

# NADPH Oxidase Pathway Is Involved in Aortic Contraction Induced by A<sub>3</sub> Adenosine Receptor in Mice

Mohammed S. El-Awady, Habib R. Ansari, Daniel Fil, Stephen L. Tilley, and S. Jamal Mustafa

Department of Physiology and Pharmacology and Center for Cardiovascular and Respiratory Sciences, West Virginia University, Morgantown, West Virginia (M.S.E., D.F., S.J.M.); Department of Biosciences, Jamia Millia Islamia, New Delhi, India (H.R.A.); and Department of Medicine, University of North Carolina, Chapel Hill, North Carolina (S.L.T.)

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## 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 A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR)-induced vascular effects and its relation to ROS and Nox1, 2, and 4 using aortic tissues from wild-type (WT) and A<sub>3</sub>AR knockout (A<sub>3</sub>KO) mice. The selective A<sub>3</sub>AR agonist 2-chloro-N<sup>6</sup>-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (Cl-IBMECA) (10<sup>-10</sup>–10<sup>-5</sup> M) induced contraction of the aorta from WT but not from A<sub>3</sub>KO mice, and this contraction was inhibited by the Nox inhibitor apocynin (10<sup>-5</sup> M) and the ROS scavengers superoxide dismutase-polyethylene glycol and catalase-polyethylene glycol (100 U/ml each). Cl-IBMECA-induced

contraction was not affected by the mast cell degranulator compound 48/80 (100 μg/ml) or the stabilizer cromolyn sodium (10<sup>-4</sup> M). In addition, Cl-IBMECA (10<sup>-7</sup> M) increased intracellular ROS generation by 35 ± 14% in WT but not in A<sub>3</sub>KO aorta, and this increase was inhibited by apocynin (10<sup>-5</sup> M), diphenyleiiodonium chloride (10<sup>-5</sup> M), and the A<sub>3</sub>AR antagonist 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate (MRS1523) (10<sup>-5</sup> M). Furthermore, Cl-IBMECA selectively increased the protein expression of the Nox2 subunit by 150 ± 15% in WT but not in A<sub>3</sub>KO 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 A<sub>3</sub>KO aortas. In conclusion, A<sub>3</sub>AR enhances ROS generation, possibly through activation of 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 (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>) (Tabrizchi and Bedi, 2001; Mustafa et al., 2009). The role of adenosine receptors (ARs) in vascular contraction and relaxation has been studied in several species, A<sub>2A</sub>AR and A<sub>2B</sub>AR showing vasorelaxant effects and A<sub>1</sub>AR showing vasoconstricting effects (Tabrizchi and Bedi, 2001; Ansari et al., 2007a, 2009). However, the physiological role of A<sub>3</sub>AR 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 A<sub>3</sub>AR leads to endothelium-dependent aortic contraction through cyclooxygenase-1 (COX-1) using A<sub>3</sub>AR knockout (A<sub>3</sub>KO) mice (Ansari et al., 2007b). A<sub>3</sub>AR has also been shown to inhibit or negatively modulate coronary flow in isolated mouse heart (Tallukder et al., 2002), to cause vasoconstriction in hamster arterioles (Shepherd et al., 1996), and to reverse vascular hyporeactivity after hemorrhagic shock in rats (Zhou et al., 2010).

NADPH oxidases (Nox) are the major source of reactive oxygen species (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 (Schröder, 2010). Among these, Nox1, Nox2, and Nox4 are of relevance in the cardiovascular system. Nox5 is not

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**ABBREVIATIONS:** AR, adenosine receptor; COX-1, cyclooxygenase-1; KO, knockout; Nox, NADPH oxidase(s); ROS, reactive oxygen species; Cl-IBMECA, 2-chloro-N<sup>6</sup>-(3-iodobenzyl)-adenosine-5'-N-methyluronamide; WT, wild type; A<sub>3</sub>KO, A<sub>3</sub>AR knockout; KH buffer, Krebs-Henseleit buffer; PE, phenylephrine; DPI, diphenyleiiodonium chloride; MRS1523, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate; PEG-SOD, superoxide dismutase-polyethylene glycol; PEG-catalase, catalase-polyethylene glycol; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; ANOVA, analysis of variance.

expressed in rodents because of gene deletion (Kawahara et al., 2007). Endothelial cells express Nox2 and Nox4, whereas vascular smooth muscle cells express Nox1 and Nox4 (Görlach et al., 2000; Cheng et al., 2001; Sorescu et al., 2004). In conditions such as ischemia-reperfusion and hypoxia, both ROS and adenosine are released, suggesting a possible interaction between them (Zatta et al., 2006; Gebremedhin et al., 2010). It is becoming increasingly clear that adenosine may exhibit some of its actions through modulation of Nox activity (Ribé et al., 2008; Carlstrom et al., 2009; Jajoo et al., 2009; Nadeem et al., 2009).

