Title page: Role of adenosine receptor(s) in the control of vascular tone in the mouse pudendal artery

Hicham Labazi, Stephen L Tilley, Catherine Ledent, and S. Jamal Mustafa

Department of Physiology and Pharmacology, Center for Cardiovascular and Respiratory Sciences and Clinical Translational Science Institute, West Virginia University, Morgantown, WV, USA: HL and SJM.

Department of Medicine, University of North Carolina, Chapel Hill, NC, USA: SLT.

Universite Libre de Bruxelles, Brussels, Belgium: CL.
Running title page: Adenosine receptors and the pudendal artery.

Corresponding author: S. Jamal Mustafa, Ph.D.
Department of Physiology and Pharmacology
WVU School of Medicine
PO Box 9229, 224 BMRF – 2nd Floor
Morgantown, WV  26506
Phone: (304) 293-1123
Fax: (304) 293-3850
E-mail: sjmustafa@hsc.wvu.edu

Ach: acetylcholine
AR: adenosine receptors
CC: corpus cavernosum
CCPA: 2-Chloro-N6-cyclopentyladenosine
CI-IBMECA: 1-[2-Chloro-6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide
CRC: Concentration-response curves
DMSO: dimethyl sulfoxide
DPCPX: 8-Cyclopentyl-1,3-dipropylxanthine
ED: erectile dysfunction
EDHF: endothelium-derived hyperpolarizing factors
K⁺: potassium
KT 5720: (9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester
L-NAME: N^G^-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
PA: pudendal artery
Phe: phenylephrine
PKA: protein kinase A
RT-PCR: Reverse transcription polymerase chain reaction
VSMC: vascular smooth muscle cells
WT: Wild type
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 A2BAR 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, A2AAR knockout, A2BAR knockout, and A2A/A2BAR double-knockout mice. Real-time reverse transcription–polymerase chain reaction was used to determine the expression of the AR subtypes. Using pharmacologic and genetic approaches, our data suggest that AR activation-mediated vasodilation in the PA is mediated by both the A2AAR and A2BAR, while neither the A1AR nor A3AR play a role in vascular tone regulation of the PA. In addition, we showed that A2AAR and A2BAR-mediated vasorelaxation requires activation of nitric oxide and potassium channels; however, only the A2AAR-mediated response requires protein kinase A activation. Our data are complemented by mRNA expression showing the expression of all AR subtypes except the A3AR. 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 neuro-vascular processes, which 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 vs. erection) (Andersson, 2001; Nunes et al., 2012; Tostes et al., 2007). 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 (de Tejada et al., 2004; Detejada et al., 1989). The erection is mainly mediated by nitric oxide (NO) released from the endothelium and non-adrenergic-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 adenosine monophosphate (AMP) into adenosine. Intracellular adenosine levels are primarily regulated by adenosine kinase, which converts adenosine to AMP, while extracellular adenosine levels are critically regulated by adenosine deaminase, which degrades adenosine to inosine (Blackburn, 2003; Ham et al., 2012; Wen et al., 2012). Recently, adenosine was described as an EDHF due to 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 that may regulate penile erection in humans and animals (Carneiro et al., 2008; Chiang et al., 1994;
Filippi et al., 2000; Headrick et al., 2013; Layland et al., 2014; Mantelli et al., 1995; Mi et al., 2008; Ning et al., 2012; Noto et al., 2001; Phatarpekar et al., 2010; Takahashi et al., 1992; Takahashi et al., 1991; Tostes et al., 2007; Vallon et al., 2009; Wen et al., 2010; Wen et al., 2012; Wen et al., 2011b). Adenosine binds to a family of four P1 G-protein coupled AR subtypes: A1, A2A, A2B, and A3. Vascular studies from our laboratory and others have demonstrated that, while the activation of the A1AR and A3AR result in vasoconstriction, the activation of the A2AAR and A2BAR result in vasodilation (Ansari et al., 2007; El-Awady et al., 2011; El-Gowelli et al., 2013; Hein et al., 2013; Kunduri et al., 2013; Nayeem et al., 2008; Sanjani et al., 2011; Tawfik et al., 2005; Teng et al., 2013). The different contributions of each of the 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 A2AAR and A2BAR mediate the CC’s vasorelaxation (Faria et al., 2006; Filippi et al., 2000; Mi et al., 2008; Moura et al., 2015; Noto et al., 2001; Tostes et al., 2007). Using quantitative RT-PCR, Mi et al. showed that the A2BAR was the predominant receptor subtype expressed in murine cavernosal smooth muscle cells, while relatively low-level expression of the A2AAR was observed. The subtypes promoting vasoconstriction, namely the A1AR and A3AR, were not detectable (Mi et al., 2008). In contrast, an earlier study by Tostes et al. suggested that activation of both the A2AAR and A2BAR mediate CC relaxation in mice (Tostes et al., 2007). While the contribution of the A1AR to erectile function plays an important role in the release of neurotransmitters, the role of the A3AR in erectile function is still not known (Chiang et al., 1994; Ning et al., 2012; Tostes et al., 2007). 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 off of 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 (Hale et al., 2009; Hannan et al., 2011; Manabe et al., 2000). 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.

