml of KH buffer. After this incubation, the aortic tissues were incubated with 100 µM 2',7'-dichlorofluorescin diacetate (DCFH-DA) for 30 min at 37°C. DCFH-DA forms a fluorescent product,
2′,7′-dichlorofluorescin (DCF) intracellularly upon oxidation with ROS (Wang and Joseph, 1999). Fluorescence caused by DCF in each well was measured and recorded for 15 min at 485 nm (excitation) and 530 nm (emission) using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, Vermont) with temperature maintained at 37°C. The background fluorescence caused by buffer and DCF were subtracted from the total fluorescence in each well caused by aortic rings in the presence of DCF. Fluorescence intensity units were then normalized by mg of wet weight tissue for each aortic ring and expressed as arbitrary fluorescence units/mg tissue.

**Immunoblotting of Nox1, 2 and 4 in mouse aorta**

The aortic tissues from both WT and A3KO mice were processed similar to the organ bath experiments and then incubated in the absence or presence of Nox inhibitor, apocynin (10⁻⁵ M) for 30 min prior to treatment with CI-IBMECA (10⁻⁷ M) for 10 min. The aortic tissues were homogenized with six volumes of ice-cold tissue lysis buffer consisting of 0.05 M Tris-buffered saline (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride. Homogenized samples were centrifuged for 30 min at 12,000g at 4°C. The protein content of the supernatant was determined using Bradford protein assay (BioRad, Hercules, CA).

Aliquots of the aortic lysates (40 µg protein/well) were separated on 10% SDS-PAGE. Prestained protein molecular markers (20–112 kDa) were run in parallel. Proteins were transferred to nitrocellulose membranes then incubated with 5% milk for 1
h to block non-specific binding sites. Membranes were then probed with either anti-gp91 phox (anti-Nox2) mouse monoclonal IgG (BD Biosciences, San Jose, CA) or anti-Nox4 rabbit polyclonal IgG (Abcam, Cambridge, MA) at a dilution of 1:1000, or anti-Nox1 rabbit polyclonal IgG (Abcam) at a dilution of 1:500, followed by incubation with secondary antibodies for 1 h (horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit at 1:10,000 dilution (Santa Cruz Biotechnology). After extensive washing, membranes were then stripped and reprobed with monoclonal anti-β-actin antibody (Santa Cruz Biotechnology) at a dilution of 1:10000. For detection of bands, the membranes were treated with enhanced chemiluminescence plus (for Nox2, Nox4 and β-actin) or advance (for Nox1) kit (GE Healthcare, Buckinghamshire, UK) for 1 min and subsequently exposed to ECL Hyperfilm. Relative band intensities were quantified by densitometry (ImageJ 1.43u, NIH), and each sample was normalized to the β-actin values. Western blot values are expressed as percentage of control after densitometric analysis.

Real-Time PCR for Nox2 in mouse aorta

Mouse aortic rings were treated as previously described, and then tissues were snap-frozen in liquid nitrogen and kept at -80°C. Total RNA was isolated from the aortic rings by using the TRI reagent (MRC Inc., Cincinnati, OH) followed by purification of the RNA in aqueous phase and removal of genomic DNA by RNeasy Plus Micro kit (Quiagen, Hilden, Germany). This was followed by conversion of 0.5 µg of total RNA into cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions in a total volume of 20 µl. Real-time
PCR was performed on ABI PRISM 7300 Detection System (Applied Biosystems) using Taqman Universal Master Mix (Applied Biosystems). In brief, the reaction volume (20 µl) included 10 µl of 2X Taqman Universal Mastermix, 4 µl of cDNA, and 1 µl 20X FAM-labeled Taqman gene expression assay. Taqman inventoried assays-on-demand gene expression products were purchased from Applied Biosystems. Mm01287743_m1 assay was used for Nox2 gene. Hs999999_s1 assay was used for Ribosomal RNA (18S rRNA) as an endogenous control. The fold difference in expression of target cDNA was determined by using the comparative CT method. The fold difference in gene expression of the target was calculated as described previously (Livak and Schmittgen, 2001).

Drugs used

Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma-Aldrich (St. Louis, MO). DCFH-DA, MRS1523, Cl-IBMECA and DPI were dissolved in dimethyl sulfoxide (DMSO), whereas apocynin, acetylcholine, PE, PEG-SOD and PEG-catalase were dissolved in distilled water. DMSO final concentration in organ bath had no effect by itself on the aortic rings.

