

# Role of Adenosine Receptor(s) in the Control of Vascular Tone in the Mouse Pudendal Artery

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## ABSTRACT

Activation of adenosine receptors (ARs) has been implicated in the modulation of renal and cardiovascular systems, as well as erectile functions. Recent studies suggest that adenosine-mediated regulation of erectile function is mainly mediated through  $A_{2B}$ AR activation. However, no studies have been conducted to determine the contribution of AR subtype in the regulation of the vascular tone of the pudendal artery (PA), the major artery supplying and controlling blood flow to the penis. Our aim was to characterize the contribution of AR subtypes and identify signaling mechanisms involved in adenosine-mediated vascular tone regulation in the PA. We used a DMT wire myograph for muscle tension measurements in isolated PAs from wild-type,  $A_{2A}$ AR knockout,  $A_{2B}$ AR knockout, and  $A_{2A}/A_{2B}$ AR double-knockout mice. Real-time reverse

transcription–polymerase chain reaction was used to determine the expression of the AR subtypes. Data from our pharmacologic and genetic approaches suggest that AR activation–mediated vasodilation in the PA is mediated by both the  $A_{2A}$ AR and  $A_{2B}$ AR, whereas neither the  $A_1$ AR nor  $A_3$ AR play a role in vascular tone regulation of the PA. In addition, we showed that  $A_{2A}$ AR- and  $A_{2B}$ AR-mediated vasorelaxation requires activation of nitric oxide and potassium channels; however, only the  $A_{2A}$ AR-mediated response requires protein kinase A activation. Our data are complemented by mRNA expression showing the expression of all AR subtypes with the exception of the  $A_3$ AR. AR signaling in the PA may play an important role in mediating erection and represent a promising therapeutic option for the treatment of erectile dysfunction.

## Introduction

Penile erection consists of multiple neurovascular processes that all simultaneously involve the nerves, blood vessels, and endothelium in the sinusoids and trabecular smooth muscle cells of the penis. Those factors, which regulate contraction and relaxation of vascular smooth muscle, determine the state of the penis (flaccidity versus erection) (Andersson, 2001; Tostes et al., 2007; Nunes et al., 2012). The flaccid state is mainly mediated by the release of norepinephrine from adrenergic nerve terminals and other vasoconstrictors, such as endothelin-1 and angiotensin II (Sáenz de Tejada et al., 1989, 2004). The erection is mainly mediated by nitric oxide (NO) released from the endothelium and nonadrenergic-noncholinergic nerves in addition to other neurotransmitters and endothelium-derived

hyperpolarizing factors (EDHFs), such as adenosine (Chiang et al., 1994; Tostes et al., 2007; Wen et al., 2011b).

Adenosine, a signaling nucleoside, is produced during conditions of metabolic stress and high cellular activity, resulting in increased oxygen supply and decreased oxygen consumption. Adenosine is mainly generated by the 5'-nucleotidases CD73 that catalyze the dephosphorylation of AMP into adenosine. Intracellular adenosine levels are primarily regulated by adenosine kinase, which converts adenosine to AMP, whereas extracellular adenosine levels are critically regulated by adenosine deaminase, which degrades adenosine to inosine (Blackburn, 2003; Ham and Evans, 2012; Wen and Xia (2012)). Recently, adenosine was described as an EDHF because of its ability to relax and hyperpolarize vascular smooth muscle cells (VSMC) (Ohta et al., 2013). The activation of adenosine receptors (ARs) is implicated in the modulation of renal and cardiovascular functions, as well as erectile function, with both in vivo and in vitro studies demonstrating that, like NO, adenosine is a potent vasodilator

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**ABBREVIATIONS:** Ach, acetylcholine; AR, adenosine receptors; CC, corpus cavernosum; CCPA, 2-chloro-*N*-6-cyclopentyladenosine; Cl-IBMECA, 1-[2-chloro-6-[[[3-iodophenyl]methyl]amino]-9H-purin-9-yl]-1-deoxy-*N*-methyl- $\beta$ -D-ribofuranuronamide; CRC, concentration-response curves; CVT 6883 (GS 6201), 3-ethyl-3,9-dihydro-1-propyl-8-[1-[[3-(trifluoromethyl)phenyl]methyl]-1*H*-pyrazol-4-yl]-1*H*-purine-2,6-dione; DKO, double-KO; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ED, erectile dysfunction; EDHF, endothelium-derived hyperpolarizing factors; K<sup>+</sup>, potassium; KO, knockout; KT 5720, (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*,3',2',1'-*kl*]pyrrolo[3,4-*ij*][1,6]benzodiazocine-10-carboxylic acid, hexyl ester; L-NAME, *N*<sup>G</sup>-nitro-L-arginine methyl ester; MRS1523, 6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylic acid propyl ester; NECA, 5'-*N*-ethylcarboxamidoadenosine; NO, nitric oxide; NOS, nitric oxide synthase; PA, pudendal artery; Phe, phenylephrine; PKA, protein kinase A; qPCR, quantitative real-time polymerase chain reaction; SCH-58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-*e*)-1,2,4-triazolo(1,5-*c*)pyrimidine; VSMC, vascular smooth muscle cells; WT, wild type.