**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 Physiological Society and National Institutes of Health regarding the care and use of laboratory animals. $A_{2A}$AR and $A_{2B}$AR single knockout (KO) mice ($A_{2A}$AR KO and $A_{2B}$AR KO mice, respectively) were generously provided by Dr. C. Ledent (Universite Libre de Bruxelles, Brussels, Belgium) and Stephen Tilley (University of North Carolina, Chapel Hill, NC), respectively. $A_{2A}$AR and $A_{2B}$AR KO mice, both backcrossed 12 generations to wild type (WT)
C57BL/6 background mice (Jackson Laboratory, Bar Harbor, ME) were bred to generate A2A/A2BAR double-KO (DKO) heterozygotes. Double heterozygotes were intercrossed, and 1/16 of the offspring were A2A/A2BAR DKO mice. A2A/A2BAR DKO breeding pairs were then established (Zhou et al., 2014). Mice were caged on a 12-h 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 physiological salt solution (130 mM NaCl, 14.9 mM NaHCO₃, 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄·7H₂O, 1.6 mM CaCl₂·2H₂O), 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 (Danish Myo Technology, Aarhus, Denmark) containing buffer at 37°C and continuously bubbled with a mixture of 95% O₂ and 5% CO₂. 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, USA). After equilibration, rings were pre-contracted with 50 mM KCl to check the contractility of the individual PA rings. Arterial and endothelial integrity were assessed by contracting with phenylephrine (Phe, 10⁻⁶ M), followed by relaxation with acetylcholine (ACh, 10⁻⁶ M). Concentration-response curves (CRC) for AR agonists (5'-N-ethylcarboxamidoadenosine [NECA], 2-Chloro-N6-cyclopentyladenosine [CCPA], and 1-[2-Chloro-6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide [Cl-IBMECA]; 10⁻⁹ to 10⁻⁵) were performed. Endothelium-dependent relaxation was assessed by measuring the dilatory response to ACh (10⁻⁹ to 10⁻⁵) in Phe-contracted vessels.
**Drugs and Solutions:** ACh, Phe, NECA, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; an A₁AR antagonist), KT 5720 (a protein kinase A [PKA] inhibitor), MRS1523 (an A₃AR antagonist), N⁢G-nitro-l-arginine methyl ester (L-NAME; a nitric oxide synthase [NOS] inhibitor), and Cl-IBMECA (an A₃ AR agonist) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CCPA (an A₁ AR agonist), SCH-58261 (a selective A₂A AR antagonist), and CVT-6883 (a selective A₂B AR antagonist) were purchased from Tocris Bioscience. 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 pre-incubated with antagonist or inhibitors 30 min 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 RNAEasy Total RNA isolation kit from Qiagen. 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 7 to 9 mice. Because of the relatively low expression of ARs, a polymerase chain reaction (PCR) PreAmplification Kit from ABI was used. Reverse transcription polymerase chain reaction (RT-PCR) 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 µL of 2X TaqMan Universal Master Mix, 1 µL of cDNA, and 1.25 µL of 20X FAM-labeled TaqMan gene expression assay Master Mix solution. For real-time RT-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 ΔCT value was determined in each experiment by subtracting the average 18S CT value from the corresponding average CT for the A1, A2A, A2B, and A3AR 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 ANOVA 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 EC50 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±SEM (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 upon A2AAR and A2BAR activation:*