Data analysis

All of the experimental values are presented as means ± SE (n = number of animals). The comparison among different groups was analyzed by ANOVA followed by Tukey’s multiple comparisons test method as a post hoc test. Comparison between two groups was assessed by unpaired t-test. P < 0.05 was taken as significant. EC50 for aortic contraction was obtained from individual curves by nonlinear regression (curve-fit)
graphic analysis. All of the statistical analyses were performed using GraphPad Prism statistical package (version 3.0, GraphPad Software, San Diego, CA).
Results

Effect of A3AR activation on contractility of WT/A3KO mice aorta

Incubation of WT aortic rings to increasing concentration of the selective A3AR agonist Cl-IBMECA produced a concentration-dependent contraction with a pEC_{50} of 8.22± 0.27 (Figure 1A and 1B). The contraction induced by Cl-IBMECA was insignificant in A3KO aortic rings. At 10^{-7} M, Cl-IBMECA increased contraction significantly by 25% in WT, while producing negligible effect (~ 6%) in A3KO mouse aorta (Figure 1A). The contraction induced by Cl-IBMECA in WT aortic rings was significantly inhibited by preincubation with the NOX inhibitor, apocynin (10^{-5} M). These results suggest that A3AR-mediated contraction is dependent on Nox activation in mouse aorta.

To further test the involvement of the ROS in the contraction induced by A3AR activation, PEG-SOD and PEG-catalase as ROS scavengers were tested against Cl-IBMECA-induced contraction (Figure 1C). Preincubation with PEG-(SOD+catalase) inhibited the contraction induced by Cl-IBMECA in WT aortic rings without affecting that of the A3KO, indicating that A3AR activation causes contraction through the release of ROS.

The role of mast cells in Cl-IBMECA-mediated contraction was also investigated. Preincubation of aortic rings with compound 48/80, a mast cell degranulator, or cromolyn sodium, a mast cell stabilizer, did not affect the baseline or Cl-IBMECA-induced contraction (data not shown). These data suggest that mast cells may not play a significant role in either controlling aortic tone or A3AR-mediated contraction in this investigation.
Effect of A3AR activation on ROS generation from WT/A3KO mice aorta

Incubation of WT aortic rings with $10^{-7}$ M Cl-IBMECA increased the amount of intracellular ROS production in WT by 35±14% compared with corresponding A3KO aortae (Figure 2A). In contrast, Cl-IBMECA did not increase ROS production in A3KO aorta. Preincubation with Nox inhibitors apocynin ($10^{-5}$ M) or DPI ($10^{-5}$ M) prevented this increase in ROS production (Figure 2A), indicating that the source of ROS activated by Cl-IBMECA is Nox. Similarly, preincubation with the selective A3AR inhibitor MRS-1523 ($10^{-7}$ M) leads to inhibition of ROS production induced by Cl-IBMECA (Figure 2B) confirming that A3AR activation induces ROS production through Nox.

Effects of A3AR activation on Nox1, 2 and 4 protein expression in WT/A3KO mice aorta

For further confirmation of the role of Nox in the contraction and ROS generation induced by A3AR activation, the protein expression levels of Nox1, 2 and 4 subunits were examined. Activation of A3AR with Cl-IBMECA ($10^{-7}$ M) selectively increased the expression of Nox2 in WT aorta by 150±15% compared to control (Figure 3A). This increase in Nox2 expression was inhibited by preincubation with apocynin ($10^{-5}$ M), while neither Cl-IBMECA alone nor with apocynin had an effect on Nox2 expression in A3KO mice aorta (Figure 3B). Conversely, Cl-IBMECA ($10^{-7}$ M) had no effect on the expression of Nox1 (Figure 4A and 4B) or Nox4 (Figure 4C and 4D) either in WT or in A3KO mice aorta. These data confirm the selective activation of Nox2 subunit by A3AR leading to ROS generation and contraction in mice aorta.
Effects of A3AR activation on Nox2 mRNA expression in WT/A3KO mice aorta

Since Nox2 protein expression was selectively increased by A3AR activation in WT but not in A3KO mice aorta, we examined the change of Nox2 mRNA expression induced by Cl-IBMECA. Interestingly, the mRNA expression of Nox2 was not affected by Cl-IBMECA in either WT or A3KO mice aorta (data not shown), suggesting that A3AR activation enhances Nox2 protein expression at a post-transcriptional level.
Discussion

This is the first study to show that NADPH oxidases (Nox) are involved in A$_3$AR-induced contraction of mouse aorta. However, other studies have shown a relationship between adenosine receptors and Nox in other tissues (Carlstrom, et al., 2009; Gebremedhin, et al., 2010), but not in aorta. Our findings show that A$_3$AR activation using the selective agonist Cl-IBMECA causes contraction of aorta in WT mice but not in A$_3$KO mice. This A$_3$AR-mediated contraction of aorta was inhibited by Nox inhibitor (apocynin), in addition to ROS scavengers PEG-SOD and PEG-catalase, and was not affected by mast cell degranulator compound 48/80 or stabilizer cromolyn sodium. We also found that A$_3$AR activation leads to increased intracellular ROS generation which was inhibited by apocynin, DPI and the selective A$_3$AR antagonist, MRS1523. Furthermore, the protein expression of Nox2 subunit was selectively increased by Cl-IBMECA in WT but not in A$_3$KO mice aorta without affecting either Nox1 or 4.