that may regulate penile erection in humans and animals (Takahashi et al., 1991, 1992; Chiang et al., 1994; Mantelli et al., 1995; Filippi et al., 2000; Noto et al., 2001; Tostes et al., 2007; Carneiro et al., 2008; Mi et al., 2008; Vallon and Osswald, 2009; Phatarpekar et al., 2010; Wen et al., 2010, 2011b, 2012; Ning et al., 2012; Headrick et al., 2013; Layland et al., 2014). Adenosine binds to a family of four P1 G protein-coupled AR subtypes: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>. Vascular studies from our laboratory and others have demonstrated that, whereas the activation of the A<sub>1</sub>AR and A<sub>3</sub>AR results in vasoconstriction, the activation of the A<sub>2A</sub>AR and A<sub>2B</sub>AR results in vasodilation (Tawfik et al., 2005; Ansari et al., 2007; Nayeem et al., 2008; El-Awady et al., 2011; Sanjani et al., 2011; El-Gowell et al., 2013; Hein et al., 2013; Kunduri et al., 2013; Teng et al., 2013). The different contribution of each AR subtype to the physiology or pathophysiology of erection has been studied in the corpus cavernosum (CC). Studies in humans and animals reported that both the A<sub>2A</sub>AR and A<sub>2B</sub>AR mediate the CC's vasorelaxation (Filippi et al., 2000; Noto et al., 2001; Faria et al., 2006; Tostes et al., 2007; Mi et al., 2008; Moura et al., 2015). Using quantitative real-time polymerase chain reaction (qPCR), Mi et al. (2008) showed that the A<sub>2B</sub>AR was the predominant receptor subtype expressed in murine cavernosal smooth muscle cells, whereas relatively low level expression of the A<sub>2A</sub>AR was observed. The subtypes promoting vasoconstriction, namely the A<sub>1</sub>AR and A<sub>3</sub>AR, were not detectable. In contrast, an earlier study by Tostes et al. (2007) suggested that activation of both the A<sub>2A</sub>AR and A<sub>2B</sub>AR mediate CC relaxation in mice. While the contribution of the A<sub>1</sub>AR to erectile function plays an important role in the release of neurotransmitters, the role of the A<sub>3</sub>AR in erectile function is still not known (Chiang et al., 1994; Tostes et al., 2007; Ning et al., 2012). Recent studies demonstrated that adenosine functions to promote penile erection; however, this research has focused solely on the CC.

In addition to the CC, an important player in regulating erectile function and blood flow to the penis is the pudendal artery (PA). The PA is an artery that branches from the internal iliac artery, providing oxygenated blood to the external genitals. The PA branches into cavernous arteries that further branch into tortuous helicine arteries, all feeding the cavernous sinuses. In the PA, the absence of capillaries allows for a rapid filling of cavernosal sinuses during erection (Hale et al., 2009). Recent studies have shown that the PA contributes approximately 70% of total pudendal-penile vascular resistance, whereas the intrapenile vasculature contributes less than 25% of total resistance in this bed (Manabe et al., 2000). In addition, it has been demonstrated that optimal erection requires vasodilation of both prepenile arteries, such as the PA, as well as intrapenile arteries (Manabe et al., 2000; Hale et al., 2009; Hannan et al., 2011). To date, no studies have characterized the contribution of adenosine and its receptors in the regulation of the PA vascular tone. In the present study, we characterized the contribution of AR subtypes to vascular tone in the PA and identified signaling mechanisms involved in adenosine-mediated vascular tone regulation.

## Materials and Methods

**Animals.** The Institutional Animal Care and Use Committee of West Virginia University approved all experimental protocols. We

followed guidelines set forth by the American Physiologic Society and National Institutes of Health regarding the care and use of laboratory animals. A<sub>2A</sub>AR and A<sub>2B</sub>AR single knockout (KO) mice (A<sub>2A</sub>AR KO and A<sub>2B</sub>AR KO mice, respectively) were generously provided by Dr. C. Ledent (Université libre de Bruxelles, Brussels, Belgium) and Stephen Tilley (University of North Carolina, Chapel Hill, NC), respectively. A<sub>2A</sub>AR and A<sub>2B</sub>AR KO mice, both backcrossed 12 generations to wild-type (WT) C57BL/6 background mice (Jackson Laboratory, Bar Harbor, ME) were bred to generate A<sub>2A</sub>/A<sub>2B</sub>AR double-KO (DKO) heterozygotes. Double heterozygotes were intercrossed, and 1/16 of the offspring were A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice. A<sub>2A</sub>/A<sub>2B</sub>AR DKO breeding pairs were then established (Zhou et al., 2014). Mice were caged on a 12-hour light/dark cycle with free access to standard chow diet, with water ad libitum.

**Muscle Tension Studies in Pudendal Arteries.** Mice were euthanized using sodium pentobarbital (50 mg/kg i.p.). PAs were excised, transferred into an ice-cold physiologic salt solution (130 mM NaCl, 14.9 mM NaHCO<sub>3</sub>, 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH<sub>2</sub>PO<sub>4</sub>, 1.17 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.6 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O), and dissected to remove loose connective tissue and fat. The arteries were then cut into 2-mm segments and mounted on a wire myograph in 5-ml chambers (DMT, Aarhus, Denmark) containing buffer at 37°C and continuously bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The tissues were stretched to a resting force of 200 mg and allowed to equilibrate for 60 minutes. Changes in isometric force were recorded using a PowerLab/8SP data acquisition system (Chart software, version 5.0; ADInstruments, Colorado Springs, CO). After equilibration, rings were precontracted with 50 mM KCl to check the contractility of the individual PA rings. Arterial and endothelial integrity were assessed by contracting with phenylephrine (Phe, 10<sup>-6</sup> M), followed by relaxation with acetylcholine (ACh, 10<sup>-6</sup> M). Concentration-response curves (CRC) for AR agonists [5'-N-ethylcarboxamidoadenosine (NECA), 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA), and 1-[2-chloro-6-[[[3-iodophenyl]methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (Cl-IBMECA), 10<sup>-9</sup> M to 10<sup>-5</sup> M] were performed. Endothelium-dependent relaxation was assessed by measuring the dilatatory response to ACh (10<sup>-9</sup> M to 10<sup>-5</sup> M) in Phe-contracted vessels.

**Drugs and Solutions.** ACh, Phe, NECA, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; an A<sub>1</sub>AR antagonist), KT 5720 [a protein kinase A (PKA) inhibitor], MRS1523 (an A<sub>3</sub>AR antagonist), N<sup>G</sup>-nitro-L-arginine methyl ester [L-NAME; a nitric oxide synthase (NOS) inhibitor], and Cl-IBMECA (an A<sub>3</sub>AR agonist) were purchased from Sigma-Aldrich/MilliporeSigma (St. Louis, MO). CCPA (an A<sub>1</sub>AR agonist), SCH-58261 (a selective A<sub>2A</sub>AR antagonist), and CVT-6883 (a selective A<sub>2B</sub>AR antagonist) were purchased from Tocris Bioscience (Minneapolis, MN). Stock solutions were prepared in deionized water or dimethyl sulfoxide (DMSO) and stored in aliquots at -20°C. Dilutions were prepared immediately before use. Rings were preincubated with antagonist or inhibitors 30 minutes prior to Phe preconstruction for the CRC. Stock solutions originally diluted in DMSO were used with a final concentration of less than 0.003% v/v in the muscle bath; this concentration has been demonstrated to have no effect on vascular reactivity.

