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High salt diet modulates vascular response in $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice: role of sEH, $PPAR\gamma$, and K_{ATP} channels

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Abstract

This study aims to investigate the signaling mechanism involved in HS-induced modulation of adenosine-mediated vascular tone in the presence or absence of adenosine A_{2A} receptor ($A_{2A}AR$). We hypothesized that HS-induced enhanced vascular relaxation through $A_{2A}AR$ and epoxyeicosatrienoic acid (EETs) is dependent on peroxisome proliferator-activated receptor gamma ($PPAR\gamma$) and ATP-sensitive potassium channels (K_{ATP} channels) in $A_{2A}AR^{+/+}$ mice, while HS-induced vascular contraction to adenosine is dependent on soluble epoxide hydrolase (sEH) that degrades EETs in $A_{2A}AR^{-/-}$ mice. Organ bath and Western blot techniques were

conducted in HS (4 % NaCl) and normal salt (NS, 0.45 % NaCl)-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mouse aorta. We found that enhanced vasodilation to $A_{2A}AR$ agonist, CGS 21680, in HS-fed $A_{2A}AR^{+/+}$ mice was blocked by PPAR γ antagonist (T0070907) and K_{ATP} channel blocker (Glibenclamide). Also, sEH inhibitor (AUDA)-dependent vascular relaxation was mitigated by PPAR γ antagonist. PPAR γ agonist (Rosiglitazone)-induced relaxation in HS- $A_{2A}AR^{+/+}$ mice was attenuated by K_{ATP} channel blocker. Conversely, HS-induced contraction in $A_{2A}AR^{-/-}$ mice was attenuated by sEH inhibitor. Overall, findings from this study that implicates the contribution of EETs, PPAR γ and K_{ATP} channels downstream of $A_{2A}AR$ to mediate enhanced vascular relaxation in response to HS diet while, role of sEH in mediating vascular contraction in HS-fed $A_{2A}AR^{-/-}$ mice.

Keywords

High salt; $A_{2A}AR$; PPAR γ ; EETs; K_{ATP} channel; Soluble epoxide hydrolase

Introduction

Adenosine, an endogenous nucleoside, is generally cytoprotective in nature and produced in response to stressful conditions. Adenosine mediates its effects via four adenosine receptors: A_1 , A_{2A} , A_{2B} , and A_3 [1]. Among many stressful conditions such as ischemia, hypoxia, injury, and inflammation, exposure to high salt (HS) diet has also been shown to elevate the generation of adenosine [2, 3]. Growing evidence suggests a link between high dietary salt intake and activation of adenosine receptors and A_{2A} receptor in particular [4–6]. In response to salt loading, enhanced vasodilation has been reported to occur through $A_{2A}AR$ [4–8].

HS-induced $A_{2A}AR$ -mediated vasodilation is dependent on epoxyeicosatrienoic acid (EETs) generation through enhanced cyp-epoxygenase activity [6, 9, 10]. Studies from our lab and others have found that compared to normal salt (NS), HS diet-fed mouse aortae produced enhanced vascular relaxation to selective adenosine A_{2A} receptor agonist (CGS-21680), and this enhanced dilation was blunted by cyp-epoxygenases inhibitor, methylsulfonyl-propargyloxyphenylhexanamide (MSPPOH) [5], and also by EETs antagonist (14,15 EEZE) [11].

EETs are 20-carbon fatty acid derived from the metabolism of arachidonic acid (AA) by cytochrome P450 epoxygenases (CYPs). The cellular levels of EETs depend not only on their production by CYPs but also on hydrolysis to dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) [12]. Inhibiting or deleting sEH has become an important approach to increase the level of EETs that cause reduction in blood pressure in mice [13, 14]. Many effects exerted by EETs overlap with adenosine function including regulation of vascular tone, inflammation, proliferation, platelet aggregation, and cardiac function [15–17]. The receptors for EETs are, however, poorly understood. EETs receptors have not been identified and cloned yet, and the mechanism by which EETs mediate their effects is largely unknown. However, there is some evidence suggesting that EETs act through receptors, and some studies suggest that EETs have affinity to already known

binding sites and receptors [18–20]. Report showing that EETs specifically bind to the plasma membrane of human U937-transformed monocytes [19] indicates the presence of EETs receptor. Given its pleiotropic effects, EETs may act through number of different receptors.

