CYP-epoxygenases contribute to A2A receptor-mediated aortic relaxation via sarcolemmal KATP channels

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Ponnoth DS, Nayeem MA, Tilley SL, Ledent C, Mustafa SJ. CYP-epoxygenases contribute to A2A receptor-mediated aortic relaxation via sarcolemmal KATP channels. Am J Physiol Regul Integr Comp Physiol 303: R1003–R1010, 2012. First published September 26, 2012; doi:10.1152/ajpregu.00335.2012.—Previously, we have shown that A2A adenosine receptor (A2AAR) mediates aortic relaxation via cytochrome P-450 (CYP)-epoxygenases. However, the signaling mechanism is not understood properly. We hypothesized that A2AAR-mediated K+ (KATP) channels play an important role in A2AR-mediated relaxation. Organ bath and Western blot experiments were done using isolated aorta from A2AKO and corresponding wild-type (WT) mice. Aortic rings from WT and A2A knockout (KO) mice were precontracted with submaximal dose of phenylephrine (PE, 10−6 M). Concentration-response curves for pinacidil, cromakalim (nonselective KATP openers), and diazoxide (mitochondrial KATP opener) were obtained. Diazoxide did not have any relaxation effect on precontracted tissue, whereas relaxation to pinacidil (48.09 ± 5.23% in WT vs. 25.41 ± 2.73% in A2AKO; P < 0.05) and cromakalim (51.19 ± 2.05% in WT vs. 38.50 ± 2.26% in A2AKO; P < 0.05) was higher in WT than A2AKO aorta. This suggested the involvement of sarcolemmal rather than mitochondrial KATP channels. Endothelium removal, treatment with SCH 58651 (A2AAR antagonist; 10−6 M), Nω-nitro-l-arginine methyl ester (l-NNAME, nitric oxide synthase inhibitor) and methylsulfonyl-proparglyoxyphenylhexanamid (MS-PPOH, CYP-epoxygenases inhibitor; 10−5 M) significantly reduced pinacidil-induced relaxation in WT compared with controls, whereas these treatments did not have any effect in A2AKO aorta. Glibenclamide (KATP channel inhibitor, 10−5 M) blocked 2-p-(2-carboxyethyl)phenethylamino-5’-N-ethylcarboxamido adenosine hydrochloride (CGS 21680, A2AAR agonist)-induced relaxation in WT and changed 5’-N-ethylcarboxamido (NECA) (nonselective adenosine analog)-induced response to higher contraction in WT and A2AKO. 5-Hydroxydecanoate (5-HD, mitochondrial KATP channel inhibitor, 10−5 M) had no effect on CGS 21680-mediated response in WT aorta. Our data suggest that A2AAR-mediated vasorelaxation occurs through opening of sarcolemmal KATP channels via CYP-epoxygenases and possibly, nitric oxide, contributing to pinacidil-induced responses.

epoxyeicosatrienoic acids; ATP-sensitive channels; adenosine

ADENOSINE is an endogenous purine nucleoside that has many physiological functions, including modulation of vascular tone through four adenosine receptor (AR) subtypes; i.e., A1, A2A, A2B, and A3 (43). It is well established that vascular contraction is mediated via activation of the A1 and A2AR, whereas activation of the A2 AR (A2A and A2B) cause relaxation (3, 13, 14, 21, 27, 41, 44, 46). Metabolites of arachidonic acid (AA) produced by the endothelium are known to regulate vascular tone. Epoxyeicosatrienoic acids (EETs) are AA-derived metabolites, formed through the action of cytochrome P-450 (CYP)-epoxygenases, including cyp2c9 that have been proposed to be endothelium-derived hyperpolarizing factor (EDHF) (12). Activation of A2A AR is related to increased activity of CYP-epoxygenase that results in vasorelaxation (8, 29). We also found an upregulation of cyp2c9 associated with the presence of A2AAR that caused endothelium-dependent relaxation (29). In studies from our laboratory, we have shown that A2AKO mice have impaired relaxation to adenosine and its analogues (29, 34), and that A2AAR mediated aortic relaxation involves EETs (29, 33).