**Real-Time Reverse Transcription-Polymerase Chain Reaction.** Total RNA was isolated from PAs of WT mice using an RNeasy Total RNA isolation kit from Qiagen (Valencia, CA). This was followed by conversion of 0.5 μg of total RNA into complementary DNA (cDNA) using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions in a total volume of 100 μl. Each sample contained PAs pooled from seven to nine mice. Because of the relatively low expression of ARs, a polymerase chain reaction (PCR) PreAmplification Kit (Applied Biosystems) was used. Reverse transcription-polymerase chain reaction (qPCR) was performed using an ABI PRISM 7300 Detection System (Applied Biosystems) with TaqMan Universal Master Mix (Applied Biosystems, Branchburg, NJ) according to manufacturer's instructions. The reaction volume (25 μl) included

12.5  $\mu$ l of 2 $\times$  TaqMan Universal Master Mix, 1  $\mu$ l of cDNA, and 1.25  $\mu$ l of 20 $\times$  FAM-labeled TaqMan Gene Expression Assay Master Mix solution. For real-time PCR of AR genes, the TaqMan-inventoried gene expression product was purchased from Applied Biosystems. The 18S ribosomal RNA was used as an endogenous control. The fold-difference in expression of target cDNA was determined using the comparative CT method. The  $\Delta$ CT value was determined in each experiment by subtracting the average 18S CT value from the corresponding average CT for the A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>AR in coronary arteries, as previously described (Teng et al., 2013).

**Statistical Analysis.** Student's *t* test was used for the comparison between two groups, and one-way analysis of variance was used for groups of three or more. CRC data were analyzed between groups at the same concentrations. In addition, an F-test was used for the estimation of EC<sub>50</sub> values obtained from best-fit analysis using a nonlinear, interactive fitting program (GraphPad Prism; Graph Pad Software Inc. San Diego CA). Data are expressed as means  $\pm$  S.E.M. (*N*), where *N* is the number of mice. Values of *P* < 0.05 were considered a statistically significant difference.

## Results

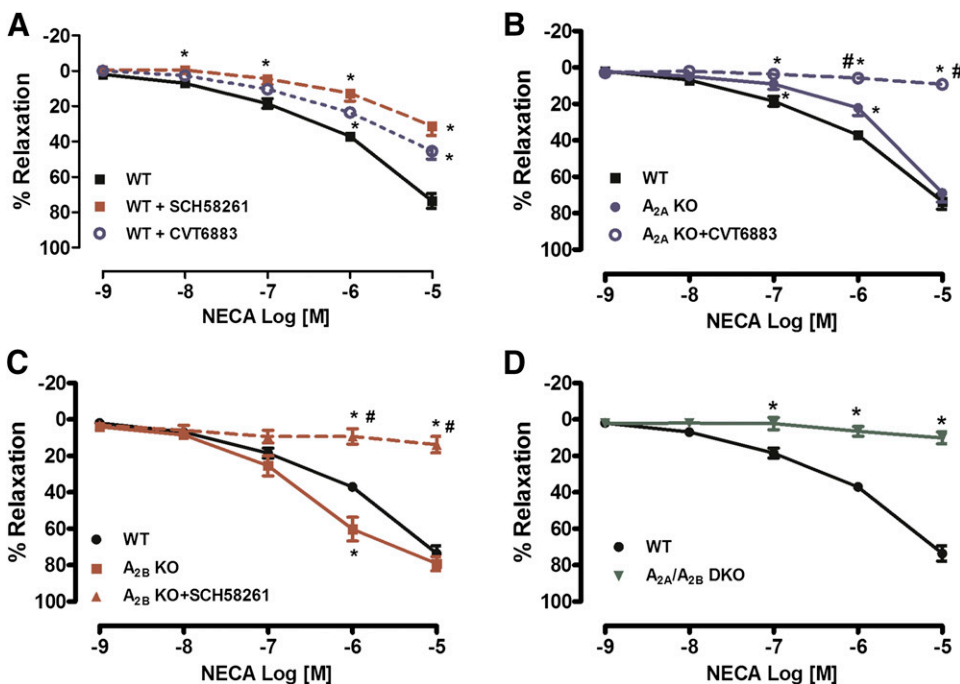
**NECA-Mediated Relaxation in the PA Is Dependent on A<sub>2A</sub>AR and A<sub>2B</sub>AR Activation.** In precontracted PA rings isolated from WT mice, NECA (a nonselective AR agonist) produced a concentration-dependent relaxation, suggesting a role for either the A<sub>2A</sub>AR or A<sub>2B</sub>AR, since both ARs are associated with vascular relaxation. Preincubation with either the A<sub>2A</sub>AR antagonist SCH-58261 or A<sub>2B</sub>AR antagonist CVT-6883 significantly decreased the NECA-mediated relaxation (Fig. 1A). Furthermore, PAs isolated from A<sub>2A</sub>AR KO mice exhibited a decreased relaxation response to NECA (EC<sub>50</sub>  $-5.88 \pm 0.10$  in WT versus  $-5.40 \pm 0.17$  in A<sub>2A</sub>AR KO mice, *P* < 0.05) (Fig. 1B). Incubation of PAs isolated from A<sub>2A</sub>AR KO mice with the A<sub>2B</sub>AR antagonist abolished the NECA-mediated vasorelaxation (Fig. 1B). Contrary to A<sub>2A</sub>AR KO mice, PAs isolated from A<sub>2B</sub>AR KO mice exhibited an increase in sensitivity to NECA compared with PAs from WT

mice ( $-5.88 \pm 0.10$  versus  $-6.50 \pm 0.15$ , *P* < 0.05) (Fig. 1C). Incubation of PAs isolated from A<sub>2B</sub>AR KO mice with the A<sub>2A</sub>AR antagonist abolished the NECA-mediated vasorelaxation (Fig. 1C). Furthermore, genetic deletion of both the A<sub>2A</sub>AR and A<sub>2B</sub>AR resulted in the absence of vasodilation in response to increased NECA concentrations (Fig. 1D). Taken together, our data suggest that both the A<sub>2A</sub>AR and A<sub>2B</sub>AR contribute to relaxation in the PA.