Many of the long-term actions of the epoxygenases pathways are common to PPARs activation. Activation of PPARs, similar to effect of EETs, causes regulation of vascular tone, vascular cell proliferation, migration, and inflammation [21, 22]. Proposed endogenous PPAR ligands have originated from AA and linoleic acid metabolism, among which possible ligands could be produced by epoxygenases pathway. Few studies have been conducted to examine the possible candidacy of epoxygenase-derived metabolites as PPAR ligands. Cyp4a metabolite of EETs has been reported to be high affinity PPAR ligands [23, 24]. Also, EETs protect against angII-induced abdominal aneurysm in cyp2j2-overexpressed mice through PPAR γ [25]. In addition, EETs-induced angiogenesis in endothelial progenitor cells in patients with acute myocardial infarction is demonstrated to be dependent on PPAR γ [26]. Liu et al. reported that PPAR γ antagonist (GW9662) blocked EETs/AUDA-mediated anti-inflammatory effects [27], suggesting that PPAR γ is an effector of EETs. The EETs-PPAR γ pathway may therefore represent a novel endogenous pathway by which transitory lipid mediators control vascular cell function. Henceforth, it would be interesting to evaluate the role of PPAR γ on A_{2A}AR-EETs-mediated regulation of vascular tone in HS and NS-fed mice.

The peroxisome proliferator-activated receptors (PPARs) are ligand activated transcriptional factors belonging to the family of three (α , β/δ , γ) nuclear receptors that are activated by lipid metabolites and play a critical role in glucose homeostasis and lipid metabolism. PPARs have the ability to bind to large number of ligands mainly because of their large ligand-binding domain [28, 29]. Accumulating evidence suggests that PPAR γ contributes in regulation of vascular function and blood pressure in addition to its well-recognized role in adipogenesis and insulin sensitivity. Thiazolidinedione (TZD), specific PPAR γ ligands, has been reported to lower blood pressure and provide cardiovascular benefits through regulating vascular function and vascular tone [30]. Studies have demonstrated that PPAR γ activation elicits relaxation in pulmonary arteries by activating K_{ATP} channels through unknown mechanism [31].

It is interesting that activation of PPAR γ , similar to effects of A_{2A}AR and EETs, causes vasodilation, anti-inflammation, and anti-proliferation. To our interest, it is evident that A_{2A}AR and EETs are involved in HS-induced vascular relaxation; however, information regarding the role of PPAR γ and K_{ATP} channels is lacking. Here in this study, the role of PPAR γ and K_{ATP} channels in HS-induced A_{2A}AR-mediated vascular relaxation and the interplay between A_{2A}AR, EETs, and sEH in the regulation of vascular tone are explored using HS and NS-fed A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice.

Materials and methods

The experimental and animal care protocols used in this study were approved by the West Virginia University Institutional Animal Care and Use Committee and carried out according

to the principles and guidelines of the Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals. Ten-week-old inbred male and female CD-1 ($A_{2A}AR^{-/-}$ and $A_{2A}AR^{+/+}$ mice) obtained from Dr. Ledent (Belgium), bred and maintained in our facility at West Virginia University were placed on a whole-grain diet containing either 0.45 % NaCl, NS, or 4 % NaCl, high salt (HS) (TD88311 and TD92100 diets; Teklad, Madison, WI). The initial characterization of $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice has been previously described by Ledent et al. [32]. In brief, to produce mice on a homogenous genetic background, first-generation heterozygotes were bred for 14 generations to mice on a CD-1 (Charles River Laboratories) outbred background, with selection for the mutant $A_{2A}AR$ gene at each generation by PCR. Fourteenth-generation heterozygotes were bred together to generate $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ (1:1, their mate controls) mice [32]. All mice were studied 4–5 weeks after assignment to either the NS or HS group.

Isometric tension muscle bath experiments

HS and NS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice were euthanized with pentobarbital sodium (100 mg/kg, i.p.). The aorta was gently removed after thoracotomy, cleaned of fat and connective tissues, and cut transversely into rings of 3–4 mm in length as described previously by us [5]. Extreme care was taken not to damage the endothelium. The rings were mounted vertically between two stainless steel wire hooks. The two rings were suspended in 10 ml organ baths containing modified Krebs–Henseleit buffer (in 118 mM of NaCl, 4.8 mM of KCl, 1.2 mM of $MgSO_4$, 1.2 mM of KH_2PO_4 , 25 mM of $NaHCO_3$, 11 mM of glucose, and 2.5 mM of $CaCl_2$). The buffer was maintained around pH 7.4 at 37 °C. The aortic rings were equilibrated for 60 min with a resting force of 1 g as described previously by us [5]. At the end of the equilibration period, tissues were contracted with KCl (50 mM) to check the viability. Aortic rings were then constricted with phenylephrine (PE, 10^{-6} M), and changes in tension were monitored continuously with a fixed range precision force transducer (TSD, 125 C, BIOPAC system). Data were recorded using MP100 WSW, BIOPAC digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC system). The vascular endothelium was tested to determine whether it was intact, as previously described by our laboratory [5] through acetylcholine (ACh, 10^{-6} M) on pre-contracted aortic rings with phenylephrine (PE). Preparations were then washed several times with Krebs–Henseleit buffer solution and allowed to equilibrate for 30 min before the experimental protocol began. For all tests, the contraction and relaxation responses are expressed as % decrease or % increase of PE-induced pre-contraction.