We have also shown that activation of A2A AR is related to increased activity of CYP-epoxygenases, the enzymes that metabolize AA to EETs, which results in vasorelaxation (8, 29), and lack of the A2AAR results in lowered aortic relaxation through 20-HETE via PKC-α/p-ERK pathway (33). However, the pathway linking A2AAR-mediated aortic relaxation via EETs is still not clear.

The endothelium modulates vascular tone through many factors, including nitric oxide (NO) and EETs. Several studies have shown that adenosine-mediated hyperpolarization of arteries involves the opening of ATP-sensitive K+ (KATP) channels and a role for A2A and A2B receptors (16, 17, 20, 28) as well as a role for NO (17, 18). In addition, EETs are potent KATP activators (48). However, the relationship among A2AAR, EETs, NO, and KATP channels has not been investigated.

The purpose of the present study was to elucidate the mechanism that contributes to A2AR-mediated aortic relaxation and to examine the relationship among EETs, NO, KATP channels, and A2AAR. We tested the hypothesis that A2AR-induced aortic relaxation is through the opening of KATP channels and, that, endothelial factors, including EETs, contribute to this mechanism. To test this hypothesis, we used A2AKO, A2BKO, and A2A2B DKO mice, in addition to various pharmacological tools.

MATERIALS AND METHODS

Animals. A2AKO mice (originally from C. Ledent, Universite Libre de Bruxelles, Brussels, Belgium) and A2A2B double knockout mice (A2A2B DKO) were obtained from Dr. Stephen Tilley, UNC Chapel Hill on C57BL/6 background. C57BL/6j (wild type, WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). A2AKO mice were purchased from The Jackson Laboratory (Bar Harbor, ME).
mice were backcrossed 12 generations to the C57BL/6 background, and the A2A-KO mice were generated and genotyped by polymerase chain reaction (PCR). Males and females (equal numbers) 14–18 wk old were used in our studies. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of West Virginia University.

**Drugs and chemicals.** Acetylcholine and phenylephrine were dissolved in distilled water. 5'-N-ethoxycarbonyl-2'-deoxyadenosine (NECA), 2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamido adenine hydrochloride (CGS 21680), pinacidil, cromakalim, and SCH 58261 were dissolved in 100% DMSO as a 10 mM stock solution; N\(^6\)-nitro-L-arginine methyl ester (l-NAME) was dissolved in 100% DMSO as a 0.1 M stock solution. Methyshethylpropargyloxy-phenylethanolamid (MS-PPOH; Cayman Chemical, Ann Arbor, MI) and 5-hydroxydecanooe (5-HD)- were dissolved 100% ethanol as 10 mM and 0.1 M stock solutions, respectively. The final concentration of DMSO in organ bath (10 ml) had no effect by itself on the aortic rings. Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (St. Louis, MO).

**Preparation of isolated mouse aorta and isotonic force measurement.** Mice were euthanized by anesthesia with pentobarbital sodium (65 mg/kg ip) followed by thoracotomy and removal of aorta that was then cut transversely into 3- to 4-mm rings. The rings were mounted vertically between two stainless steel wire hooks and then suspended in 10-ml organ baths containing Krebs-Henseleit buffer. The Krebs-Henseleit buffer (pH 7.4) containing (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 11 glucose, and 2.5 CaCl\(_2\) was maintained at 37°C with continuous bubbling of 95% O\(_2\) and 5% CO\(_2\). For measurement of isotonic force response, aortic rings were equilibrated for 90 min with a resting force of 1 g and change of the bathing solution at 15-min intervals. The resting force of 1 g has been used earlier in our laboratory and was determined using a length-tension relationship (3, 32, 46). At the end of the equilibration period, tissues were contracted with 50 mM KCl to check the contractility of individual aortic rings twice, which were washed out with Krebs-Henseleit buffer. Aortic rings were then constricted with phenylephrine (PE) at submaximal concentration (10\(^{-6}\) M) to obtain a steady contraction, and changes in tension were monitored continuously with fixed range precision force transducer (TSD, 125 C, BIOPAC system) connected to the differential amplifier (DA100E, BIOPAC system). The data were recorded using MP100, BIOPAC digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC system).