The question arises whether A<sub>3</sub>AR-induced contraction in mouse aorta is mediated through ROS from Nox. Our data indicate that A<sub>3</sub>AR activation using Cl-IBMECA leads to aortic contraction in wild-type (WT) but not in A<sub>3</sub>AR knock-out (A<sub>3</sub>KO) mice. In addition, Cl-IBMECA induced intracellular ROS generation through selective activation of the Nox2 subunit in WT but not in A<sub>3</sub>KO mice without affecting Nox1 or 4. The Nox inhibitor apocynin inhibited A<sub>3</sub>AR-induced aortic contraction, ROS generation, and Nox2 activation in the aorta of WT mice but not in A<sub>3</sub>KO mice.

## Materials and 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. A<sub>3</sub>KO (male/female) mice were generated, as described previously (Salvatore et al., 2000) and backcrossed 12 generations to the C57BL/6 background. The corresponding WT (C57BL/6) mice were purchased from Harlan (Indianapolis, IN). A<sub>3</sub>KO 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 i.p.) 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 to 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 MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 11 mM glucose, and 2.5 mM CaCl<sub>2</sub>. The KH buffer was maintained at 37°C with continuous bubbling of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The aortic rings were equilibrated for 90 min with a resting force of 1 g, with changes of the bathing solution at 15-min intervals. The changes in isometric tension were monitored continuously with a fixed-range precision force transducer (TSD, 125 C; BIOPAC Systems, Inc., Goleta, CA) connected to a differential amplifier (DA 100B; BIOPAC Systems, Inc.). The data were recorded on a digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC Systems, Inc.).

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<sup>-7</sup> 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, because we previously showed that activation of A<sub>3</sub>AR leads to endothelium-dependent aortic contraction because of the higher expression of A<sub>3</sub>AR in endothelium compared with that in aortic smooth muscle in mouse (Ansari et al., 2007b). The cumulative concentration-response curves for the selective A<sub>3</sub>AR agonist Cl-IBMECA (10<sup>-10</sup>–10<sup>-5</sup> M) were run in parallel in aortic rings from both WT and A<sub>3</sub>KO mice. Inhibitors and antagonists [apocynin, 10<sup>-5</sup> M; DPI, 10<sup>-5</sup> M; 3-propyl-6-ethyl-5-[(ethyl-

thio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS1523), 10<sup>-5</sup> M; superoxide dismutase-polyethylene glycol (PEG-SOD), and catalase-polyethylene glycol (PEG-catalase), 100 U/ml each; cromolyn sodium, 10<sup>-4</sup> M; and compound 48/80, 100 µg/ml] were added 30 min before aortic contraction with PE and were present throughout the experiments.

**ROS Generation in Mouse Aorta.** For reactive oxygen species generation, mouse aortic tissues were cut into 3- to 4-mm-lengths and were first preincubated 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'-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C. DCFH-DA forms a fluorescent product, 2',7'-dichlorofluorescein (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, Winooski, VT) with temperature maintained at 37°C. The background fluorescence caused by buffer and DCF was subtracted from the total fluorescence in each well caused by aortic rings in the presence of DCF. Fluorescence intensity units were then normalized by milligrams of wet weight tissue for each aortic ring and expressed as arbitrary fluorescence units per milligram of tissue.

**Immunoblotting of Nox1, 2, and 4 in Mouse Aorta.** The aortic tissues from both WT and A<sub>3</sub>KO mice were processed similar to the organ bath experiments and then incubated in the absence or presence of the Nox inhibitor apocynin (10<sup>-5</sup> M) for 30 min before treatment with Cl-IBMECA (10<sup>-7</sup> M) for 10 min.