CGS 21680, AUDA, and Rosiglitazone-induced vascular response in $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice fed HS and NS diet

The responsiveness of pre-contracted aortic rings from $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice fed HS and NS diet to the selective sEH inhibitor, 12-(3-adamantan-1-yl-ureido)-do-decanoic acid (AUDA), selective $A_{2A}AR$ agonist, 2-*p*-(2-carboxyethyl) phenethylamino-5'-*N*-ethylcarboxamido adenosine hydrochloride (CGS 21680), or selective PPAR γ agonist, Rosiglitazone, were obtained by cumulative addition of these drugs to the organ bath in 1-log increments to obtain a concentration-response curve (CRC) as previously described [28, 29]. All concentration-response determinations were run in parallel on pairs of rings from either HS ($A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$) or NS ($A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$).

Effects of PPAR γ antagonists on 5'-N-ethylcarboxamidoadenosine (NECA), CGS 21680, and AUDA-induced vascular response in A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice fed HS and NS diet

Selective PPAR γ antagonist (T0070907; 0.1 μ M) was added 30 min before contraction of the tissue with PE and was present throughout the experiment. These experiments were performed in parallel on four rings from the same aorta with two serving as control and two treated with T0070907.

Effects of sEH inhibitor on NECA-induced vascular response in A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice fed HS and NS diet

Selective sEH inhibitor, 12-(3-adamantan-1-yl-ureido)-do-decanoic acid (AUDA; 10 μ M), was added 30 min before contraction of the tissue with PE and was present throughout the experiment. These experiments were performed in parallel on four rings from the same aorta with two serving as control and two treated with AUDA.

Effects of K_{ATP} channels inhibitor and A_{2A}AR antagonist on CGS 21680, Rosiglitazone-induced vascular response in A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice fed HS and NS

Selective K_{ATP} channel inhibitor (Glibenclamide, 10 μ M) and selective A_{2A}AR antagonist (SCH-58261; 1 μ M) were added 30 min before contraction of the tissue with PE and were present throughout the experiment. These experiments were performed in parallel on four rings from the same aorta with two serving as control and two treated with Glibenclamide or SCH-58261.

Western blot analysis

Aortae from HS and NS-fed A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice were isolated, and each sample was homogenized with 130 μ l RIPA buffer (Cell Signaling Technology Inc) on wet ice. The samples were transferred to dry ice for 5 min and then thawed on wet ice. After thawing, lysates were sonicated and the samples were vortexed and centrifuged for 5 min at 12,000 rpm at 4 °C. Then, the supernatant was 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 μ g of total protein per lane was 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-ECL) by electroelution. Protein transfer was confirmed by employing pre-stained molecular weight markers (Bio-Rad Laboratories, Hercules, CA). Following blocking with either 5 % nonfat dry milk or BSA, the nitrocellulose membranes were incubated with primary antibodies for sEH and PPAR γ (Santa Cruz Biotechnology, Santa Cruz, CA). 1:5000 primary antibody concentration was used for sEH antibody, and 1:1000 primary antibody concentration was used for PPAR γ antibody. β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 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-mouse IgG. The membranes were developed using enhanced chemiluminescence (Amersham 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.

Chemicals, drugs, and antibodies

Phenylephrine hydrochloride and acetylcholine chloride were dissolved in distilled water. NECA, CGS 21680, SCH-58261, Glibenclamide (Sigma Chemicals, St. Louis, MO), Rosiglitazone (Cayman chemicals, Ann Arbor, MI) were dissolved in 100 % DMSO as 10 mM stock solutions, which were followed by serial dilutions in distilled water. T0070907 (Cayman chemicals) and AUDA (gift from Dr. Morisseau, UC Davis) were dissolved in DMSO. sEH (Santa Cruz Biotechnology) was used for Western blot experiments.