**Contraction/relaxation experiments.** After equilibration, the responsiveness and stability of individual rings were checked by administration of PE (10\(^{-6}\) M). The integrity of the vascular endothelium was assessed pharmacologically by acetylcholine (10\(^{-6}\) M) to produce relaxation of PE precontracted rings. Aortic rings were then washed several times with Krebs-Henseleit solution and allowed to equilibrate for 30 min before the experimental protocol began. At the end of concentration response, tissues were finally relaxed with the use of sodium nitroprusside (10\(^{-5}\) M). In experiments where the involvement of the endothelium in KATP-mediated vascular relaxation was assessed pharmacologically by acetylcholine (10\(^{-6}\) M), pinacidil, cromakalim, 10\(^{-6}\) M) and CGS 21680 responses were determined in PE-precontracted tissues in parallel as described above. The effect of SCH 58261 (A2AKR antagonist, 10\(^{-5}\) M), l-NAME (NO synthase inhibitor, 10\(^{-4}\) M) and, MS-PPOH (CYP-epoxygenase inhibitor, 10\(^{-5}\) M) on pinacidil response was also studied in PE-precontracted tissues in parallel as described above. Drugs were added 30 min before contraction of the tissue with PE and were present throughout the experiments.

**Effects of endothelium removal, SCH 58261, l-NAME, and MS-PPOH on pinacidil response.** Pinacidil concentration-response (10\(^{-11}\)–10\(^{-5}\) M) was run in WT and A2A-KO aorta in which endothelium was removed. This was done to determine the contribution of endothelium in KATP-mediated vascular relaxation. The effect of SCH 58261 (A2AKR antagonist, 10\(^{-6}\) M), l-NAME (NO synthase inhibitor, 10\(^{-4}\) M) and, MS-PPOH (CYP-epoxygenase inhibitor, 10\(^{-5}\) M) on pinacidil response was also studied in PE-precontracted tissues in parallel as described above. Drugs were added 30 min before contraction of the tissue with PE and were present throughout the experiments.

**Western blot analysis.** Aorta from WT and A2A-KO mice were isolated, and each sample was homogenized with 130 μl RIPA buffer (Cell Signaling Technology) on wet ice. The samples were transferred to dry ice for 5 min and then thawed on wet ice. After thawing was completed, the samples were vortexed and centrifuged for 5 min at 12,000 rpm at 4°C. Lysates were sonicated and stored at −80°C. Protein was measured using Bio-Rad assay based on the Bradford dye procedure with bovine serum albumin (BSA) as a standard. The protein mixture was divided into aliquots and stored at −80°C. At the time of analysis, samples were thawed and 30 μg of total protein per lane were loaded on a slab gel. Proteins were separated by SDS-PAGE using 10% acrylamide gels (1-mm thick). After electrophoresis, the proteins on the gel were transferred to nitrocellulose membrane (Hybond-P Membrane; Amersham Biosciences) according to the instructions of the manufacturer. The membranes were incubated with primary antibodies for the KATP channel subunits Kir (Kir6.1, Santa Cruz Biotechnology, Santa Cruz, CA) and SUR 1 (Millipore, CA). The SUR antibody detects SUR 1/2 at 70 kDa and was used at a dilution of 1:1000, while 1:500 dilution was used for the Kir subunit. β-Actin antibody (Santa Cruz Biotechnology) was used as an internal control to normalize the target protein expression in each lane. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG. The membranes were developed using enhanced chemiluminescence (Amerham BioSciences) and exposed to X-ray film for the appropriate time. The data are presented as the ratio of target protein expression to β-actin. The films were scanned using EPSON Perfection V-30 series scanner, and ImageJ software was used to quantify the data.

**Real-time PCR for expression of Kir 6.1 and SUR 2 genes.** The aortic tissues from WT and A2A-KO mice were processed for total RNA isolation using the TRI reagent (MRC, 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 High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μl. Real-time PCR was then performed using an ABI PRISM 7300 Detection System (Applied Biosystems) using Taqman Universal Mastermix (Applied Biosystems, Branchburg, NJ) according to the instructions of the manufac-
RESULTS