The aortic tissues were homogenized with 6 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 a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Aliquots of the aortic lysates (40 µg of 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 and incubated with 5% milk for 1 h to block nonspecific 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, Inc., Santa Cruz, CA). After extensive washing, membranes were then stripped and reprobed with monoclonal anti-β-actin antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:10,000. For detection of bands, the membranes were treated with a ECL Plus (for Nox2, Nox4, and β-actin) or Advance (for Nox1) Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 1 min and subsequently exposed to ECL Hyperfilm. Relative band intensities were quantified by densitometry (ImageJ 1.43u; National Institutes of Health, Bethesda, MD), and each sample was normalized to the β-actin values. Western blot values are expressed as a percentage of control after densitometric analysis.

**Real-Time PCR for Nox2 in Mouse Aorta.** Mouse aortic rings were treated as described previously, and then tissues were snap-frozen in liquid nitrogen and kept at -80°C. Total RNA was isolated from the aortic rings by using TRI reagent (MRC Inc., Cincinnati, OH) followed by purification of the RNA in aqueous phase and removal of genomic DNA by an RNeasy Plus Micro Kit (QIAGEN, 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 an ABI PRISM 7300 Detection System (Applied Biosystems) using TaqMan Universal Master Mix (Applied Biosystems). In brief, the reaction volume (20  $\mu$ l) included 10  $\mu$ l of 2 $\times$  TaqMan Universal Master Mix, 4  $\mu$ l of cDNA, and 1  $\mu$ l of 20 $\times$  FAM-labeled TaqMan gene expression assay. TaqMan inventoried Assays-on-Demand gene expression products were purchased from Applied Biosystems. A Mm01287743\_m1 assay was used for the Nox2 gene. An 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 C<sub>T</sub> 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, whereas apocynin, acetylcholine, PE, PEG-SOD, and PEG-catalase were dissolved in distilled water. Dimethyl sulfoxide final concentration in the organ bath had no effect by itself on the aortic rings.

**Data Analysis.** All of the experimental values are presented as means  $\pm$  S.E.M. ( $n$  = number of animals). The comparison among different groups was analyzed by ANOVA followed by the Tukey multiple comparisons test method as a post hoc test. Comparison between two groups was assessed by an unpaired  $t$  test.  $P < 0.05$  was taken as significant. The EC<sub>50</sub> 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 (version 3.0; GraphPad Software Inc., San Diego, CA).