Statistical analysis

Statistical data are reported as mean $\pm$ SEM. One-way analysis of variance (ANOVA) was used to compare difference among groups and two-way ANOVA for repeated measure, followed by Tukey post hoc test to compare the vascular responses to antagonist SCH-58261, Glibenclamide, AUDA, and T0070907. Differences were considered significant if $p < 0.05$. Further, densitometry of Western blot analysis (sEH) data was expressed as mean $\pm$ SEM in arbitrary units. All the statistical analyses were performed using Graph Pad Prism statistical package.

Results

Effects of sEH inhibitor (AUDA) on NECA-dependent vascular response in HS and NS diet-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

HS-induced vascular response to NECA was significantly different in $A_{2A}AR^{+/+}$ versus $A_{2A}AR^{-/-}$ mice ($p < 0.05$; Fig. 1a, b). HS diet enhanced relaxation ($+17.34 \pm 2.50$ %) to NECA (10^{-6} M) in $A_{2A}AR^{+/+}$ mice compared to NS diet, whereas HS diet produced contraction (-56.77 ± 3.49 %) to NECA in $A_{2A}AR^{-/-}$ mice ($p < 0.05$; Fig. 1a, b). Previous study from our lab has shown downregulation of cyp-epoxygenases enzyme that produce EETs in HS-fed $A_{2A}AR^{-/-}$ mice [35]. Hence, we examined if increase in EETs using sEH inhibitor could improve vascular response from contraction to relaxation in $A_{2A}AR^{-/-}$ mice. AUDA significantly attenuated NECA (10^{-6} M)-dependent contraction (-56.77 ± 3.49 and -53.31 ± 7.27 %) in HS and NS-fed $A_{2A}AR^{-/-}$ mice, respectively (-14.72 ± 3.24 and -22.26 ± 3.63 %; $p < 0.05$; Fig. 1b). These results suggest that pharmacological inhibition of sEH using AUDA to increase EETs availability can reverse vascular contraction to NECA in $A_{2A}AR^{-/-}$ mice. But, AUDA did not further enhance relaxation in HS $A_{2A}AR^{+/+}$ group.

Effects of PPAR γ antagonist (T0070907) on CGS 21680, NECA, and AUDA-dependent vascular response in HS and NS diet-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

Selective $A_{2A}AR$ agonist, CGS 21680, demonstrated concentration-dependent vascular relaxation in both HS and NS-fed $A_{2A}AR^{+/+}$ mice with a significant difference ($p < 0.05$; Fig. 2a). HS-induced relaxation ($+27.59 \pm 3.04$ %) to CGS 21680 (10^{-6} M) was significantly diminished by PPAR γ antagonist, T0070907 (10^{-7} M), in $A_{2A}AR^{+/+}$ mice to $+10.60 \pm 1.84$ % ($p < 0.05$; Fig. 2a). However, relaxation response to CGS 21680 in NS-fed $A_{2A}AR^{+/+}$ mice (Fig. 2a) and contraction to NECA in NS/HS-fed $A_{2A}AR^{-/-}$ mice (Fig. 2b) were not affected by PPAR γ antagonist. This indicates that HS-induced $A_{2A}AR$ -enhanced relaxation which is dependent on PPAR γ in $A_{2A}AR^{+/+}$ compared to NS-fed mice.

We investigated the role of PPAR γ in AUDA-induced vascular response in NS/HS-fed A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice (Fig. 3). In Fig. 3, potent sEH inhibitor, AUDA produced concentration-dependent vascular relaxation ($+4.14 \pm 2.31$ % at 10^{-6} M; $p < 0.05$) in HS-fed A_{2A}AR^{+/+} mice compared to NS-fed A_{2A}AR^{+/+} mice (-3.94 ± 2.44 %). Selective PPAR γ antagonist, T0070907 (10^{-7} M), completely blunted AUDA-dependent vascular response (from $+4.14 \pm 2.31$ to -7.07 ± 2.98 % at 10^{-6} M; $p < 0.05$) in HS-fed A_{2A}AR^{+/+} mice (Fig. 3). No significant difference was noted with PPAR γ antagonist in AUDA-induced response in NS-fed A_{2A}AR^{+/+} mice (Fig. 3). These data suggest that AUDA-induced relaxation in HS-fed A_{2A}AR^{+/+} mice is dependent on PPAR γ .