We used the pharmacological selective A$_3$AR agonist Cl-IBMECA, in addition to the A$_3$KO mice to confirm that A$_3$AR activation induces aortic contraction in WT mice. The role of A$_3$AR in the control of vascular tone has also been demonstrated by our lab (Ansari, et al., 2007b). In another study, infusion of adenosine in A$_3$KO mice has been shown to cause a significant decrease in blood pressure compared to WT mice (Zhao, et al., 2000). In addition, the effects of A$_3$AR-activation on vascular tone has also been demonstrated through inhibition or negative modulation of coronary flow in isolated mouse heart (Talukder, et al., 2002), vasoconstriction in hamster arterioles (Shepherd, et
al., 1996) and reversal of vascular hyporeactivity after hemorrhagic shock in rats (Zhou, et al., 2010).

It should be noted that mast cells can be stimulated by A3AR to release histamine and thromboxane, leading to either vasoconstriction (Shepherd, et al., 1996), or a short-lasting hypotension in conscious rats (Van Schaick, et al., 1996). Our data shows that neither mast cell degranulator (compound 48/80) or stabilizer (cromolyn sodium) affected A3AR-induced contraction, suggesting that mast cells may not play a significant role in our model.

In the present work, apocynin significantly reduced the contraction induced by Cl-IBMECA, suggesting that A3AR-induced contraction in mouse aorta involves Nox. We have shown previously that COX-1 plays a role in this A3AR response (Ansari, et al., 2007b), therefore it is likely that there may be a relationship between Nox and COX-1. Nox activity has been shown to be activated through arachidonic acid pathway in cardiac fibroblasts (Colston, et al., 2005). In addition, ROS derived from Nox can induce COX-2 protein in human neutrophils (Vega, et al., 2006), suggesting an interaction between Nox and COX pathways. Taken together, these data along with our data indicate that A3AR may play an important role in the regulation of vascular tone, mainly through endothelium-dependent pathways.

A3ARs have been shown to be involved in the modulation of diseases involving ROS generation such as ischemia-reperfusion injury (Maddock, et al., 2003; Zatta, et al., 2006), suggesting that A3AR effects may be related to ROS generation. It has been shown that adenosine constricts renal arterioles of WT but not Nox(-/-)KO mice (Carlstrom, et al., 2009), while its cerebral vasodilation in rat involves Nox (Gebremedhin, et al., 2010).
Furthermore, A3ARs have been shown to modulate Nox activity in monocytes (Broussas, et al., 1999) and in prostate cancer cells (Jajoo, et al., 2009). Our results show that A3AR induces ROS production that was inhibited by apocynin and DPI. In addition, Cl-IBMECA induced selective protein expression of Nox2 subunit only in WT but not in A3KO animals without affecting Nox1 or 4, suggesting that A3AR induces ROS generation, possibly through the activation of Nox2.

We previously demonstrated that activation of A3AR leads to endothelium-dependant aortic contraction (Ansari, et al., 2007b). However, since Nox isoforms are expressed in both endothelium and vascular smooth muscle, therefore, further studies are needed to differentiate the role of each cell type in A3AR-induced ROS generation.

Interestingly, the protein expression of Nox2 was increased by Cl-IBMECA, but the mRNA was not affected, suggesting that activation of A3AR enhances Nox2 protein expression through post-transcriptional response, such as changes in protein translation and/or turnover. This may also partly explain the rapid change in Nox2 protein expression by short-time exposure to Cl-IBMECA. However, a direct correlation between this rapid change in Nox2 protein and vascular contraction requires further confirmation. Rapid changes in gene and protein expression patterns have been shown within minutes of tissue ischemia following surgical tumor excision (Spruessel, et al., 2004). In addition, up-regulation of different cellular proteins has been shown due to changes in the translational level or protein turnover without changes in mRNA expression (Burd, et al., 2008; Yoshimura, et al., 2008).