In pre-constricted PA rings isolated from WT mice, NECA (a non-selective AR agonist) produced a concentration-dependent relaxation, suggesting a role for either the A2AAR or A2BAR, since both ARs are associated with vascular relaxation. Pre-incubation with either the A2AAR antagonist SCH-58261 or A2BAR antagonist CVT-6883 significantly decreased the NECA-mediated relaxation (Figure 1A). Furthermore, PAs isolated from A2AAR KO mice exhibited a decreased relaxation response to NECA (EC50 -5.88±0.10 in WT vs. -5.40±0.17 in A2AAR KO mice, p < 0.05) (Figure 1B). Incubation of PAs isolated from A2AAR KO mice with the A2BAR antagonist abolished the NECA-mediated vasorelaxation (Figure 1B). Contrary to A2AAR KO mice, PAs isolated from A2BAR KO mice exhibited an increase in sensitivity to
NECA as compared to PAs from WT mice (-5.88±0.10 vs. -6.50±0.15, p < 0.05) (Figure 1C). Incubation of PAs isolated from A2BAR KO mice with the A2AAR antagonist abolished the NECA-mediated vasorelaxation (Figure 1C). Furthermore, genetic deletion of both the A2AAR and A2BAR resulted in the absence of vasodilation in response to increased NECA concentrations (Figure 1D). Taken together, our data suggest that both the A2AAR and A2BAR contribute to relaxation in the PA.

**Neither A1AR nor A3AR activation resulted in vasoconstriction in the PA:**

Activation of either A1AR or A3AR is known to result in vasoconstriction. Cumulative addition of CCPA (an A1AR agonist) or Cl-IBMECA (an A3AR agonist) did not cause vasoconstriction in PAs isolated from WT mice. Furthermore, both CCPA and Cl-IBEMCA caused vasorelaxation of PAs at high concentrations (10^{-6} M and 10^{-5} M), possibly due to non-selectivity of the agonists; however, the vasodilatory response to CCPA and Cl-IBMECA was significantly lower in comparison to that elicited by NECA (Figure 2).

**Neither A1AR nor A3AR is required for AR-mediated vasorelaxation:**

Since A1AR or A3AR agonists were shown to activate either the A2AAR or A2BAR at high concentrations, we used PAs isolated from A2A/A2BAR DKO mice (Figure 3). In pre-constricted PAs isolated from A2A/A2BAR DKO mice, while NECA did not affect the vascular relaxation, both CCPA and Cl-BMECA caused dilation at the highest dose (10^{-5} M) (Figure 3A). Incubation of PAs isolated from A2A/A2BAR DKO mice with both the A1AR antagonist DPCPX and the A3AR antagonist MRS-1593 did not affect CCPA and Cl-BMECA mediated relaxation at the 10^{-5} M concentration, suggesting that the dilation mediated by CCPA and Cl-BMECA at the 10^{-5} M concentration is not dependent upon ARs (Figure 3B and 3C).
Genetic deletion of A$_{2A}$AR or A$_{2B}$AR resulted in decreased endothelium-mediated vasorelaxation:

Recently, adenosine was described as an EDHF due to its ability 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 (Figure 4A). However, PAs isolated from A$_{2A}$ KO or A$_{2B}$ KO mice exhibited a decrease in EC$_{50}$ in response to ACh when compared to WT mice: (EC$_{50}$ -7.78±0.06 in WT vs. -7.29±0.06 in A$_{2A}$ KO, p < 0.05) and (EC$_{50}$ -7.78±0.06 in WT vs. -7.26±0.10 in A$_{2B}$ KO, p < 0.05) (Figure 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$_{2B}$ KO mice (Figure 5A). Pre-incubation with either a NOS inhibitor (L-NAME), protein kinase A (PKA) inhibitor (KT 5720) or potassium channel inhibition (5mM KCl) significantly decreased A$_{2A}$-mediated vasorelaxation in PAs isolated from A$_{2A}$ KO mice. To elucidate the mechanisms of A$_{2B}$AR-mediated relaxation, we used PAs isolated from A$_{2A}$ KO mice (Figure 5B). Pre-treatment with the either L-NAME or increased extracellular K$^+$ significantly decreased the A$_{2B}$-mediated vasorelaxation, while treatment with KT 5720 did not affect relaxation in PAs isolated from A$_{2A}$ 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 (RT-PCR) was used to determine mRNA expression of the AR subtypes in PAs isolated from
WT mice. Of the 4 AR subtypes, only the A₁R, A₂AAR, and A₂BAR were expressed, with the A₂AAR having the highest mRNA expression level compared to the A₁R and A₂BAR (Figure 6).