Pinacidil, cromakalim, and 5-HD response in WT and A2AKO mouse aorta. A2AKO mice had significantly lower vascular relaxation (25.41 ± 2.73%, n = 7) compared with WT (48.09 ± 5.23%, n = 7; P < 0.05; Fig. 1A; representative tracings Fig. 2A). To confirm whether this effect was also seen with another KATP opener and not limited just to pinacidil, we used cromakalim for a concentration-response curve (Fig. 1B; representative tracings Fig. 2B). Cromakalim produced greater relaxation in WT aorta compared with that of A2AKO (51.19 ± 2.05% vs. 38.50 ± 2.26% at 10⁻⁶ M; P < 0.05, n = 4). Diazoxide, the mitochondrial K<sub>ATP</sub> opener, did not produce any relaxation in either WT or A2AKO aorta (Fig. 1C; representative tracings Fig. 2C). This suggested the involvement of sarcolemmal K<sub>ATP</sub> channels in the pinacidil- and cromakalim-mediated relaxation responses.

Effect of glibenclamide on pinacidil, NECA, and CGS 21680-induced vascular response. Glibenclamide (10⁻⁵ M, K<sub>ATP</sub> inhibitor) significantly reduced the pinacidil-mediated relaxation (Fig. 3A; representative tracings Fig. 2D) in both WT (from relaxation of 48.09 ± 5.23% to contraction of 3.55 ± 5.87% at 10⁻⁶ M; P < 0.05) and A2AKO (from relaxation of 25.41 ± 2.73% to contraction of −5.14 ± 4.76% at 10⁻⁶ M; P < 0.05) mice. Glibenclamide decreased NECA-mediated relaxation in WT (from 1.31 ± 3.24% to contraction of −35.38 ± 6.62% at 10⁻⁶ M; P < 0.05, n = 6–8; Fig. 3B) and increased the contraction seen in A2AKO (from −15.57 ± 5.11% to −56.45 ± 6.97% at 10⁻⁶ M; P < 0.05, n = 6–12; Fig. 3B). Glibenclamide also decreased the relaxation response to CGS 21680 (selective A<sub>2A</sub>AR agonist; Fig. 3C) in WT (from 19.02 ± 2.75% to 4.74 ± 6.13% at 10⁻⁶ M; P < 0.05, n = 4). We also used 5-HD (mitochondrial K<sub>ATP</sub> inhibitor, 10⁻⁴ M) in CGS 21680 response to determine the contribution of mitochondrial K<sub>ATP</sub> channels in A2AR-induced response. 5-HD did not have any effect on CGS 21680-mediated response in WT (data not shown), suggesting that the A2AR-mediated response involved sarcolemmal K<sub>ATP</sub> channels.

Pinacidil response in A<sub>2B</sub>KO, A<sub>2A</sub>Δ<sub>B</sub> DKO aorta, and effect of SCH 58261 in WT aorta. Pinacidil response was also studied in A<sub>2B</sub>KO and A<sub>2A</sub>Δ<sub>B</sub> DKO aorta (Fig. 4A) to confirm the contribution of the A2AR in K<sub>ATP</sub>-mediated response. A<sub>2B</sub>KO had a similar relaxation response to the WT (41.13 ± 4.71% vs. 48.09 ± 5.23% at 10⁻⁶ M in WT), whereas the A<sub>2A</sub>Δ<sub>B</sub> DKO had a similar relaxation response to the A2AKO (19.17 ± 3.73% vs. 25.41 ± 2.73 at 10⁻⁶ M in A2AKO). This suggested that presence of A2AR was essential for maximum relaxation to K<sub>ATP</sub> opener pinacidil. Furthermore, we used SCH 58261 (A2AR antagonist; Fig. 4B) in WT and A2AKO and then performed a pinacidil concentration response: when the A2AR was blocked in WT, the response produced was similar to A2AKO, whereas there were no differences observed between SCH 58261-treated and nontreated A2AKO aorta.

Effect of removal of endothelium, NO synthase inhibitor (l-NAME), and CYP-epoxygenase inhibitor (MS-PPOH) on pinacidil response. We determined the contribution of the endothelium to pinacidil-mediated relaxation by performing a concentration response in WT and A2AKO aorta (Fig. 5A), in which the endothelium had been removed (−E). Removal of the endothelium reduced the relaxation observed in WT (from 48.09 ± 5.23% to 23.40 ± 2.96% at 10⁻⁶ M) to the same level as intact endothelium A2AKO aorta, whereas removal of the endothelium had no significant effect in A2AKO (25.41 ± 2.73% vs. 24.240 ± 3.79% at 10⁻⁶ M), suggesting the A2A receptor-K<sub>ATP</sub> mediated relaxation was dependent on endothelium.