Rosiglitazone-dependent vascular response in HS and NS diet-fed A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice

Selective PPAR γ agonist, Rosiglitazone, yielded concentration-dependent relaxation ($+6.02 \pm 2.60$ % at 10^{-6} M; $p < 0.05$) in HS-fed A_{2A}AR^{+/+} mice whereas contraction (-10.62 ± 3.00 %; 10^{-6} M) in NS-fed A_{2A}AR^{+/+} mice (Fig. 4a). On the other hand, Rosiglitazone (10^{-6} M) produced significant contraction in NS and HS-fed A_{2A}AR^{-/-} mice (-14.49 ± 2.02 and -18.47 ± 3.48 %; Fig. 4a), respectively. This interesting finding implies that HS-induced relaxation to Rosiglitazone requires A_{2A}AR, suggesting a link between A_{2A}AR and PPAR γ .

Effect of A_{2A}AR antagonist (SCH 58261) on Rosiglitazone-dependent vascular response in HS and NS diet-fed A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice

To pharmacologically confirm if HS-induced relaxation to Rosiglitazone is dependent on A_{2A}AR, we used selective A_{2A} receptor antagonist, SCH 58261 for Rosiglitazone CRC (Fig. 4b). SCH 58261 ($1 \mu\text{M}$) changed at 10^{-6} M, the Rosiglitazone-dependent response from -9.08 ± 2.51 and $+5.71 \pm 3.73$ % in NS and HS-fed A_{2A}AR^{+/+} aortae to -20.67 ± 2.81 and -10.53 ± 2.22 % ($p < 0.05$; Fig. 4b), respectively.

Effects of K_{ATP} channels inhibitor (Glibenclamide) on CGS 21680 and Rosiglitazone-dependent response in HS and NS diet-fed A_{2A}AR^{+/+} mice

Since K_{ATP} channels is also involved in vasodilation [16], we investigated the role of K_{ATP} channels in CGS 21680 and Rosiglitazone-induced relaxation in NS and HS-fed A_{2A}AR^{+/+} mice (Figs. 5, 6). Relaxation to NECA at 10^{-6} M in HS-fed A_{2A}AR^{+/+} mice was abolished by K_{ATP} channel inhibitor, Glibenclamide ($10 \mu\text{M}$), and the response changed from $+20.59 \pm 2.87$ to $+4.47 \pm 4.86$ % ($p < 0.05$; Fig. 5). There was no change in CGS21680-induced response in NS-fed A_{2A}AR^{+/+} mice with or without Glibenclamide (Fig. 5).

Similarly, Glibenclamide also blocked Rosiglitazone-induced vascular relaxation in HS-fed A_{2A}AR^{+/+} mice from $+3.50 \pm 2.87$ to -8.13 ± 1.78 % at 10^{-6} M ($p < 0.05$; Fig. 6), suggesting the role of K_{ATP} channels in Rosiglitazone-dependent relaxation.

sEH protein expression in HS and NS-fed A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice

sEH (~62 kDa) protein expression observed in both NS-fed A_{2A}AR^{-/-} and HS-fed A_{2A}AR^{-/-} mice (133.10 ± 16.48 and 129.4 ± 7.29 %) was ~33.1 and ~29 % higher

compared to their control counterparts NS and HS-fed $A_{2A}AR^{+/+}$ mouse aortae (99.96 ± 0.03 and 110.20 ± 5.19 %, $p < 0.05$ Fig. 7), respectively.

Discussion

Previously [11], we have reported a clear difference in vascular reactivity between $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice on a HS diet (high salt diet exacerbates contraction in the absence of $A_{2A}AR$ in $A_{2A}AR^{-/-}$ mice); HS produced enhanced relaxation to NECA through $A_{2A}AR$ -EETs pathway in $A_{2A}AR^{+/+}$ mice whereas contraction in $A_{2A}AR^{-/-}$ mice. The present study has undertaken the investigation of underlying mechanism involved in HS-induced modulation of vascular tone in the presence and absence of $A_{2A}AR$ using $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice fed HS/NS diet. The major findings in this study shed light upon the role of important mediators downstream of $A_{2A}AR$ and EETs in mediating HS-induced change in vascular reactivity to sEH inhibitor, PPAR γ agonist and antagonist in wild-type, and $A_{2A}AR$ -deficient mice. Results from this study identified PPAR γ and K_{ATP} channels as key signaling mediators downstream of $A_{2A}AR$ -EETs pathway that contributes to enhanced vascular relaxation in HS-fed $A_{2A}AR^{+/+}$ mice. Most importantly, this study revealed significant reversal of the contraction in HS- $A_{2A}AR^{-/-}$ mice by the use of sEH inhibitor in vitro, and use of sEH inhibitor in vivo needs to be further explored. Moreover, interesting interactions between sEH and $A_{2A}AR^{-/-}$ mice, and PPAR γ and $A_{2A}AR$ have been elucidated in this study.