**Discussion:**

Using pharmacological and genetic approaches, our study is the first to investigate transcriptional expression and characterize the contribution of the four AR subtypes to vascular tone regulation of the PA. Herein, we showed that: 1) of the four AR subtypes, only the A₁R, A₂AAR, and A₂BAR were expressed in the PA, 2) although the A₁AR was expressed in the PA, only the A₂AAR and A₂BAR played a role in vascular tone regulation of the PA, and 3) both A₂AAR and A₂BAR-mediated vasorelaxation requires activation of NOS and K⁺ channels; however, only A₂AAR-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 pre-penile 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 (Hale *et al.*, 2009; Manabe *et al.*, 2000). In the first set of experiments, we observed that the adenosine analogue NECA caused a concentration-dependent vasorelaxation in murine PAs. As we already know, A₂AAR and A₂BAR activation results in vasorelaxation, while A₁AR and A₃AR activation results in vasoconstriction. In this study, our results suggested that NECA-induced relaxation is mediated through A₂AAR and A₂BAR activation. Indeed, pre-incubation of PA rings with specific A₂AAR (SCH-58261) or A₂BAR (CVT-6883) antagonists significantly decreased the NECA-mediated relaxation, suggesting that both A₂AAR and A₂BAR activation are required for the response to NECA in PAs (Figure 1A).
Furthermore, PAs isolated from A2A KO mice exhibited a decreased relaxation to NECA, which was abolished using an A2BAR antagonist. Contrary to A2A KO mice, PAs isolated from A2B KO mice exhibited an increase in sensitivity to NECA as compared to PAs from WT mice; however, when incubated with an A2AR antagonist, the relaxation response to NECA was completely abolished. The difference in PA responses using pharmacological inhibition, as compared to other genetic KO models, can be explained by the fact that global gene deletion usually results in compensatory up-regulation of other genes or other compensatory signaling pathways. Regarding A2A and A2B ARs; while 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 A2A AR gene did not affect the expression of A2B 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 A2BAR possesses the lowest affinity for adenosine and NECA. Our results were confirmed using PAs isolated from A2A/A2BAR DKO mice, where NECA failed to cause vasorelaxation in the PA rings. Our data confirm the important role played by both the A2AAR and A2BAR in CC relaxation and, thus, erection. In fact, several studies reported the role of both the A2AAR and A2BAR in mediating CC vasorelaxation (Faria et al., 2006; Filippi et al., 2000; Noto et al., 2001; Tostes et al., 2007). In mice, a recent study using a genetic approach by Mi and colleagues showed high A2BAR expression in the CC compared to expression of the A2AAR. They also showed that vasorelaxation of the CC is mediated solely through the activation of the A2BAR, while the A2AAR did not seem to play a role (Mi et al., 2008). However, Tostes et. al. showed that both A2AAR antagonists (SCH-58261) and A2BAR antagonist (MRS-1706) resulted in
decreased CC relaxation to the non–specific 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, where that both the A2AAR and A2BAR contribute to relaxation of the CC (Tostes et al., 2007). In addition, other groups also have shown the contribution of the A2AAR to vasorelaxation in the CC (Faria et al., 2006; Filippi et al., 2000; Mantelli et al., 1995; Moura et al., 2015; Noto et al., 2001).

A1AR and A3AR 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 A1AR and A3AR in the regulation of PA vascular tone, we performed CRCs to both an A1AR agonist (CCPA) and A3AR agonist (Cl-IBMECA). Both agonists failed to mediate vasoconstriction in PAs, suggesting that neither A1AR nor A3AR 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 A2AAR and A2BAR (Teng et al., 2011). To further rule out the role of A1AR and A3AR, we performed the CRC to an A1AR agonist (CCPA) and A3AR agonist (Cl-IBMECA) in PAs isolated from A2A/A2BAR DKO mice. To our surprise, both agonists (CCPA and Cl-IBMECA) were able to mediate vasorelaxation in PAs from A2A/A2BAR DKO mice at high concentrations (10^{-5} M; Figure 4A). Pre-incubation of PAs isolated from A2A/A2BAR DKO mice with both A1R and A3AR antagonists (DPCPX and MRS1523, respectively) did not affect vasorelaxation mediated by either CCPA or Cl-IBMECA at 10^{-5} M, suggesting that relaxations to both CCPA and Cl-IBMECA are not mediated by AR activation and may be due to non-specific agonist effects (Figure 4B and 4C).