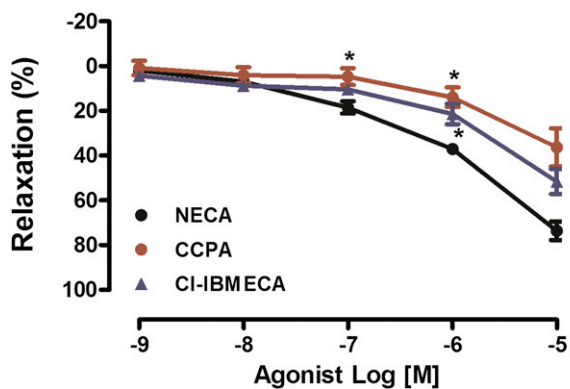
**Neither A<sub>1</sub>AR Nor A<sub>3</sub>AR Activation Resulted in Vasoconstriction in the PA.** Activation of either A<sub>1</sub>AR or A<sub>3</sub>AR is known to result in vasoconstriction. Cumulative addition of CCPA (an A<sub>1</sub>AR agonist) or Cl-IBMECA (an A<sub>3</sub>AR agonist) did not cause vasoconstriction in PAs isolated from WT mice. Furthermore, both CCPA and Cl-IBMECA caused vasorelaxation of PAs at high concentrations (10<sup>-6</sup> M and 10<sup>-5</sup> M), possibly owing to nonselectivity of the agonists; however, the vasodilatory response to CCPA and Cl-IBMECA was significantly lower in comparison with that elicited by NECA (Fig. 2).

**Neither A<sub>1</sub>AR nor A<sub>3</sub>AR is Required for AR-Mediated Vasorelaxation.** Since A<sub>1</sub>AR or A<sub>3</sub>AR agonists were shown to activate either the A<sub>2A</sub>AR or A<sub>2B</sub>AR at high concentrations, we used PAs isolated from A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice (Fig. 3). In precontracted PAs isolated from A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice, NECA did not affect the vascular relaxation, whereas both CCPA and Cl-BMECA caused dilation at the highest dose (10<sup>-5</sup> M) (Fig. 3A). Incubation of PAs isolated from A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice with both the A<sub>1</sub>AR antagonist DPCPX and the A<sub>3</sub>AR antagonist MRS-1593 did not affect CCPA- and Cl-BMECA-mediated relaxation at the 10<sup>-5</sup> M concentration, suggesting that the dilation mediated by CCPA and Cl-BMECA at the 10<sup>-5</sup> M concentration is not dependent on ARs (Fig. 3, B and C).

**Genetic Deletion of A<sub>2A</sub>AR or A<sub>2B</sub>AR Resulted in Decreased Endothelium-Mediated Vasorelaxation.** Recently, adenosine was described as an EDHF owing to its ability



**Fig. 1.** Adenosine receptor (AR)-mediated relaxation in the pudendal artery (PA) is mediated through activation of both the A<sub>2A</sub>AR and A<sub>2B</sub>AR. (A) NECA induced a concentration-dependent relaxation, and treatment with either an A<sub>2A</sub>AR antagonist (SCH-58261; 1  $\mu$ M) or A<sub>2B</sub>AR antagonist (CVT-6883; 1  $\mu$ M) resulted in a decrease in NECA-mediated vasorelaxation. (B) Genetic deletion of the A<sub>2A</sub>AR resulted in a decrease in NECA-mediated relaxation compared with wild type (WT), and treatment with an A<sub>2B</sub>AR antagonist (CVT-6883) completely abolished the NECA response. (C) Genetic deletion of the A<sub>2B</sub>AR resulted in significantly altered NECA-mediated relaxation compared with WT, and treatment with an A<sub>2A</sub>AR antagonist (SCH-58261) abolished the NECA response. (D) In A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice, the vasorelaxation response to the adenosine receptor agonist NECA was completely abolished. Data are represented as mean  $\pm$  S.E.M. (*N* = 5–7). \**P* < 0.05 versus WT and #*P* < 0.05 versus corresponding KO (A<sub>2A</sub>AR KO or A<sub>2B</sub>AR KO).



**Fig. 2.**  $A_1$ AR and  $A_3$ AR agonists caused vasorelaxation in PAs isolated from WT mice. A CRC to NECA, CCPA, and CI-IBMECA was performed in PAs isolated from WT mice. Data are represented as mean  $\pm$  S.E.M. ( $N = 5-7$ ). \* $P < 0.05$  versus NECA.

