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High salt diet exacerbates vascular contraction in the absence of adenosine A_{2A} receptor

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Abstract

High salt (4%NaCl, HS) diet modulates adenosine-induced vascular response through adenosine A_{2A}-receptor ($A_{2A}AR$). Evidence suggests $A_{2A}AR$ stimulates cyp450-epoxygenases, leading to epoxyeicosatrienoic acids (EETs) generation. The aim of this study was to understand the vascular reactivity to HS and underlying signaling mechanism in the presence or absence of $A_{2A}AR$. Therefore, we hypothesized that HS enhances adenosine-induced relaxation through EETs in $A_{2A}AR^{+/+}$, but exaggerates contraction in $A_{2A}AR^{-/-}$. Organ-bath and Western-blot experiments were conducted in HS and normal salt (NS, 0.18% NaCl)-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice aortae. HS produced concentration-dependent relaxation to non-selective adenosine analog, NECA in $A_{2A}AR^{+/+}$, whereas contraction was observed in $A_{2A}AR^{-/-}$ mice and this was attenuated by A_1AR antagonist (DPCPX). CGS-21680 (selective $A_{2A}AR$-agonist) enhanced relaxation in HS-$A_{2A}AR^{+/+}$ *vs.* NS-$A_{2A}AR^{+/+}$, that was blocked by EETs antagonist (14,15-EEZE). Compared to NS, HS significantly upregulated expression of vasodilators $A_{2A}AR$ and cyp2c29, while vasoconstrictors A_1AR and cyp4a in $A_{2A}AR^{+/+}$ were downregulated. In $A_{2A}AR^{-/-}$ mice, however, HS significantly downregulated the expression of cyp2c29, while A_1AR and cyp4a were upregulated compared to $A_{2A}AR^{+/+}$ mice. Hence, our data suggest that in $A_{2A}AR^{+/+}$, HS enhances $A_{2A}AR$-induced relaxation through increased cyp-expoxygenases-derived EETs and decreased A_1AR levels, whereas in $A_{2A}AR^{-/-}$, HS exaggerates contraction through decreased cyp-epoxygenases and increased A_1AR levels.

Keywords

HS; NS; $A_{2A}AR$; cyp4a; cyp2c29; A_1AR; relaxation; contraction

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Introduction

Adenosine, a breakdown product of ATP, acts as a local modulator with generally cytoprotective function in response to stressful conditions such as ischemia, hypoxia, injury, and inflammation (23, 26). Besides many physiological effects elicited by extracellular adenosine such as inhibition of renin release, platelet aggregation, inflammation, lipolysis and sympathetic neurotransmission (34), its effect on vasculature is crucial for vasoregulation (14) and physiological control of mean arterial pressure (MAP), (1, 19). Adenosine mediates its effects via four adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3 (10). Activation of A_{2A}AR and A_{2B}AR are known to mediate vasodilation in different vessels (6, 8, 16, 28-29, 32), whereas A_1AR and A_3AR are believed to be involved in vasoconstriction (12, 14). Adenosine receptors especially A_1AR and A_{2A}AR are also involved in the regulation of blood pressure (BP). A_{2A}AR causes renal vasodilation (13) and attenuates tubuloglomerular feedback (5). A_{2A}AR knockout mice as well as rats treated with selective A_{2A}AR antagonist displayed an elevated BP (27), (19). A_1AR is involved in water and salt retention (24), mediating tubuloglomerular feedback response (35), constriction of afferent arterioles (38), lowering of heart rate and suppression of renin release (2).

Growing evidence suggests a link between high dietary salt intake and activation of adenosine receptors and A_{2A} receptor in particular (22, 29-30). Data demonstrating upregulation of A_{2A}AR expression in response to salt loading (21, 29), accompanied by enhanced A_{2A}AR mediated vasodilation (6, 21-22, 29-30) further support the role of adenosine A_{2A}AR during high salt (HS) diet. Stimulation of A_{2A}AR is reported to result in an eNOS-independent vasodilation (30) and reduction in MAP (1). A_1AR, responsible for vasonstriction, is shown to be downregulated in HS treated animals (21, 29). In addition, elevated levels of adensoine generation have been detected upon the exposure to HS diet in rats (33, 39).