sEH inhibitor, AUDA, increases or stabilizes the vasoactive EETs [33]. HS produced greater relaxation to CGS 21680 compared to NS diet in $A_{2A}AR^{+/+}$ mice (Fig. 2a). We observed that AUDA enhanced vascular relaxation to NECA in NS-fed wild-type mice (Fig. 1a) to the level shown in HS-fed mice, suggesting that sEH inhibition causes higher relaxation through higher EETs level; however, there was no difference in vascular response to CGS 21680 in HS-fed $A_{2A}AR^{+/+}$ mice with AUDA as reported earlier by us [10].

Cyp-epoxygenase enzyme (cyp2c29) responsible for EETs production is demonstrated to be lower in $A_{2A}AR^{-/-}$ mice on a HS diet [11] implying possible reduced availability of EETs in $A_{2A}AR$ -deficient mice. Of note, studies have shown that reduced EETs can impair endothelial dilator response in obesity and diabetes [34, 35]. In HS/NS-fed $A_{2A}AR^{-/-}$ mice, AUDA was able to substantially reverse the NECA-induced vascular contraction (Fig. 1b), indicating beneficial use of sEH inhibitor in improving impaired vascular reactivity by elevating EETs in HS/NS-fed $A_{2A}AR^{-/-}$ mice. Hence, it is logical that sEH inhibitor provides beneficial effects through elevating EETs which have vasodilatory and natriuretic properties. AUDA treatment has been reported to reduce ischemic infarct size in stroke-prone spontaneously hypertensive rats [36]. Another sEH inhibitor *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclo-hexyloxy]-benzoic acid (t-AUCB) is shown to be protective against ischemia reperfusion injury [37]. Moreover, Imig et al. 2005 demonstrated the blood pressure lowering effect in rats with salt-sensitive hypertension and angiotensin II-induced hypertension [13, 38]. Also, we observed higher sEH expression in $A_{2A}AR^{-/-}$ mice compared to $A_{2A}AR^{+/+}$ mice on HS and NS diet (Fig. 7) which further contributes to increased EETs conversion into diols (DHETs) in $A_{2A}AR$ null mice leading to lower EETs level.

$A_{2A}AR$ -mediated enhanced vascular relaxation in HS-fed $A_{2A}AR^{+/+}$ mice is dependent on EETs, and EETs are shown to act on $PPAR\gamma$ [27]. Therefore, we tested if $A_{2A}AR$ -elicited vascular relaxation is dependent on $PPAR\gamma$. We found that $PPAR\gamma$ antagonist, T0070907, significantly blocked HS-induced relaxation to CGS 21680 (Fig. 2a), indicating the involvement of $PPAR\gamma$ downstream of $A_{2A}AR$ to produce enhanced vascular relaxation in mice in response to HS diet. This finding is in agreement with our previous published work [39] and others [27, 40] and confirms the contribution of $PPAR\gamma$ in $A_{2A}AR$ -mediated vascular response.

Also, AUDA concentration–response curve indirectly represents EETs-dependent vascular response because sEH inhibition increases EETs level [26]. AUDA produced enhanced relaxation in HS-fed $A_{2A}AR^{+/+}$ mice compared to NS diet, and this enhanced relaxation was blocked by $PPAR\gamma$ antagonist (Fig. 3) suggesting that EETs-mediated vascular relaxation is dependent on $PPAR\gamma$. Altogether, from our results, it became evident that $PPAR\gamma$ contributes downstream of $A_{2A}AR$ and EET to produce greater vascular relaxation upon exposure to HS diet.

Consistent with the presence of $PPAR\gamma$ in vascular tissues, substantial evidence suggest the participation of $PPAR\gamma$ in regulation of vascular function and blood pressure [41]. $PPAR\gamma$ activation is reported to increase blood flow via vasodilation [31, 42]. We examined vascular response to $PPAR\gamma$ in HS and NS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice using selective $PPAR\gamma$ agonist, Rosiglitazone. We found that Rosiglitazone produced significantly greater relaxation in HS compared to NS-fed $A_{2A}AR^{+/+}$ mice (Fig. 4a). Interestingly, Rosiglitazone produced greater contraction response in both the HS and NS-fed $A_{2A}AR^{-/-}$ mice compared to their respective $A_{2A}AR^{+/+}$ mice (Fig. 4b), suggesting an interesting interaction between $A_{2A}AR$ and $PPAR\gamma$. In agreement with our finding, He et al., also showed a positive feedback regulation between $A_{2A}AR$ and $PPAR\gamma$ to produce anti-inflammatory effects [43].