Vascular Nox are activated within minutes of stimulation (Seshiah, et al., 2002), with different species of ROS produced, mainly superoxide anions (O$_2^-$). This O$_2^-$ can
enhance vasoconstriction by rapidly converting nitric oxide (NO) to the much less vasodilator peroxynitrite (Koppenol, et al., 1992). The ROS indicator DCFH-DA used in this study is non-specific as it can detect several ROS such as $O_2^-$, hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (Biziukin, et al., 1995). However, since most Nox isoforms produce $O_2^-$ as a main ROS, therefore our data indicates that $A_3$AR possibly enhances $O_2^-$ production through Nox2, which may play an important role in the vasoconstriction induced by $A_3$AR in the mouse aorta. Further studies are needed to identify the various ROS produced by the activation of $A_3$AR.

In the literature, some concerns were raised about the selectivity of Nox inhibitors such as apocynin (Schluter, et al., 2008) and DPI (Stuehr, et al., 1991), which may affect the results obtained with these inhibitors in our study. Therefore, using these inhibitors solely may confer some arguments about their specific targets. To address this, our studies used not only these inhibitors, but also ROS generation and Nox subunits protein expression as tools to confirm the relationship between $A_3$AR and Nox.

Since ROS are involved in several diseases and $A_3$AR modulators have been tested in some diseases such as renal cancer (Jajoo, et al., 2009), therefore, a better understanding of the relationship between $A_3$AR and ROS generation, possibly through Nox, may result in potential therapeutic targets in cardiovascular pathophysiological situations involving higher oxidative stress. In addition, this study shows that Nox inhibitors can be used to attenuate the vasoconstrictor responses to $A_3$AR, therefore enhancing the vasodilator responses of adenosine through $A_{2A/B}$ AR (relaxing receptors) could be beneficial in conditions such as hypertension and coronary artery diseases.
In conclusion, A3AR activation induces contraction of the mouse aorta that is dependent on ROS generation, possibly mediated through Nox2.
Authorship contributions

Participated in research design: M. S. El-Awady, H. R. Ansari, and S. J. Mustafa.

Conducted Experiments: M. S. El-Awady, H. R. Ansari and D. Fil.

Contributed new reagents or analytical tools: S. L. Tilley.

Performed data analysis: M. S. El-Awady and H. R. Ansari.

Wrote or contributed to the writing of the manuscript: M. S. El-Awady, H. R. Ansari, and S. J. Mustafa.

Other: S. J. Mustafa is the principal investigator who acquired funding for this study.
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Pharmacol Sin* **31**:413-420.
Footnote

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Legends for Figures

Figure 1: Vascular responses to Cl-IBMECA in WT and A3KO mouse aorta. (A): Tracing showing the responses to Cl-IBMECA in WT and A3KO mouse aortic rings. (B): effect of apocynin on Cl-IBMECA-induced contraction in WT and A3KO mouse aorta. (C): effect of PEG-(SOD + Catalase) on Cl-IBMECA-induced contraction in WT and A3KO mouse aorta. Data are expressed as mean ± SEM (n=6).

* p<0.05 compared to A3 AR KO control group using one way ANOVA followed by Tukey multiple comparisons post hoc test.

Figure 2: Effects of apocynin and DPI (A) and MRS1523 (B) on Cl-IBMECA-induced ROS generation in WT and A3KO mouse aorta. Data are expressed as mean ± SEM (n=4). *p<0.05 compared to their corresponding WT control group using unpaired t-test.

Figure 3: Effects of Cl-IBMECA on protein expression of Nox2 subunit in WT (A) and A3KO (B) mouse aorta. Data are expressed as mean ± SEM (n=3). * p<0.05 compared to control; $ p< 0.05 compared to Cl-IBMECA alone using One way ANOVA followed by Tukey multiple comparisons post hoc test.

Figure 4: Effects of Cl-IBMECA on protein expression of Nox1 in WT (A), Nox1 in A3KO (B), Nox4 in WT (C) and Nox4 in A3KO (D) mouse aorta. Data are expressed as mean ± SEM (n=3).
Fig. 1

A

B

C
**A**

Control | CI-IBMECA (10^{-7}M) | Apocynin (10^{-5}M)+ CI-IBMECA (10^{-7}M)
--- | --- | ---
Nox2 | Nox2 | β-actin

**C-57**

Nox2 Protein expression (% of control)

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* $p < 0.05$

**B**

Control | CI-IBMECA (10^{-7}M) | Apocynin (10^{-5}M)+ CI-IBMECA (10^{-7}M)
--- | --- | ---
Nox2 | Nox2 | β-actin

**A_{3}KO**

Nox2 Protein expression (% of control)

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Fig. 4

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<th>Apocynin (10^{-5}M) + CHBMECA (10^{-7}M)</th>
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