Apart from adenosine, another important vasoregulatory factor associated with HS diet is cyp-epoxygenase enzyme that belongs to cytochrome P450s (cyp450) family (9, 15). Two major subtypes of cyp450 enzymes include cyp-epoxygenases and ω-hydroxylases that play an important role in regulating vascular tone (9, 15, 18). It is well documented that cyp-epoxygenases catalyze the metabolism of arachidonic acid (AA) into epoxyeicosatrienoic acids (EETs), a potent vasodilator and natriuretic agent (3), whereas, ω-hydroxylase metabolizes AA to 20-hydroxyeicosatetraenoic acid (20-HETE), a potent vasoconstrictor (11, 28-29) and natriuretics. HS diet is associated with increase in renal cyp-expoxygenases activity as a protective mechanism against rise in BP (4, 20-21). Inhibition of cyp-epoxygenases activity with clotrimazole is shown to contribute to the surge in MAP and development of salt sensitivity in rats fed HS diet, whereas salt itself was unable to increase blood pressure (25). Unlike EETs, the production of 20-HETE is found to be lower in glomeruli isolated from kidneys of rats fed HS diet than in kidneys of rats fed low salt diet (17). Increased levels of 20-HETE is shown to promote vasoconstriction that contributes to the progression of hypertension (11, 37).

Emerging evidence indicates a close relationship between HS-induced activation of A_{2A}AR and EETs generation through enhanced cyp-epoxygenase activity (7, 30-31). In previous

study, we found that non-selective adenosine analaog (NECA) and selective adenosine A_{2A} receptor agonist (CGS-21680) showed enhanced dilation in aortae from mice fed HS compared to mice fed low salt diet and the enhanced dilation in HS diet fed mice was blunted by cyp epoxygenase inhibitor, methylsulfonyl-propargyloxyphenylhexanamide (MSPPOH) (29). Moreover, Liclican et al., (21) reported that inability to upregulate the adenosine A_{2A}AR–EETs pathway in response to salt loading confers salt sensitive hypertension in rats.

Although there is some indication as to the possible adaptive role of A_{2A}AR receptor and cyp epoxygenases mediated vasodilation during HS diet, studies evaluating the ramifications of HS diet in terms of vascular response and biochemical signaling resulting from the A_{2A}AR gene deletion are lacking. On the basis of these prior investigations, it is important to examine the effects of HS diet on vascualr reacitivity in the absence of A_{2A}AR in order to directly evaluate the role of A_{2A}AR and more importantly to understand the underlying mechanism involved in vascular pathophysiological changes. Therefore, using HS and NS fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice, we aim to determine the role of A_{2A}AR on vascular response and its downstream mechanism.

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-weeks-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 0.18% NaCl, normal salt (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 have been previously described by Ledent et al., (19). 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 (19). 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 (28-29). 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 equlibrated for 60 min with a resting force of 1 g as described previously by us (28-29). 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 acqusition 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 (28-29) through acetylcholine (ACh, 10^{-6} M) on precontracted 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 precontraction.

Adenosine agonist-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 non-selective adenosine analog, 5'-N-ethylcarboxamidoadenosine (NECA), the selective A_{2A} receptor agonist, 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamido adenosine hydrochloride (CGS 21680), or selective A_1 receptor agonist, 2-chloro-N(6)-cyclopentyladenosine (CCPA) 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 A_1AR and $A_{2A}AR$ antagonists, and cyp4a inhibitor on NECA and 20-HETE-induced vascular response in $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice fed HS and NS diet—Selective A_1AR antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 10 μM) or selective $A_{2A}AR$ antagonist (SCH 58261; 10 μM) or selective cyp4a inhibitor, dibromo-dodecenyl-methylsulfimide (DDMS; 10 μM) 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 either DPCPX or SCH 58261 or DDMS respectively.

Effects of EETs antagonist on CGS 21680-induced vascular response in $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice fed HS and NS—Selective EET antagonist, 14,15-epoxyeicosa-5(z)-enoic acid (14,15-EEZE;10 μM) 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 14,15-EEZE.