Corresponding to Rosiglitazone response in $A_{2A}AR$ -deficient mice, we found that pharmacological inhibition of $A_{2A}AR$ using selective A_{2A} receptor antagonist, SCH 58261 also attenuated Rosiglitazone-induced relaxation in HS and contraction in NS-fed $A_{2A}AR^{+/+}$ mice, further validating the possible interaction between $A_{2A}AR$ and $PPAR\gamma$. Overall, these results suggest that $A_{2A}AR$ might positively regulate $PPAR\gamma$.

Supporting the data published in earlier studies from our lab [44], we found that HS-induced increased vascular relaxation to CGS 21680 is blocked by K_{ATP} channel inhibitor, Glibenclamide (Fig. 5), indicating the role of K_{ATP} channels in $A_{2A}AR$ -mediated vascular response. Also, $PPAR\gamma$ agonist, Rosiglitazone-elicited enhanced vascular relaxation, was attenuated by Glibenclamide (Fig. 6), implicating the contribution of K_{ATP} channel in $PPAR\gamma$ -mediated vascular relaxation. Taken together, these data suggest that $A_{2A}AR$ -EETs- $PPAR\gamma$ pathway produces vascular relaxation through K_{ATP} channel which is consistent with our earlier reports [10, 39], suggesting that $PPAR\gamma$ elicits relaxation in both mouse vascular and human pulmonary arteries by activating K_{ATP} channels [31].

PPAR γ -dependent effects mostly occur through regulation of gene expression. However, these A_{2A}AR-EETS-PPAR γ -mediated changes in vascular tone through K_{ATP} channels are quick response that could not correlate with changes in gene expression that generally takes longer time. There is some evidence indicating that PPAR γ -dependent alteration in vascular tone could occur through non-genetic pathway possibly via phosphorylation. Report also suggests that PPAR γ activation works through rapid phosphorylation or stimulation of ion channels that regulate vascular tone. In smooth muscle cells, PPAR γ activation inhibits the L-type Ca²⁺ current [45], thereby reducing vascular contraction. TZDs may promote vascular relaxation through stimulation of k_{ca} channels; however, the mechanism remains unclear [46]. In addition, Pioglitazone is shown to promote activation of myosin light chain phosphatase [47] and also inhibition of Rho kinase activity in vasculature [48]. Hence, A_{2A}AR-EETs-mediated vascular relaxation depend on PPAR γ signaling leading to the opening of K_{ATP} channels, the mechanism of K_{ATP} channels opening may be through non-genomic action of PPAR γ , and that needs to be further explored. In vitro organ bath data can be extrapolated into in vivo situations under high salt diet-fed conditions in mice by measuring blood pressure in live animals through tail-cuff method or telemetry.

Conclusion

The principle finding of this study identifies important signaling molecules involved in HS-induced altered vascular reactivity to adenosine receptors agonist (NECA) in the presence and absence of A_{2A}AR. EETs, PPAR γ , and K_{ATP} channels are recognized as key downstream signaling molecules contributing to HS-induced A_{2A}AR-mediated vascular relaxation, while increased sEH is identified as essential mediator involved in HS-elicited vascular contraction in A_{2A}AR null mice (Fig. 8). Most importantly, our data shed light on possible strategy to reverse HS-induced contraction to adenosine receptors agonist (NECA) observed in A_{2A}AR null mice by increasing the level of EETs through sEH inhibition.

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Fig 1.

a Effects of sEH inhibition with AUDA (10^{-5} M) on NECA-induced vascular responses in aortic rings isolated from HS and NS-fed $A_{2A}AR^{+/+}$ mice. Values are mean $\pm$ SE. * $p < 0.05$ between HS- $A_{2A}AR^{+/+}$ versus NS- $A_{2A}AR^{+/+}$, # $p < 0.05$ between NS- $A_{2A}AR^{+/+}$ versus NS- $A_{2A}AR^{+/+}$ with AUDA, and * $p > 0.05$ between HS- $A_{2A}AR^{+/+}$ versus HS- $A_{2A}AR^{+/+}$ with AUDA, $n = 6$. On the y-axis, positive and negative values represent relaxation and contraction, respectively. **b** Effects of sEH inhibition AUDA (10^{-5} M) on NECA-induced vascular responses in aortic rings isolated from HS and NS-fed $A_{2A}AR^{-/-}$ mice. Values are mean $\pm$ SE. # $p < 0.05$ between NS- $A_{2A}AR^{-/-}$ versus NS- $A_{2A}AR^{-/-}$ with AUDA and \$ $p < 0.05$ between HS- $A_{2A}AR^{-/-}$ versus HS- $A_{2A}AR^{-/-}$ with AUDA, $n = 6$