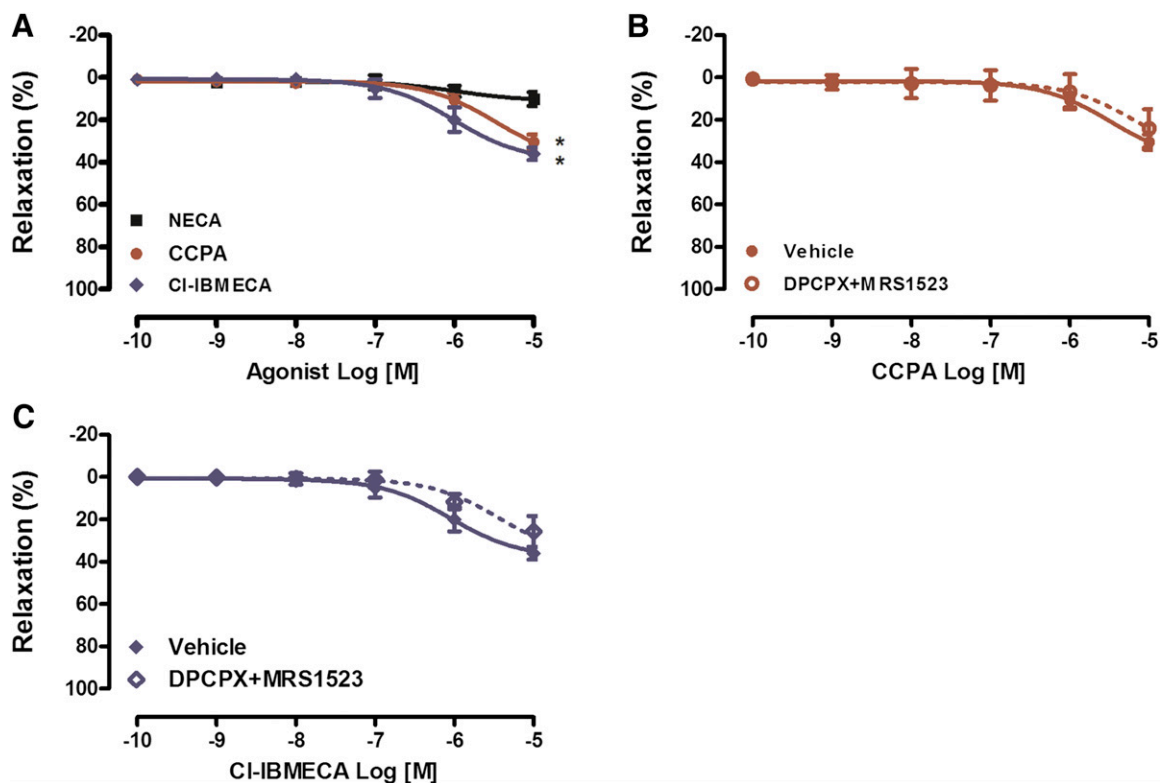
to relax and hyperpolarize VSMCs. To test the effect of either  $A_{2A}$ AR or  $A_{2B}$ AR deletion on the endothelium-mediated dilation, increased concentration of ACh resulted in a concentration-dependent relaxation in PAs isolated from WT,  $A_{2A}$ AR KO, and  $A_{2B}$ AR KO mice (Fig. 4A). However, PAs isolated from  $A_{2A}$ AR KO or  $A_{2B}$ AR KO mice exhibited a decrease in  $EC_{50}$  in response to ACh compared with WT mice:  $EC_{50} -7.78 \pm 0.06$  in WT versus  $-7.29 \pm 0.06$  in  $A_{2A}$ AR KO,  $P < 0.05$ ; and  $EC_{50} -7.78 \pm 0.06$  in WT versus  $-7.26 \pm 0.10$  in  $A_{2B}$ AR KO,  $P < 0.05$  (Fig. 4B). Taken together, our data suggest that both the  $A_{2A}$ AR and  $A_{2B}$ AR may play a role in the endothelium-mediated relaxation.

**Mechanism of  $A_{2A}$ AR- and  $A_{2B}$ AR-Mediated Relaxation in the PA.** To look at the mechanisms of  $A_{2A}$ AR-mediated relaxation, we used PAs isolated from  $A_{2B}$ AR KO mice (Fig. 5A). Preincubation with either an NOS inhibitor (L-NAME), protein kinase A inhibitor (KT 5720), or potassium channel inhibition (5 mM KCl) significantly decreased  $A_{2A}$ -mediated vasorelaxation in PAs isolated from  $A_{2A}$ AR KO mice. To elucidate the mechanisms of  $A_{2B}$ AR-mediated relaxation, we used PAs isolated from  $A_{2A}$ AR KO mice (Fig. 5B). Pretreatment with the either L-NAME or increased extracellular  $K^+$  significantly decreased the  $A_{2B}$ -mediated vasorelaxation, whereas treatment with KT 5720 did not affect relaxation in PAs isolated from  $A_{2A}$ AR KO mice. Taken together, our data suggest that both  $A_{2A}$ AR- and  $A_{2B}$ AR-mediated vasorelaxation requires activation of NO and  $K^+$  channels; however, only the  $A_{2A}$ AR-mediated response requires PKA activation.

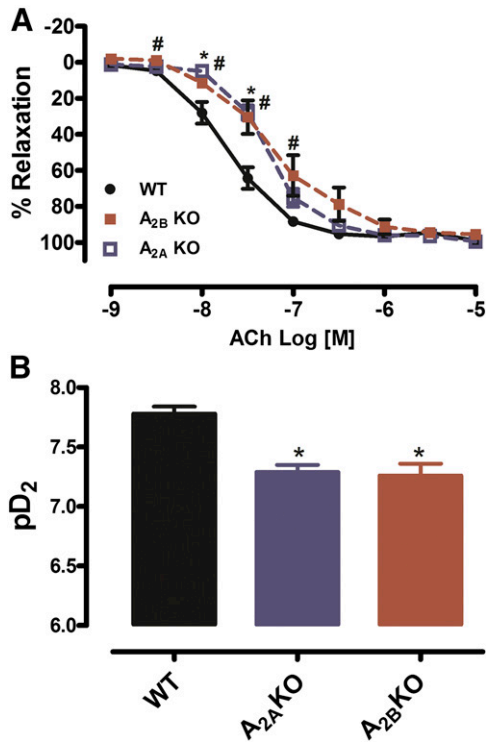
**Message RNA Expression of ARs in the PA.** In support of our observations made in the functional studies, semi-quantitative real-time PCR was used to determine mRNA expression of the AR subtypes in PAs isolated from WT mice. Of the four AR subtypes, only the  $A_1$ R,  $A_{2A}$ AR, and  $A_{2B}$ AR were expressed, with the  $A_{2A}$ AR having the highest mRNA expression level compared with the  $A_1$ R and  $A_{2B}$ AR (Fig. 6).