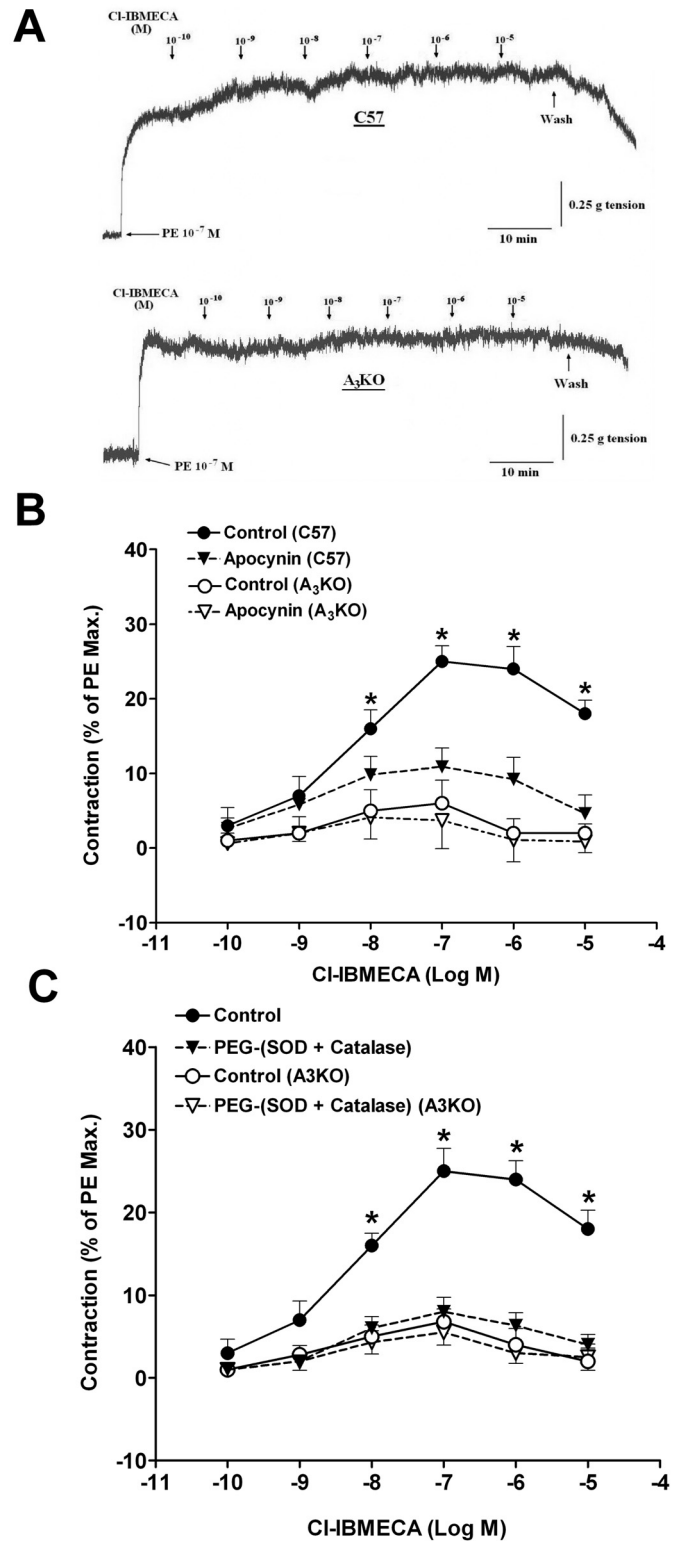
## Results

**Effect of A<sub>3</sub>AR Activation on Contractility of WT/A<sub>3</sub>KO Mouse Aorta.** Incubation of WT aortic rings with increasing concentrations of the selective A<sub>3</sub>AR agonist Cl-IBMECA produced a concentration-dependent contraction with a pEC<sub>50</sub> of  $8.22 \pm 0.27$  (Fig. 1, A and B). The contraction induced by Cl-IBMECA was insignificant in A<sub>3</sub>KO aortic rings. At 10<sup>-7</sup> M, Cl-IBMECA increased contraction significantly by 25% in WT, while producing a negligible effect (~6%) in A<sub>3</sub>KO mouse aorta (Fig. 1A). The contraction induced by Cl-IBMECA in WT aortic rings was significantly inhibited by preincubation with the Nox inhibitor, apocynin (10<sup>-5</sup> M). These results suggest that A<sub>3</sub>AR-mediated contraction is dependent on Nox activation in mouse aorta.

To further test the involvement of the ROS in the contraction induced by A<sub>3</sub>AR activation, PEG-SOD, and PEG-catalase as ROS scavengers were tested against Cl-IBMECA-induced contraction (Fig. 1C). Preincubation with PEG-(SOD + catalase) inhibited the contraction induced by Cl-IBMECA in WT aortic rings without affecting that of the A<sub>3</sub>KO, indicating that A<sub>3</sub>AR 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 control of aortic tone or A<sub>3</sub>AR-mediated contraction in this investigation.

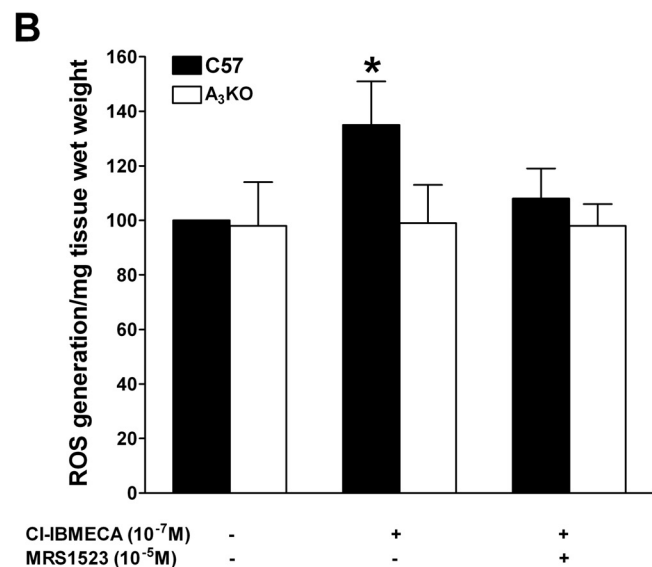
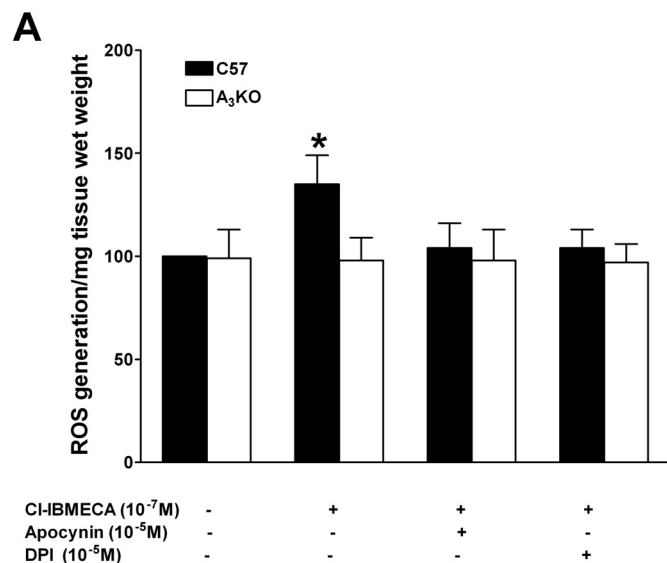
**Effect of A<sub>3</sub>AR Activation on ROS Generation from WT/A<sub>3</sub>KO Mouse Aorta.** Incubation of WT aortic rings with 10<sup>-7</sup> M Cl-IBMECA increased the amount of intracellular ROS production in WT by  $35 \pm 14\%$  compared with that in