Recently, adenosine was described as an EDHF, with CD73 as an EDHF synthase, due to its ability to relax and hyperpolarize VSMCs (Ohta et al., 2013). We looked at the effect of the
genetic deletion of either A2AAR or A2BAR on endothelium-mediated vasorelaxation in PAs. CRCs to ACh in PAs isolated from A2AAR KO and A2BAR KO mice resulted in a rightward shift of CRC in A2AAR KO and A2BAR KO mice and decreased sensitivity to ACh when compared to WT mice, suggesting that both receptors may play a role in endothelium-mediated relaxation (Figure 5A and 5B). Our data suggest that the A2AAR KO and A2BAR 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 A2AR mediated relaxation, we used PAs isolated from A2BAR KO mice to study A2AAR-mediated relaxation and A2AAR KO mice to study A2BAR-mediated relaxation in the PA (Figure 5). Previous studies suggested a role for PKA, the NO signaling pathway, and potassium (K+) channels in mediating adenosine-mediated vasorelaxation (El-Gowelli et al., 2013; Hein et al., 2013; Mi et al., 2008; Sanjani et al., 2011). 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+ resulted in a decrease in NECA-mediated vasorelaxation in PAs (Figure 5A). Our data suggest that A2AAR-mediated relaxation in the PA is dependent upon PKA, NO, and K+ channel activation. On the other hand, we found that A2BAR-mediated relaxation in the PA is dependent upon NO and K+ channel activation, but not PKA, as PKA inhibition did not result in decreased NECA-mediated vasorelaxation in PAs isolated from A2AAR KO mice. In fact, a recent study showed that A2BAR contributed to penile erection via an alternative signaling pathway, which is dependent upon PI3K/AKT signaling (Wen et al., 2011b).
Several studies have looked at the contribution of adenosine to erectile function both \textit{in vivo} and \textit{in vitro}. However, limited studies have tried to characterize AR expression in the erectile system. Using RT-PCR, Mi et al. demonstrated that the A$_{2B}$AR is the predominant receptor, with relatively low expression of the A$_{2A}$AR, while mRNA levels of both the A$_{1}$AR and A$_{3}$AR were non-detectable in CC smooth muscle cells (Mi et al., 2008). 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 RT-PCR. We found that A$_{1}$AR, A$_{2A}$AR, and A$_{2B}$AR were expressed in PAs (with higher expression of A$_{2A}$AR compared to A$_{1}$AR and A$_{2B}$AR), while A$_{3}$AR mRNA levels were not detectable (Figure 6). These results are in accordance with our functional data in the case of A$_{2A}$AR and A$_{2B}$AR; however, it did not translate to vascular response in the case of A$_{1}$AR, where there was a lack of response to the A$_{1}$AR agonist CCPA (Figure 3B). Although unexpected, this was not surprising, as previous studies also showed a lack of A$_{2A}$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, while the mRNA levels are important to determine, it was difficult to assess the protein expression and level due to the size of the tissue, which would be more functionally relevant. Although A$_{1}$AR activation did not affect vascular reactivity, A$_{1}$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). Similar to recent studies using CC smooth muscle cells and CC fibroblast cells, we were unable to detect A$_{3}$AR expression in the
PA, suggesting that A3AR 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), while increased adenosine levels and signaling through the A2BAR are associated with priapism (Mi et al., 2008; Wen et al., 2011a). The use of adenosine as treatment for 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 et al. suggested A2BAR activation as a potential therapeutic pathway for ED (Wen et al., 2012); 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, A2BAR 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 A2BAR signaling in hypertension together with its association with penile fibrosis will make the use of A2BAR agonist as a potential therapeutic target for ED, at least in this model, to be counterproductive. An alternative therapeutic target could be the use of an A2AAR agonist, since our study showed that both A2AAR and A2BAR signaling contributed to the regulation of PA vascular tone, in addition to studies showing the involvement of A2AAR signaling in mediating human CC relaxation. Furthermore, a recent study demonstrated a synergic effect of a novel A2AAR 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, A2AAR 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 A2AAR agonists use in ED treatment.

**Acknowledgements:** The authors would like to acknowledge Dr. Brandi Talkington for help with the paper editing

**Author Contributions:**

- Participated in research design: Labazi.
- Conducted experiments: Labazi.
- Contributed new agents or analytic tools: Tilley, Ledent.
- Performed data analysis: Labazi.
- Wrote or contributed to the writing of the manuscript: Labazi and Mustafa.
References:


Faria M, Magalhaes-Cardoso T, Laufente-de-Carvalho JM, Correia-de-Sa P (2006). Corpus cavernosum from men with vasculogenic impotence is partially resistant to adenosine relaxation due to endothelial A(2B) receptor dysfunction. *Journal of Pharmacology and Experimental Therapeutics* **319**(1): 405-413.