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 μL 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 μg 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 prestained 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 cyp2c29 (Dr. Zeldin, NIEHS /NIH), cyp4a (Santa Cruz Biotecnology, Santa Cruz, CA), A_1AR (Sigma Chemicals) and A_{2A}AR (Alpha Diagnostics). 1:1000 primary antibody concentration was used for all antibodies. 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 & antibodies—Phenylephrine hydrochloride and acetylcholine chloride were dissolved in distilled water. NECA, CGS 21680, SCH-58261, CCPA, DPCPX, indomethacin, and L-NAME (Sigma Chemicals, St. Louis, MO) were dissolved in 100% DMSO as 10 mM stock solutions, which were followed by serial dilutions in distilled water. 14,15-EEZE, DDMS and 20-HETE (Dr. Falck) were dissolved in 100% ethanol. Cyp2c9 antibody (Dr. Zeldin, NIEHS /NIH), cyp4a antibody (Santa Cruz Biotecnology), A_1AR (Sigma Chemicals, St. Louis, MO) and A_{2A}AR (Alpha Diagnostics) were used for Western-blot experiments.

Statistical Analysis—Statistical data are reported as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare difference among groups, and two ways ANOVA for repeated measure, followed by Tukey post hoc test to compare the vascular responses to antagonist SCH, DPCPX, 14,15-EEZE, DDMS. Differences were considered significant if $p<0.05$. Further, densitometry of Western-blot analysis (cyp4a, cyp2c29, A_1AR and A_{2A}AR) data was expressed as mean ± SEM in arbitrary units. All the statistical analyses were performed using Graph Pad Prism statistical package.

RESULTS

Effects of SCH 58261 on NECA-dependent vascular response in HS and NS diet fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

NECA produced enhanced vascular relaxation in HS-fed $A_{2A}AR^{+/+}$ mice compared to NS-fed $A_{2A}AR^{+/+}$ mice ($p<0.05$; Fig. 1a). HS–induced vascular response to NECA was significantly different in $A_{2A}AR^{+/+}$ vs. $A_{2A}AR^{-/-}$ mice ($p<0.05$; Fig. 1a). HS diet induced higher relaxation (+17.34 ± 2.50%) to NECA (10^{-6} M) in $A_{2A}AR^{+/+}$ mice whereas, HS diet caused contraction (−56.77 ± 3.49%) in $A_{2A}AR^{-/-}$ mice ($P<0.05$; Fig. 1a). Interestingly no significant difference was observed between aortic responses in HS-fed $A_{2A}AR^{-/-}$ mice and

NS-fed $A_{2A}AR^{-/-}$ mice (p>0.05; Fig. 1a). To pharmacologically confirm if HS-mediated relaxation is dependent on $A_{2A}AR$, we used selective A_{2A} receptor antagonist, SCH 58261 for NECA CRC (Fig.1b). SCH 58261 (1 μM) blocked NECA (10^{-6} M) dependent relaxation response (+17.34 ± 2.50%) in HS-fed $A_{2A}AR^{+/+}$ aortae into contraction (−12.20 ± 3.62%, p>0.05; Fig. 1b).

Effects of L-NAME, indomethacin and 14,15-EEZE on CGS 21680-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 relaxation in both HS and NS-fed $A_{2A}AR^{+/+}$ mice with a significant difference (p<0.05; Fig 2a), but no significant difference was noted between HS and NS diet fed $A_{2A}AR^{-/-}$ mice (p>0.05; Fig. 2a). CGS 21680 (10^{-6}M) produced significantly greater relaxation (+21.4 ± 1.34%) in HS-fed $A_{2A}AR^{+/+}$ mice than in NS-fed $A_{2A}AR^{+/+}$ mice (+12.96 ± 1.52; p<0.05; Fig 2a) suggesting an increased $A_{2A}AR$ activity with HS diet.

Relaxation response to CGS 21680 in both HS and NS-fed $A_{2A}AR^{+/+}$ mice was neither affected by cycloxygenase inhibitor, indomethacin (1 μM) (p>0.05) nor by e-NOS inhibitor, L-NAME (100 μM) (p>0.05; **not shown**). However, EETs antagonist, 14,15-EEZE (10 μM) completely abolished CGS 21680-dependent relaxation (from +21.4 ± 1.34% to contraction −1.08 ± 2.95 % at 10^{-6} M; p<0.05) in HS-fed $A_{2A}AR^{+/+}$ mice (Fig. 2b). Relaxation to CGS 21680 (10^{-6} M) in NS-fed $A_{2A}AR^{+/+}$ mice was also significantly blocked by 14,15-EEZE (10 μM) but to a lower extent compared to HS-fed $A_{2A}AR^{+/+}$ mice (p<0.05; Fig. 2b). These data suggest that $A_{2A}AR$ mediated relaxation in HS–fed $A_{2A}AR^{+/+}$ mice is independent of NO and cycloxygenase but dependent on EETs.