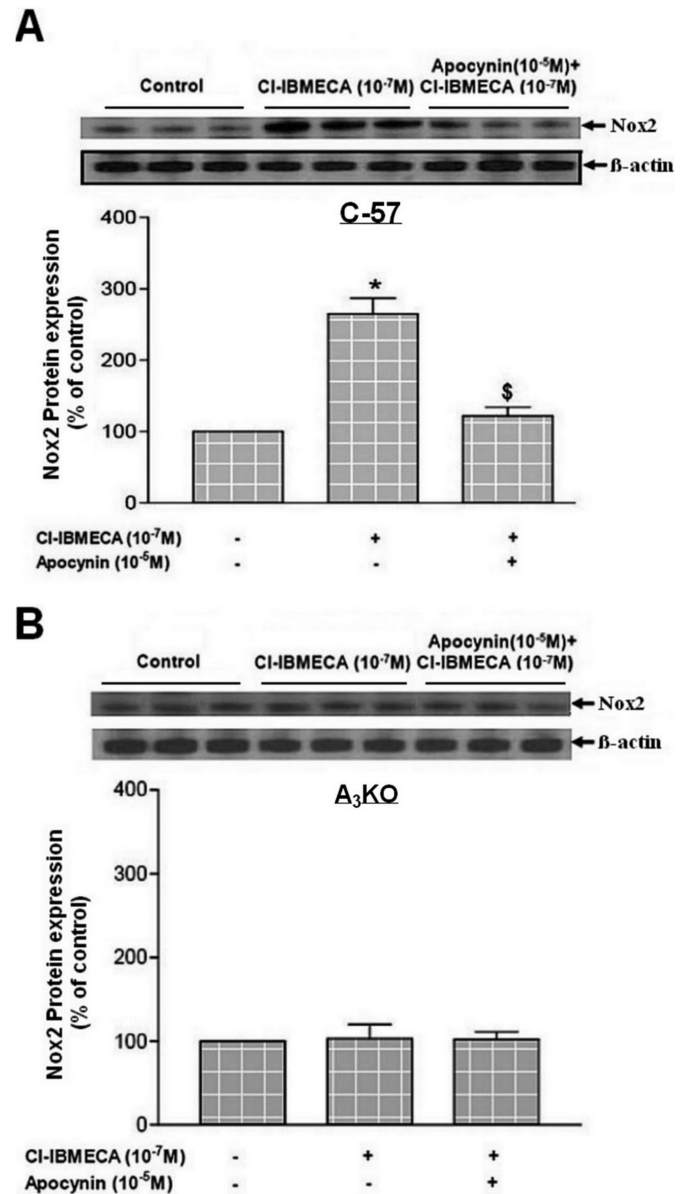
**Fig. 1.** Vascular responses to Cl-IBMECA in WT and A<sub>3</sub>KO mouse aorta. A, tracing showing the responses to Cl-IBMECA in WT and A<sub>3</sub>KO mouse aortic rings. B, effect of apocynin on Cl-IBMECA-induced contraction in WT and A<sub>3</sub>KO mouse aorta. C, effect of PEG-(SOD + catalase) on Cl-IBMECA-induced contraction in WT and A<sub>3</sub>KO mouse aorta. Data are expressed as the mean  $\pm$  S.E.M. ( $n$  = 6). \*,  $p < 0.05$  compared with the A<sub>3</sub>AR KO control group using one-way ANOVA followed by a Tukey multiple comparison post hoc test.