In the intact endothelium of WT and A2AKO aortic tissue, the relaxation to pinacidil was inhibited by l-NAME (10⁻⁴ M; Fig. 5B) and MS-PPOH (10⁻⁵ M; Fig. 5C), respectively. With l-NAME, the WT response significantly decreased from 48.09 ± 5.23% to 25.41 ± 2.73% (at 10⁻⁶ M; P < 0.05), whereas in the A2AKO, there was no significant decrease.

**Fig. 1.** Concentration responses to pinacidil [A, ATP-sensitive K⁺ channel (K<sub>ATP</sub>) opener; n = 7 mice], cromakalim (B, K<sub>ATP</sub> opener; n = 4 mice), and diazoxide (C, mitochondrial K<sub>ATP</sub> opener; n = 4 mice) in wild-type (WT) and A2AKO knockout (KO) mouse aorta (C57BL/6J background). Values are expressed as means ± SE; *P < 0.05 compared with WT.
Similarly, with MS-PPOH, the relaxation response to pinacidil was significantly lowered ($P < 0.05$) in WT from $48.09 \pm 5.23\%$ to $25.95 \pm 3.70\%$ at $10^{-6} \text{M}$ with no difference observed in $A_{2\text{A}}$KO ($25.41 \pm 2.73\%$ vs. $20.48 \pm 2.80\%$ at $10^{-6} \text{M}; P < 0.05$). We also performed these experiments with cromakalim and the findings were comparable with pinacidil. (data not shown). These data suggest the involvement of endothelial factors (NO and EETs) in $A_{2\text{A}}$AR-KATP-mediated relaxation.

Expression of Kir 6.1 and SUR 1/2 subunits in $A_{2\text{A}}$KO and WT mouse aorta. The protein and gene expressions of the KATP channel subunits Kir and SUR were determined in WT and $A_{2\text{A}}$KO aorta (Fig. 6). There were no significant differences in either the protein ($100 \pm 21.18\%$ in WT vs. $111.81 \pm 12.81\%$) or gene expressions ($100 \pm 21.18\%$ in WT vs. $111.81 \pm 12.81\%$) of Kir and SUR between WT and $A_{2\text{A}}$KO aorta. These data suggest the involvement of endothelial factors (NO and EETs) in $A_{2\text{A}}$AR-KATP-mediated relaxation.

Fig. 2. Representative tracings for concentration responses to pinacidil ($A$, $n = 7$), cromakalim ($B$, $n = 4$), diazoxide ($C$, $n = 4$), and effect of glibenclamide on pinacidil ($D$, $n = 5$) in WT and $A_{2\text{A}}$KO mice aorta. PE, phenylephrine.

Fig. 3. Effect of glibenclamide ($10^{-5} \text{M}$) on pinacidil ($A$), 5’-N-ethylcarboxamide (NECA, nonselective adenosine analog) ($B$), and CGS 21680 (selective $A_{2\text{A}}$AR agonist) ($C$) concentration responses in WT and $A_{2\text{A}}$KO aorta. Values are expressed as means $\pm$ SE ($n = 4$–12 mice). *$P < 0.05$ compared with WT, #*$P < 0.05$ compared with $A_{2\text{A}}$KO.
smooth muscle cells in resistance and conduit vessels (36) and are inhibited by intracellular ATP (5, 36). Pharmacologically, these channels are inhibited by sulfonyleurea drugs such as glibenclamide (5, 36) and activated by potassium channel openers (PCO) like pinacidil. Opening of K_{ATP} channels hyperpolarizes membrane potential that results in vasorelaxation (9). The opening of K_{ATP} channels is known to be one of the signaling mechanisms by which adenosine receptors induce vasorelaxation in several vascular beds (15, 19, 22, 31, 42, 45). We used pinacidil to determine the involvement of K_{ATP} channels. We found concentration-dependent relaxation to pinacidil in both A2A KO and WT mice aorta, but the A2A KO had significantly lower relaxation compared with WT (Fig. 1A). While we expected pinacidil to cause relaxation in the WT aorta, the finding of lower relaxation in the A2A KO upon direct activation of the K_{ATP} channels was intriguing and noteworthy. We used a different PCO, cromakalim, to determine whether this response was unique to pinacidil or a general feature of PCOs in A2A KO aorta. Our data showed that cromakalim also had the same effect; i.e., the A2A KO had significantly lower relaxation compared with the WT (Fig. 1B). Diazoxide, which is a mitochondrial K_{ATP} activator (11), had no relaxation effect in either WT or A2A KO (Fig. 1C), suggesting the involvement of sarcolemmal K_{ATP} channels. Glibenclamide (K_{ATP} channels inhibitor) blocked the pinacidil-induced response, confirming that pinacidil acted by opening K_{ATP} channels (Fig. 3A).