Effects of DDMS on NECA-dependent response in HS and NS diet fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

In Fig. 3, contraction to NECA (10^{-6} M) in NS-fed $A_{2A}AR^{+/+}$ mice was blocked by cyp4a inhibitor DDMS (10 μM), and the response changed from a contraction of −14.42 ± 7.74% to a relaxation of +10.0 ± 3.83% (p<0.05; Fig. 3a). Also, in NS-fed $A_{2A}AR^{-/-}$ mice, DDMS (10 μM) attenuated NECA-induced contraction (from −48.27 ± 2.83% to −22.1 ± 4.37% at 10^{-6} M; p<0.05; Fig. 3b), suggesting the role of 20-HETE, an arachidonic acid metabolite of cyp4a, in NECA-dependent contraction. Interestingly, NECA-induced contraction in HS-fed $A_{2A}AR^{-/-}$ mice was no different with 10 μM DDMS treatment (without treatment: 57.59 ± 2.84% vs. DDMS treated: 47.03 ± 4.38% at 10^{-6} M; p>0.05; Fig. 3b). This indicates that 20-HETE does contribute to the contraction in NS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice but not in HS-fed $A_{2A}AR^{-/-}$ mice.

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

Since A_1 adenosine receptor is also involved in vasoconstriction (12), we investigated the role of A_1AR in NECA-induced contraction in NS-$A_{2A}AR^{+/+}$ mice (Fig. 4a). Selective A_1 antagonist, DPCPX (10 μM) blocked NECA (10^{-6} M)-dependent contraction (from −14.42 ± 7.74% to relaxation +14.96 ± 3.75%; p<0.05; Fig. 4a) in NS-fed $A_{2A}AR^{+/+}$ mice. But, DPCPX did not have any significant effect on DPCPX treated HS-fed $A_{2A}AR^{+/+}$ mice

compared to non-treated HS-fed $A_{2A}AR^{+/+}$ mice ($p>0.05$; Fig. 4a). Further, DPCPX reversed NECA (10^{-6} M)-mediated contraction from 51.1 ± 3.01% to +1.02 ± 3.18% in NS-fed $A_{2A}AR^{-/-}$ ($p<0.05$; Fig. 4b) and 57.29 ± 2.84% to 1.32 ± 5.47% in HS-fed $A_{2A}AR^{-/-}$ ($p<0.05$; Fig. 4b). No difference was found between 10 μM DPCPX and 0.1 μM DPCPX (data not shown).

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

Selective A_1 agonist, CCPA yielded higher contraction in $A_{2A}AR^{-/-}$ mice compared to $A_{2A}AR^{+/+}$ mice regardless of salt content in the diet (Fig. 4c). NS-fed $A_{2A}AR^{-/-}$ and HS-fed $A_{2A}AR^{-/-}$ mice showed robust vascular contraction (77.73 ± 4.26% and 52.08 ± 4.29%) to CCPA (10^{-6} M) compared to their controls NS-fed $A_{2A}AR^{+/+}$ and HS-fed $A_{2A}AR^{+/+}$ (43.05 ± 7.51% and 29.31 ± 4.81%; $p<0.05$; Fig. 4c), respectively. Interestingly, CCPA (10^{-6} M) produced significantly lower contraction in HS-fed $A_{2A}AR^{-/-}$ mice (52.08 ± 4.29%) compared to NS-fed $A_{2A}AR^{-/-}$ mice (77.73 ± 4.26%; $p<0.05$; Fig. 4c).

Effects of DPCPX on 20-HETE-dependent vascular response in HS and NS diet fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

We investigated the role of A_1AR in 20-HETE-induced contraction in NS/HS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice (Fig. 5a, b). Selective A_1 antagonist, DPCPX was unable to block 20-HETE (10^{-6} M)-induced contraction (Fig. 5a, b) in NS/HS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice, suggesting that 20-HETE is downstream of A_1AR.