corresponding A<sub>3</sub>KO aortas (Fig. 2A). In contrast, Cl-IBMECA did not increase ROS production in A<sub>3</sub>KO aorta. Preincubation with Nox inhibitors apocynin (10<sup>-5</sup> M) or DPI (10<sup>-5</sup> M) prevented this increase in ROS production (Fig. 2A), indicating that the source of ROS activated by Cl-IBMECA is Nox. Likewise, preincubation with the selective A<sub>3</sub>AR inhibitor MRS1523 (10<sup>-7</sup> M) leads to inhibition of ROS production induced by Cl-IBMECA (Fig. 2B), confirming that A<sub>3</sub>AR activation induces ROS production through Nox.

**Effects of A<sub>3</sub>AR Activation on Nox1, 2, and 4 Protein Expression in WT/A<sub>3</sub>KO Mouse Aorta.** For further confirmation of the role of Nox in the contraction and ROS generation induced by A<sub>3</sub>AR activation, the protein expression levels of Nox1, 2, and 4 subunits were examined. Activation of A<sub>3</sub>AR with Cl-IBMECA (10<sup>-7</sup> M) selectively increased the expression of Nox2 in WT aorta by 150 ± 15% compared with that in the control (Fig. 3A). This increase in Nox2 expression was inhibited by preincubation with apocynin (10<sup>-5</sup> M),



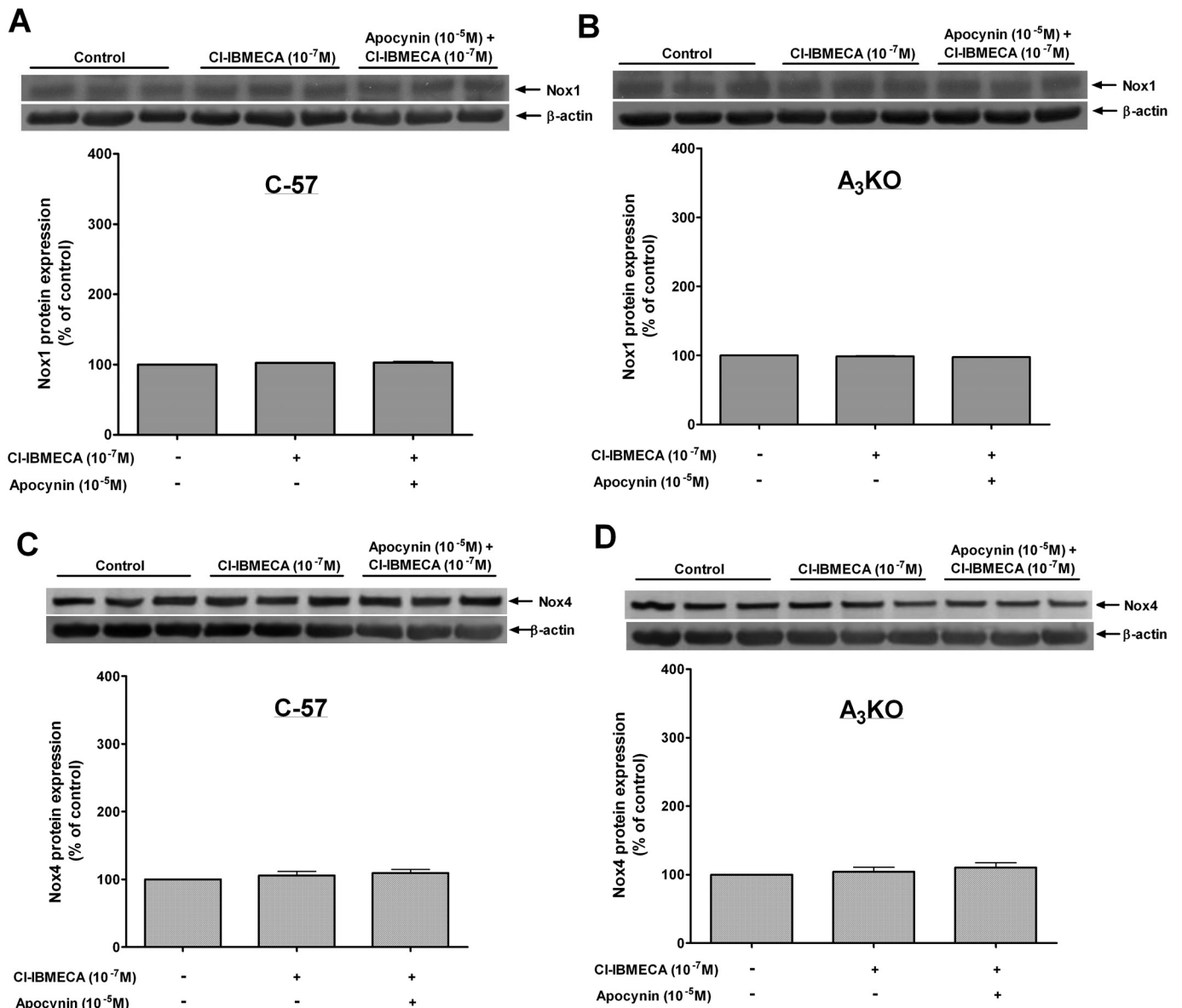
**Fig. 2.** Effects of apocynin and DPI (A) and MRS1523 (B) on Cl-IBMECA-induced ROS generation in WT and A<sub>3</sub>KO mouse aorta. Data are expressed as the mean ± S.E.M. ( $n = 4$ ). \*,  $p < 0.05$  compared with the corresponding WT control group using an unpaired  $t$  test.