Vascular relaxation to adenosine is induced via opening of K_{ATP} channels through the A2A and A2B receptors (16, 17, 20, 28). Our laboratory has also recently shown that A2A and A2B receptors contribute to coronary vasodilation via K_{ATP} channels (40). In this study, we found that nonselective adenosine analog NECA-induced relaxation was lower in WT aorta with the use of glibenclamide, whereas the contraction response seen in the A2A KO was significantly higher (Fig. 3B). This confirmed that K_{ATP} channels contributed to adenosine-mediated relaxation, as blocking of the channels either reduced relaxation (WT) or increased contraction (A2A KO) to NECA. To delineate the selectivity of the A2AAR-induced response by K_{ATP} channels, we used glibenclamide with the selective A2A AR agonist CGS 21680 in WT aorta and found that glibenclamide blocked the relaxation to CGS 21680 (Fig. 3C). These findings were in accordance with other studies, which found that glibenclamide blocked adenosine and A2A AR-mediated vasorelaxation in other vascular beds (15, 16). On the other hand, the mitochondrial K_{ATP} inhibitor 5-HD had no effect on CGS 21680 response. These data indicated that activation of A2A AR induced aortic relaxation via the opening of sarcolemmal K_{ATP} channels.

Previous studies have shown that activation of both the A2A AR and the A2B AR may contribute to adenosine-mediated responses in arterioles, as well as conduit vessels like the aorta (3, 29, 34). The A2A and A2B receptors have been shown to mediate vascular relaxation via K_{ATP} channels. We also ran concentration curves to pinacidil in A2B KO and A2A/2B DKO aorta to determine the contribution of the A2AAR and A2BAR to pinacidil-mediated response and also to investigate whether absence of the A2B AR resulted in similarly lower aortic relaxation to pinacidil as observed in the A2A KO. The A2B KO had the same relaxation response as the WT while the DKO had the same response as the A2A KO; i.e., lower compared with the WT (Fig. 4A). This suggested that the presence of the A2A and
not the A2B AR was essential to the difference in pinacidil response that we had observed. To further confirm this, we blocked the A2AAR in WT pharmacologically using SCH 58261, which is a selective A2AAR antagonist; use of SCH 58261 produced the same effect as knocking down of the A2AAR (Fig. 4B).

The endothelium and endothelium-derived factors play vital roles in adenosine-mediated vascular responses (3, 4, 27, 29, 34). These effects are either completely endothelium dependent or partially endothelium dependent in various isolated vessels (3, 4, 30, 39, 49). Several labs, including ours, have reported that the A2AAR contributes to vascular relaxation through endothelium-dependent mechanisms (1, 25–27, 29, 34, 37, 38) in aorta and other vascular tissues. We found that upregulation of CYP-epoxygenase (CYP2C29) is associated with the presence of A2AAR in the endothelium-dependent relaxation to adenosine agonists (NECA and CGS 21680) in the aorta (29) and also that basal levels of CYP-epoxygenases are significantly lower in A2AKO aorta compared with those of WT (29, 33). These observations were accompanied by findings that showed inhibition of CYP-epoxygenases by MS-PPOH abolished A2AAR-induced relaxation in intact aorta, whereas upon removal of the endothelium, MS-PPOH did not have any effect (33).