## Discussion

Using pharmacological and genetic approaches, our study is the first to investigate transcriptional expression and to characterize the contribution of the four AR subtypes to



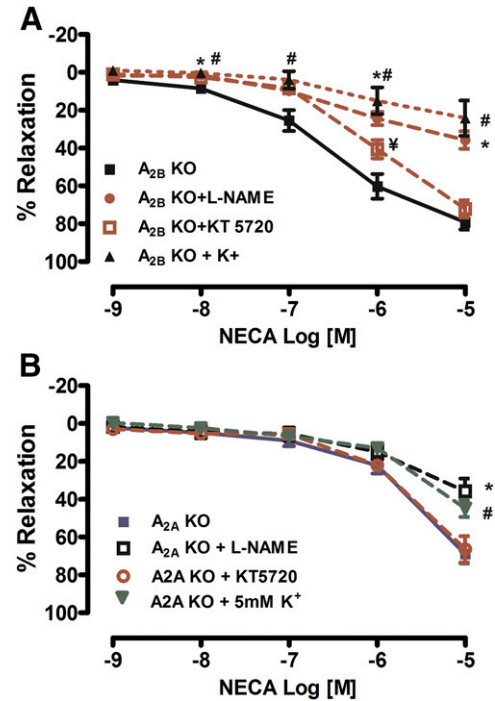
**Fig. 3.** Neither  $A_1$ AR nor  $A_3$ AR is required for the AR-mediated vasorelaxation in PAs. (A) CRC to NECA, CCPA and CI-IBMECA in  $A_{2A}/A_{2B}$ AR DKO mice. (B) CRC to CCPA in  $A_{2A}/A_{2B}$ AR DKO mice in the presence of vehicle or the  $A_1$ AR antagonist DPCPX (0.1  $\mu$ M) and the  $A_3$ AR antagonist MRS1523 (1  $\mu$ M). (C) CRC to CI-IBMECA in  $A_{2A}/A_{2B}$ AR DKO mice in the presence of vehicle or DPCPX and MRS1523. Data are represented as mean  $\pm$  S.E.M. ( $N = 5-8$ ). \* $P < 0.05$  versus NECA.



**Fig. 4.** Genetic deletion of either the A<sub>2A</sub>AR or A<sub>2B</sub>AR resulted in decreased sensitivity of the PA to endothelium-mediated relaxation. (A) A<sub>2A</sub>AR KO and A<sub>2B</sub>AR KO mice exhibit decreases in ACh-mediated relaxation ( $*P < 0.05$  A<sub>2A</sub> KO versus WT and  $^{\#}P < 0.05$  A<sub>2B</sub> KO versus WT). (B) Representative graph of EC<sub>50</sub> (expressed as potency or pD<sub>2</sub>) from both A<sub>2A</sub>AR KO and A<sub>2B</sub>AR KO mice exhibited a decrease in EC<sub>50</sub> ( $*P < 0.05$  KOs versus WT). Data are represented as mean  $\pm$  S.E.M. ( $N = 5-7$ ).

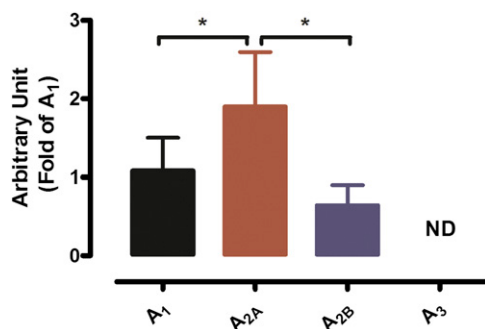
vascular tone regulation of the PA. Herein, we showed that: 1) of the four AR subtypes, only the A<sub>1</sub>R, A<sub>2A</sub>AR, and A<sub>2B</sub>AR were expressed in the PA; 2) although the A<sub>1</sub>R was expressed in the PA, only the A<sub>2A</sub>AR and A<sub>2B</sub>AR played a role in vascular tone regulation of the PA; and 3) both A<sub>2A</sub>AR- and A<sub>2B</sub>AR-mediated vasorelaxation requires activation of NOS and K<sup>+</sup> channels; however, only A<sub>2A</sub>AR-mediated responses requires PKA activation. In this paper, we sought to fill a gap in the understanding of AR signaling in erectile function and define the roles of AR subtypes in prepenile vascular tone regulation.

Numerous studies have investigated the contribution of adenosine to erectile function, looking at the effect of AR activation in the CC. However, this study is the first to characterize the contribution of ARs in the regulation of PA vascular tone, the major artery supplying and controlling blood flow to the penis (Manabe et al., 2000; Hale et al., 2009). In the first set of experiments, we observed that the adenosine analog NECA caused a concentration-dependent vasorelaxation in murine PAs. As we already know, A<sub>2A</sub>AR and A<sub>2B</sub>AR activation results in vasorelaxation, whereas A<sub>1</sub>AR and A<sub>3</sub>AR activation results in vasoconstriction. In this study, our results suggested that NECA-induced relaxation is mediated through A<sub>2A</sub>AR and A<sub>2B</sub>AR activation. Indeed, preincubation of PA rings with specific A<sub>2A</sub>AR (SCH-58261) or A<sub>2B</sub>AR (CVT-6883) antagonists significantly decreased the NECA-mediated relaxation, suggesting that both A<sub>2A</sub>AR and A<sub>2B</sub>AR activation are required for the response to NECA in PAs (Fig. 1A). Furthermore, PAs isolated from A<sub>2A</sub> KO mice exhibited a decreased relaxation to NECA, which was



**Fig. 5.** Both A<sub>2A</sub>AR- and A<sub>2B</sub>AR-mediated vasorelaxation is dependent on nitric oxide (NO) in PAs. (A) Treatment with a nitric oxide synthase (NOS) inhibitor (L-NAME; 100  $\mu$ M), increased K<sup>+</sup> (5 mM KCl), or with a protein kinase A (PKA) inhibitor (KT 5720; 0.1  $\mu$ M) significantly decreased A<sub>2A</sub>AR-mediated vasorelaxation from A<sub>2B</sub>AR KO mice. (B) Treatment with either the NOS inhibitor or increased K<sup>+</sup> significantly decreased the A<sub>2B</sub>AR-mediated vasorelaxation, whereas treatment with KT 5720 (0.1  $\mu$ M) did not affect relaxation in A<sub>2A</sub>AR KO mice. Data are represented as mean  $\pm$  S.E.M. ( $N = 4-7$ ).  $*P < 0.05$  L-NAME versus vehicle,  $^{\#}P < 0.05$  K<sup>+</sup> versus vehicle and  $^{\Delta}P < 0.05$  KT5720 versus vehicle.