**Fig. 3.** Effects of Cl-IBMECA on protein expression of Nox2 subunit in WT (A) and A<sub>3</sub>KO (B) mouse aorta. Data are expressed as the mean ± S.E.M. ( $n = 3$ ). \*,  $p < 0.05$  compared with control; \$,  $p < 0.05$  compared with Cl-IBMECA alone using one-way ANOVA followed by a Tukey multiple comparisons post hoc test.

whereas neither Cl-IBMECA alone nor with apocynin had an effect on Nox2 expression in A<sub>3</sub>KO mice aorta (Fig. 3B). On the other hand, Cl-IBMECA (10<sup>-7</sup> M) had no effect on the expression of Nox1 (Fig. 4, A and B) or Nox4 (Fig. 4, C and D) either in WT or in A<sub>3</sub>KO mice aorta. These data confirm the selective activation of the Nox2 subunit by A<sub>3</sub>AR, leading to ROS generation and contraction in mouse aorta.

**Effects of A<sub>3</sub>AR Activation on Nox2 mRNA Expression in WT/A<sub>3</sub>KO Mouse Aorta.** Because Nox2 protein expression was selectively increased by A<sub>3</sub>AR activation in WT but not in A<sub>3</sub>KO mouse aorta, we examined the change in Nox2 mRNA expression induced by Cl-IBMECA. Of interest, the mRNA expression of Nox2 was not affected by Cl-IBMECA in either WT or A<sub>3</sub>KO mouse aorta (data not shown), suggesting that A<sub>3</sub>AR activation enhances Nox2 protein expression at a post-transcriptional level.



**Fig. 4.** Effects of Cl-IBMECA on protein expression of Nox1 in WT (A), Nox1 in A<sub>3</sub>KO (B), Nox4 in WT (C), and Nox4 in A<sub>3</sub>KO (D) mouse aorta. Data are expressed as the mean  $\pm$  S.E.M. ( $n = 3$ ).

## Discussion

This is the first study to show that Nox are involved in A<sub>3</sub>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<sub>3</sub>AR activation using the selective agonist Cl-IBMECA causes contraction of aorta in WT mice but not in A<sub>3</sub>KO mice. This A<sub>3</sub>AR-mediated contraction of aorta was inhibited by a Nox inhibitor (apocynin), in addition to ROS scavengers PEG-SOD and PEG-catalase, and was not affected by the mast cell degranulator compound 48/80 or the stabilizer cromolyn sodium. We also found that A<sub>3</sub>AR activation leads to increased intracellular ROS generation, which was inhibited by apocynin, DPI, and the selective A<sub>3</sub>AR antagonist, MRS1523. Furthermore, the protein expression of Nox2 subunit was selectively increased by Cl-IBMECA in WT but not in A<sub>3</sub>KO mice aorta without affecting either Nox1 or 4.