Fig 2.

a Effects of PPAR γ inhibition with T0070907 (10⁻⁷ M) on CGS-induced vascular response in HS and NS-fed A_{2A}AR^{+/+} aortic rings. Values are mean $\pm$ SE. * p < 0.05 between HS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{+/+} and \$ p < 0.05 between HS-A_{2A}AR^{+/+} versus HS-A_{2A}AR^{+/+} with T0070907, n = 4–6. **b** Effects of PPAR γ inhibition with T0070907 (10⁻⁷ M) on NECA-induced vascular response in NS and HS-fed A_{2A}AR^{-/-} aortic rings. Values are mean $\pm$ SE, n = 4–6

Fig 3.

Effects of PPAR γ inhibition with T0070907 (10^{-7} M) on sEH inhibitor (AUDA)-induced vascular response in NS and HS-fed A_{2A}AR^{+/+} aortic rings. Values are mean $\pm$ SE. \$ p < 0.05 between HS-A_{2A}AR^{+/+} versus HS-A_{2A}AR^{+/+} with T0070907 and * p < 0.05 between HS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{+/+}, n = 6

Fig 4.

a PPAR γ activation with Rosiglitazone-mediated vascular responses in aortic rings of A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice fed NS and HS diet. Values are mean $\pm$ SE. * p < 0.05 between HS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{+/+}, \$ p < 0.05 between HS-A_{2A}AR^{+/+} versus HS-A_{2A}AR^{-/-}, and # p < 0.05 between NS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{-/-}, n = 6. **b** Effects of A_{2A}AR antagonist with SCH 58261 (10⁻⁶ M) on Rosiglitazone-induced vascular response in NS and HS-fed A_{2A}AR^{+/+} aortic rings. Values are mean $\pm$ SE. * p < 0.05 between HS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{+/+}, # p < 0.05 between NS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{+/+} with SCH 58261, and \$ p < 0.05 between HS-A_{2A}AR^{+/+} versus HS-A_{2A}AR^{+/+} with SCH 58261, n = 4–6

Fig 5.

Effects of K_{ATP} channels inhibition with Glibenclamide (10^{-5} M) on CGS 21680-induced vascular response in NS and HS-fed A_{2A}AR^{+/+} aortic rings. Values are mean $\pm$ SE. \$ $p < 0.05$ between HS-A_{2A}AR^{+/+} versus HS-A_{2A}AR^{+/+} with Glibenclamide, and * $p < 0.05$ between HS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{+/+}, $n = 4-6$

Fig 6.

Effects of K_{ATP} channels inhibition with Glibenclamide (10^{-5} M) on Rosiglitazone-induced vascular response in NS and HS-fed A_{2A}AR^{+/+} aortic rings. Values are mean $\pm$ SE. \$ $p < 0.05$ between HS-A_{2A}AR^{+/+} versus HS-A_{2A}AR^{+/+} with Glibenclamide and * $p < 0.05$ between HS-A_{2A}AR^{+/+} versus NS-A_{2A}AR^{+/+}, $n = 4-6$

Fig 7.

Representative Western blot and densitometric analysis for sEH (~ 62 kDa) protein in aortas of HS and NS-fed A_{2A}AR^{+/+} and A_{2A}AR^{-/-} mice. Values are mean ± SE. \$*p* < 0.05 between HS-A_{2A}AR^{+/+} versus HS-A_{2A}AR^{-/-} and #*p* < 0.05 between NS-A_{2A}AR^{+/+} versus NS - A_{2A}AR^{-/-}, *n* = 4

Fig 8.

The schematic diagram represents the high salt (HS) diet-induced altered vascular reactivity to adenosine receptors agonist (NECA) in the presence and absence of $A_{2A}AR$. Cyp-epoxygenases, EETs, $PPAR\gamma$, and K_{ATP} channels are recognized as key downstream signaling molecules contributing to HS-induced $A_{2A}AR$ -enhanced vascular relaxation, while increased sEH is identified as essential mediator involved in HS-elicited vascular contraction in $A_{2A}AR$ -null mice