abolished using an A<sub>2B</sub>AR antagonist. Contrary to A<sub>2A</sub> KO mice, PAs isolated from A<sub>2B</sub> KO mice exhibited an increase in sensitivity to NECA compared with PAs from WT mice; however, when incubated with an A<sub>2A</sub>AR antagonist, the relaxation response to NECA was completely abolished. The difference in PA responses using pharmacological inhibition, compared with other genetic KO models, can be explained by the fact that global gene deletion usually results in compensatory upregulation of other genes or other compensatory signaling pathways. Regarding A<sub>2A</sub> and A<sub>2B</sub> ARs, although it has been shown that a single gene KO results in upregulation of the other AR in mesenteric artery (Teng et al., 2013), this was not the case in aorta, where KO of A<sub>2A</sub>AR gene did not affect the expression of A<sub>2B</sub>AR (Ponnoth et al., 2009). This also confirms the importance of pharmacological studies in addition to studies using gene deletion. In addition, the differences in the affinity of the receptors to the agonist could also explain the different response to NECA in the PA isolated from the KO mice, since A<sub>2B</sub>AR possesses the lowest affinity for adenosine and NECA. Our results were confirmed using PAs isolated from A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice, where NECA failed to cause vasorelaxation in the PA rings. Our data confirm the important role played by both the A<sub>2A</sub>AR and A<sub>2B</sub>AR in CC relaxation and, thus, erection. In fact, several studies reported the role of both the A<sub>2A</sub>AR and A<sub>2B</sub>AR in mediating CC vasorelaxation (Filippi et al., 2000; Noto et al., 2001; Faria et al., 2006; Tostes et al., 2007). In mice, a recent study using a genetic approach by Mi and colleagues (2008) showed high



**Fig. 6.** Messenger RNA expression of A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> AR subtypes as determined by real-time reverse-transcriptase PCR using total RNA extracted from WT mouse PAs. Values were normalized by the corresponding 18S ribosomal RNA of each sample. Results are represented as mean  $\pm$  S.E. M. ( $N = 7$ ), with each  $N$  representing pooled PAs from seven to nine mice. \* $P < 0.05$ .

A<sub>2B</sub>AR expression in the CC compared with expression of the A<sub>2A</sub>AR. They also showed that vasorelaxation of the CC is mediated solely through the activation of the A<sub>2B</sub>AR, whereas the A<sub>2A</sub>AR did not seem to play a role. However, Tostes et al. (2007) showed that both A<sub>2A</sub>AR antagonist SCH-58261 and A<sub>2B</sub>AR antagonist MRS-1706 resulted in decreased CC relaxation to the nonspecific agonist 2-chloroadenosine, with the combination of both antagonists resulting in almost total inhibition of the CC's relaxation, supporting our data in the present study, whereas both the A<sub>2A</sub>AR and A<sub>2B</sub>AR contribute to relaxation of the CC. In addition, other groups also have shown the contribution of the A<sub>2A</sub>AR to vasorelaxation in the CC (Mantelli et al., 1995; Filippi et al., 2000; Noto et al., 2001; Faria et al., 2006; Moura et al., 2015).

A<sub>1</sub>AR and A<sub>3</sub>AR activation has been shown to mediate vascular contraction (Ansari et al., 2007; El-Awady et al., 2011; Kunduri et al., 2013). To test the contribution of the A<sub>1</sub>AR and A<sub>3</sub>AR in the regulation of PA vascular tone, we performed CRCs to both an A<sub>1</sub>AR agonist (CCPA) and A<sub>3</sub>AR agonist (CI-IBMECA). Both agonists failed to mediate vasoconstriction in PAs, suggesting that neither A<sub>1</sub>AR nor A<sub>3</sub>AR contribute to the vascular tone in PAs. However, both drugs at high concentrations caused PA relaxation. These results were not surprising, as both drugs at high concentrations activate other receptors, including A<sub>2A</sub>AR and A<sub>2B</sub>AR (Teng and Mustafa, 2011). To further rule out the role of A<sub>1</sub>AR and A<sub>3</sub>AR, we performed the CRC to an A<sub>1</sub>AR agonist (CCPA) and A<sub>3</sub>AR agonist (CI-IBMECA) in PAs isolated from A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice. To our surprise, both agonists (CCPA and CI-IBMECA) were able to mediate vasorelaxation in PAs from A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice at high concentrations ( $10^{-5}$  M; Fig. 3A). Preincubation of PAs isolated from A<sub>2A</sub>/A<sub>2B</sub>AR DKO mice with both A<sub>1</sub>R and A<sub>3</sub>AR antagonists (DPCPX and MRS1523, respectively) did not affect vasorelaxation mediated by either CCPA or CI-IBMECA at  $10^{-5}$  M, suggesting that relaxations to both CCPA and CI-IBMECA are not mediated by AR activation and may be attributable to nonspecific agonist effects (Fig. 3, B and C).

Recently, adenosine was described as an EDHF, with CD73 as an EDHF synthase, owing to its ability to relax and hyperpolarize VSMCs (Ohta et al., 2013). We looked at the effect of the genetic deletion of either A<sub>2A</sub>AR or A<sub>2B</sub>AR on endothelium-mediated vasorelaxation in PAs. CRCs to ACh in PAs isolated from A<sub>2A</sub>AR KO and A<sub>2B</sub>AR KO mice resulted in a

rightward shift of CRC in A<sub>2A</sub>AR KO and A<sub>2B</sub>AR KO mice and decreased sensitivity to ACh compared with WT mice, suggesting that both receptors may play a role in endothelium-mediated relaxation (Fig. 4, A and B). Our data suggest that the A<sub>2A</sub>AR KO and A<sub>2B</sub>AR KO partially contributed to the endothelium-mediated vasorelaxation in the PA, corroborating the role of adenosine as an EDHF.