Fig. 5. A: concentration response to pinacidil in WT and A2AKO in the presence and absence (∼E) of endothelium. B: effect of nitro-L-arginine methyl ester (L-NAME) (nitric oxide synthase inhibitor; 10⁻⁴ M) in WT and A2AKO aorta. C: effect of methylsulfonyl-propargyloxyphenylhexanamide (MS-PPOH) [cytochrome P-450 (CYP)-epoxygenases inhibitor; 10⁻⁵ M] in WT and A2AKO aorta. Values are expressed as means ± SE (n = 5–9 mice). *P < 0.05 compared with WT. The control responses are same for WT and A2AKO in A, B, and C.

Fig. 6. A: expression of Kir 6.1 subunit in WT and A2AKO aorta and densitometric analysis. B: gene expression of Kir 6.1 subunit in WT and A2AKO aorta. C: expression of SUR 1/2 subunit in WT and A2AKO aorta and densitometric analysis. D: gene expression of SUR 2 subunit in WT and A2AKO aorta. Western blot data are presented as means (% ratio to respective control) ± SE (n = 3–4 mice). All the gene expressions were normalized to 18s rRNA and expressed as a ratio to one control tissue (means ± SE; n = 6–8).
We removed the endothelium and found that the WT response to pinacidil in tissues without endothelium was the same as the A2AKO tissue with the endothelium intact (Fig. 5A). Previously, we have also found that A2AKO have less ACh-mediated response and the A2AAR-mediated vascular relaxation was endothelium dependent as removal of endothelium abolished response to A2AAR agonist CGS 21680 (33, 34). One of the possible factors that may cause the endothelium-dependent response is CYP-epoxygenase, which generates AA-derived metabolites in the form of EETs in the endothelium. These EETs have been proposed to act as EDHFs (12). To determine whether CYP-epoxygenases play any role in the pinacidil-induced response, we used MS-PPOH, CYP-epoxygenases inhibitor. We observed that in the WT, pinacidil-induced relaxation was significantly reduced with MS-PPOH (Fig. 5C). These data confirmed that endothelial CYP-epoxygenases were involved in pinacidil-induced relaxation via the opening of KATP channels. Other labs have also reported that genases were involved in pinacidil-induced relaxation via the mechanism that exists between the activation of A2AAR and tigations are necessary to delineate a more detailed signaling pathway. Studies have also shown a role for NO in the A2AAR-KATP mediation of A2AAR-induced aortic relaxation and that A2AKO have less KATP-mediated response. Based on the observations that A2AKO had lower KATP-mediated relaxation, we measured expression of the KATP subunits Kir 6.1 and SUR 1/2 by Western blot and the Kir 6.1 and SUR 2 subunits by real-time RT-PCR to understand if differential expression of the channel was a possible explanation for the functional data. There were no differences in either the protein or gene expressions of the KATP subunits in WT and A2AKO aorta (Fig. 6). There could be two explanations for this; we have found that the endothelial response is less in the A2AKO and also found that CYP-epoxygenases are lower (29, 33, 34). It is possible that due to some dysfunction in the endothelium as well as lower basal levels or release of endothelium-derived factors like EETs and NO that A2AKO have lower pinacidil response, despite having unaltered expression of KATP channels. Studies have shown that differences in basal NO release account for differences in vasodilation to pinacidil (10). More studies will have to be done, however, to understand this difference in pinacidil-response.

Perspectives and Significance

The A2A receptor is believed to mediate adenosine-induced relaxation in the mouse aorta (35, 46), and we have shown that the endothelium is involved in this response. We have also shown that the endothelial factor (e.g., EETs) plays a role in mediating A2AAR-induced aortic relaxation and that A2AKO mouse aorta has lower basal levels of CYP-epoxygenases compared with WT. In summary, the present study shows that the A2AAR-induced aortic relaxation involves the opening of sarcolemmal KATP channels and that both nitric oxide and CYP-epoxygenases contribute to this relaxation. Further investigations are necessary to delineate a more detailed signaling mechanism that exists between the activation of A2AAR and the opening of KATP channels. Given the importance of the A2AAR in modulating vascular tone under conditions like hypoxia and ischemia, this can lead to better approaches for the treatment of vascular diseases.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions


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