We used the pharmacological selective A<sub>3</sub>AR agonist Cl-IBMECA, in addition to A<sub>3</sub>KO mice to confirm that A<sub>3</sub>AR activation induces aortic contraction in WT mice. The role of A<sub>3</sub>AR in the control of vascular tone has also been demonstrated by our laboratory (Ansari et al., 2007b). In another study, infusion of adenosine in A<sub>3</sub>KO mice has been shown to cause a significant decrease in blood pressure compared with that in WT mice (Zhao et al., 2000). In addition, the effects of A<sub>3</sub>AR activation on vascular tone have 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 A<sub>3</sub>AR 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 show that neither the mast cell degranulator (compound 48/80) nor stabilizer (cromolyn sodium) affected  $A_3AR$ -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  $A_3AR$ -induced contraction in mouse aorta involves Nox. We have shown previously that COX-1 plays a role in this  $A_3AR$  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 the 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  $A_3AR$  may play an important role in the regulation of vascular tone, mainly through endothelium-dependent pathways.

$A_3AR$ s 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  $A_3AR$  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), whereas its cerebral vasodilation in rat involves Nox (Gebremedhin et al., 2010). Furthermore,  $A_3AR$ s 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  $A_3AR$  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  $A_3KO$  animals without affecting Nox1 or 4, suggesting that  $A_3AR$  induces ROS generation, possibly through the activation of Nox2.

We previously demonstrated that activation of  $A_3AR$  leads to endothelium-dependent aortic contraction (Ansari et al., 2007b). However, because Nox isoforms are expressed in both endothelium and vascular smooth muscle, further studies are needed to differentiate the role of each cell type in  $A_3AR$ -induced ROS generation.

Of interest, the protein expression of Nox2 was increased by Cl-IBMECA, but the mRNA was not affected, suggesting that activation of  $A_3AR$  enhances Nox2 protein expression through post-transcriptional response, such as changes in protein translation and/or turnover. This finding may also partly explain the rapid change in Nox2 protein expression by short-term 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 after surgical tumor excision (Spruessel et al., 2004). In addition, up-regulation of different cellular proteins due to changes in the translational level or protein turnover without changes in mRNA expression has been shown (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 to the much less vasodilator peroxynitrite (Koppenol et al., 1992). The ROS indicator DCFH-DA used in this study is nonspecific

because it can detect several ROS such as  $O_2^-$ , hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals (Biziukin et al., 1995). However, because most Nox isoforms produce  $O_2^-$  as a main ROS; therefore, our data indicate that  $A_3AR$  possibly enhances  $O_2^-$  production through Nox2, which may play an important role in the vasoconstriction induced by  $A_3AR$  in the mouse aorta. Further studies are needed to identify the various ROS produced by the activation of  $A_3AR$ .

In the literature, some concerns were raised about the selectivity of Nox inhibitors such as apocynin (Schlüter et al., 2008) and DPI (Stuehr et al., 1991), which may affect the results obtained with these inhibitors in our study. Therefore, use of these inhibitors solely may present some arguments about their specific targets. To address this issue, our studies used not only these inhibitors but also ROS generation and Nox subunit protein expression as tools to confirm the relationship between  $A_3AR$  and Nox.

Because ROS are involved in several diseases and  $A_3AR$  modulators have been tested in some diseases such as renal cancer (Jajoo et al., 2009), a better understanding of the relationship between  $A_3AR$  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  $A_3AR$ ; therefore, enhancement of 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,  $A_3AR$  activation induces contraction of the mouse aorta that is dependent on ROS generation, possibly mediated through Nox2.

#### Authorship Contributions

*Participated in research design:* El-Awady, Ansari, and Mustafa.  
*Conducted experiments:* El-Awady, Ansari, and Fil.  
*Contributed new reagents or analytic tools:* Tilley.  
*Performed data analysis:* El-Awady and Ansari.  
*Wrote or contributed to the writing of the manuscript:* El-Awady, Ansari, and Mustafa.

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**Address correspondence to:** Dr. S. Jamal Mustafa, Department of Physiology and Pharmacology, West Virginia University, Morgantown, WV 26505. E-mail: smustafa@hsc.wvu.edu