Sanjani MS, Teng BY, Krahn T, Tilley S, Ledent C, Mustafa SJ (2011). Contributions of A(2A) and A(2B) adenosine receptors in coronary flow responses in relation to the K-ATP channel using A(2B) and


Footnotes:

This study was supported by NIH grants [Grants HL027339, HL094447 and U54GM104942] to S.J. Mustafa and Sexual Medicine Society of North America Mini-Grant to H. Labazi [Grant 1006727R].
Legends for Figures:

**Figure 1:** Adenosine receptor (AR)-mediated relaxation in the pudental artery (PA) is mediated through activation of both the A2AAR and A2BAR. A) NECA induced a concentration-dependent relaxation, and treatment with either an A2AAR antagonist (SCH-58261; 1µM) or A2BAR antagonist (CVT-6883; 1µM) resulted in a decrease in NECA-mediated vasorelaxation; B) Genetic deletion of the A2AAR resulted in a decrease in NECA-mediated relaxation compared to wild type (WT), and treatment with an A2BAR antagonist (CVT-6883) completely abolished the NECA response; C) Genetic deletion of the A2BAR resulted in significantly altered NECA-mediated relaxation compared to WT, and treatment with an A2AAR antagonist (SCH-58261) abolished the NECA response; D) In A2A/A2BAR DKO mice, the vasorelaxation response to the adenosine receptor agonist NECA was completely abolished. Data are represented as mean ± SEM. (N = 5–7). *p < 0.05 vs. WT and # p < 0.05 vs. corresponding KO (A2AAR KO or A2BAR KO).

**Figure 2:** A1AR and A3AR agonists caused vasorelaxation in PAs isolated from WT mice. A concentration response curve (CRC) to NECA, CCPA, and Cl-IBMECA was performed in PAs isolated from WT mice. Data are represented as mean ± SEM. (N = 5–7). *p < 0.05 vs. NECA.

**Figure 3:** Neither A1AR nor A3AR is required for the AR-mediated vasorelaxation in PAs. A) CRC to NECA, CCPA and Cl-IBMECA in A2A/A2BAR DKO mice; B) CRC to CCPA in A2A/A2BAR DKO mice in the presence of vehicle or the A1AR antagonist DPCPX (0.1 µM) and the A3AR antagonist MRS1523 (1 µM); C) CRC to Cl-IBMECA in A2A/A2BAR DKO mice in the presence of vehicle or DPCPX and MRS1523. Data are represented as mean ± SEM. (N = 5–8). *p < 0.05 vs. NECA.
Figure 4: Genetic deletion of either the A2AAR or A2BAR resulted in decreased sensitivity of the PA to endothelium-mediated relaxation, A) A2AAR KO and A2BAR KO mice exhibit decreases in ACh-mediated relaxation B) Representative graph of EC50 from both A2AAR KO and A2BAR KO mice exhibited a decrease in EC50. Data are represented as mean ± SEM. (N = 5–7). *p < 0.05 KOs vs. WT.

Figure 5: Both A2AAR and A2BAR-mediated vasorelaxation is dependent upon nitric oxide (NO) in PAs. A) Treatment with a nitric oxide synthase (NOS) inhibitor (L-NAME; 100µM), increased K+ (5mM KCl), or a protein kinase A (PKA) inhibitor (KT 5720; 0.1µM) significantly decreased A2AAR-mediated vasorelaxation from A2BAR KO mice. B) Treatment with the either the NOS inhibitor or increased K+ significantly decreased the A2BAR-mediated vasorelaxation, while treatment with KT 5720 (0.1µM) did not affect relaxation in A2AAR KO mice. Data are represented as mean ± SEM. (N = 4–7). *p < 0.05 treatment vs. vehicle.

Figure 6: Messenger RNA expression of A1, A2A, A2B and A3 AR subtypes as determined by real time reverse-transcriptase polymerase chain reaction 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 ± SEM (N = 7), with each N representing pooled PAs from 7–9 mice. *p < 0.05.
Figure 2

![Graph showing relaxation (%) against Agonist Log [M] for NECA, CCPA, and CI-IBMECA.](image-url)
Figure 3A

Figure 3B

Figure 3C