Next, we sought to elucidate the mechanism of AR-mediated relaxation in the PA. To dissect the signaling pathways for A<sub>2</sub>AR-mediated relaxation, we used PAs isolated from A<sub>2B</sub>AR KO mice to study A<sub>2A</sub>AR-mediated relaxation and A<sub>2A</sub>AR KO mice to study A<sub>2B</sub>AR-mediated relaxation in the PA (Fig. 5). Previous studies suggested a role for PKA, the NO signaling pathway, and potassium (K<sup>+</sup>) channels in mediating adenosine-mediated vasorelaxation (Mi et al., 2008; Sanjani et al., 2011; El-Gowell et al., 2013; Hein et al., 2013). Our data showed that PKA inhibition caused a rightward shift of the CRC, resulting in a decrease in relaxation to NECA. In addition, inhibition of NOS and increasing extracellular K<sup>+</sup> resulted in a decrease in NECA-mediated vasorelaxation in PAs (Fig. 5A). Our data suggest that A<sub>2A</sub>AR-mediated relaxation in the PA is dependent on PKA, NO, and K<sup>+</sup> channel activation. On the other hand, we found that A<sub>2B</sub>AR-mediated relaxation in the PA is dependent on NO and K<sup>+</sup> channel activation, but not PKA, as PKA inhibition did not result in decreased NECA-mediated vasorelaxation in PAs isolated from A<sub>2A</sub>AR KO mice. In fact, a recent study showed that A<sub>2B</sub>AR contributed to penile erection via an alternative signaling pathway, which is dependent on PI3K/AKT signaling (Wen et al., 2011b).

Several studies have looked at the contribution of adenosine to erectile function both in vivo and in vitro. However, limited studies have tried to characterize AR expression in the erectile system. Using qPCR, Mi et al. (2008) demonstrated that the A<sub>2B</sub>AR is the predominant receptor, with relatively low expression of the A<sub>2A</sub>AR, whereas mRNA levels of both the A<sub>1</sub>AR and A<sub>3</sub>AR were nondetectable in CC smooth muscle cells. A similar pattern of AR expression was found in another study using primary CC fibroblast cells (Wen et al., 2011b). However, the expression level of ARs at the whole tissue level or other cell types in the CC (i.e., endothelial cells) was not evaluated. In the present study, we used isolated PAs to evaluate the expression pattern of AR subtypes using qPCR. We found that A<sub>1</sub>AR, A<sub>2A</sub>AR, and A<sub>2B</sub>AR were expressed in PAs (with higher expression of A<sub>2A</sub>AR compared with A<sub>1</sub>AR and A<sub>2B</sub>AR), whereas A<sub>3</sub>AR mRNA levels were not detectable (Fig. 6). These results are in accordance with our functional data in the case of A<sub>2A</sub>AR and A<sub>2B</sub>AR; however, it did not translate to vascular response in the case of A<sub>1</sub>AR, where there was a lack of response to the A<sub>1</sub>AR agonist CCPA (Fig. 3B). Although unexpected, this was not surprising, as previous studies also showed a lack of A<sub>2A</sub>AR-mediated vascular response despite the presence of this receptor in rat and mouse mesenteric arteries (Rubino et al., 1995; Teng et al., 2013). In addition, one limitation of the present study is that, whereas the mRNA levels are important to determine, it was difficult to assess the protein expression and level owing to the size of the tissue, which would be more functionally relevant. Although A<sub>1</sub>AR activation did not affect vascular reactivity, A<sub>1</sub>AR may still contribute to the overall PA vascular tone, as it was shown to play an important role in the release of neurotransmitters (Chiang et al., 1994; Ning et al., 2012). As

in recent studies using CC smooth muscle cells and CC fibroblast cells, we were unable to detect A<sub>3</sub>AR expression in the PA, suggesting that A<sub>3</sub>AR may not play an important role in erectile function (Mi et al., 2008; Wen et al., 2010).

Early studies looked at the potential role of adenosine signaling in erectile function at the level of the penile tissue; however, our study is the first one to look at its role in the regulation of PA vascular tone, which is an important regulator of blood flow to the penis. Decreased adenosine levels or signaling results in erectile dysfunction (ED), whereas increased adenosine levels and signaling through the A<sub>2B</sub>AR are associated with priapism (Mi et al., 2008; Wen et al., 2011a). The use of adenosine as treatment of ED has been previously evaluated; however, the rapid degradation of adenosine may interfere with its ability to maintain erection (Chiang et al., 1994; Kilic et al., 1994).

Wen and Xia (2012) suggested A<sub>2B</sub>AR activation as a potential therapeutic pathway for ED; however, further studies looking at the change of AR expression in diseases associated with vasculogenic ED, such as hypertension and diabetes, are crucial. In fact, using an angiotensin II model of hypertension, the same group demonstrated that in this model, which was also shown to be associated with ED, A<sub>2B</sub>AR expression and signaling increased in the kidney, resulting in progression of hypertension associated with increased renal fibrosis (Jin et al., 2008; Labazi et al., 2013; Zhang et al., 2013). Increased A<sub>2B</sub>AR signaling in hypertension together with its association with penile fibrosis will make the use of A<sub>2B</sub>AR agonist as a potential therapeutic target for ED, at least in this model, counterproductive. An alternative therapeutic target could be the use of an A<sub>2A</sub>AR agonist, since our study showed that both A<sub>2A</sub>AR and A<sub>2B</sub>AR signaling contributed to the regulation of PA vascular tone, in addition to studies showing the involvement of A<sub>2A</sub>AR signaling in mediating human CC relaxation. Furthermore, a recent study demonstrated a synergic effect of a novel A<sub>2A</sub>AR agonist and sildenafil (a PDE5 inhibitor), suggesting that combined treatment may reduce side effects and increase efficacy in ED patients who do not respond to sildenafil alone (Moura et al., 2015). Together, A<sub>2A</sub>AR activation may represent an alternative clinical target for the treatment of ED, although future studies looking at the change of AR expression and signaling in diseases associated with vasculogenic ED would be critical to evaluate the safety and efficacy of A<sub>2A</sub>AR agonists use in ED treatment.

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#### Authorship Contributions

Participated in research design: Labazi.

Conducted experiments: Labazi.

Contributed new reagents or analytic tools: Tilley, Ledent.

Performed data analysis: Labazi.

Wrote or contributed to the writing of the manuscript: Labazi, Mustafa.

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