$A_{2A}AR$, cyp2c29, cyp4a and A_1AR expression in HS and NS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

The expression of $A_{2A}AR$ (~45 kDa) protein was upregulated by ~30% in HS-fed $A_{2A}AR^{+/+}$ mouse aortae (129.7 ± 1.63%) compared with the control NS-fed $A_{2A}AR^{+/+}$ mouse aortae (100.1 ± 2.16%, $p<0.05$; Fig. 6).

Also, cyp2c29 (~55 kDa) protein showed increased expression by ~64 % in HS-fed $A_{2A}AR^{+/+}$ mouse aortae (164.2 ± 18.50%) compared to the control NS-fed $A_{2A}AR^{+/+}$ mouse aorta (100 ± 5.55%; $p<0.05$, Fig. 7). Significantly diminished level of cyp2c29 (~54%) was observed in HS-fed $A_{2A}AR^{-/-}$ mice (59.8 ± 5.20%; Fig. 7) compared to HS-fed $A_{2A}AR^{+/+}$ mice (164.2 ± 18.50%; $p<0.05$; Fig. 7).

Substantial elevation in the level of cyp4a (~58 kDa; ~74 %) protein was found in NS-fed $A_{2A}AR^{-/-}$ mice (173.8 ± 12.90%) compared to NS-fed $A_{2A}AR^{+/+}$ (100.9 ± 1.34%; $p<0.05$, Fig. 8). On the contrary, cyp4a expression was decreased by ~46 % in HS-fed $A_{2A}AR^{+/+}$ mice (54.1 ± 7.88%; $p<0.05$; Fig. 8) compared to the NS-fed $A_{2A}AR^{+/+}$ mouse aortae (100.9 ± 1.34%). Cyp4a level was ~50% higher in HS-fed $A_{2A}AR^{-/-}$ mouse aortae (103.9 ± 6.53%; $p<0.05$) compared to HS-fed $A_{2A}AR^{+/+}$ mice (54.1 ± 7.88%; Fig. 8).

A_1AR (~37 kDa) protein expression observed in both NS-fed $A_{2A}AR^{-/-}$ and HS-fed $A_{2A}AR^{-/-}$ mice (151.60 ± 26.85% and 120.1 ± 11.94%) was ~51.6% and ~45% higher compared to their control counterparts NS and HS-fed $A_{2A}AR^{+/+}$ mouse aortae (100.01 ± 0.04% and 82.84 ± 9.24%, Fig. 9) respectively. Also, A_1AR expression was down-regulated

by ~17% in HS-fed $A_{2A}AR^{+/+}$ mouse (82.84 ± 9.24%) compared to NS-fed $A_{2A}AR^{+/+}$ mouse aortae (100.0 ± 0.04%, $p<0.05$; Fig. 9).

Discussion

Our study utilized $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice on a HS/NS diet to directly investigate functional and biochemical consequences of HS diet in the presence and absence of $A_{2A}AR$. The major findings in this study show clear differences in vascular reactivity and signaling cascade between $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice on a HS diet. Results from this study confirm that HS diet enhances vascular relaxation through increased $A_{2A}AR$ and cyp-epoxygenases pathway in $A_{2A}AR^{+/+}$ mice. Moreover, our novel findings suggest that HS diet potentiates contraction through reduced cyp-epoxygenases pathway and increased A_1AR in $A_{2A}AR^{-/-}$ mice. One of the interesting findings from our study demonstrates the contribution of 20-HETE in A_1AR mediated contraction in NS fed. This suggests the involvement of different signaling mediators downstream of A_1AR in vascular contraction observed in NS vs. HS fed $A_{2A}AR^{-/-}$ mice. Hence, we speculate that HS diet in $A_{2A}AR^{-/-}$ mice might induce some pathways downstream of A_1AR other than 20-HETE to cause contraction that needs to be explored in future.

Adenosine-induced vascular response in HS/NS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

As reported earlier by us (28) in mice and in rats pre-glomerular vessels (21-22), in $A_{2A}AR^{+/+}$ mice, we found enhanced relaxation to NECA (Fig.1a) which was blocked by selective A_{2A} receptor antagonist, SCH 58261 and increased $A_{2A}AR$ protein levels with HS diet compared to NS diet (Fig. 6). However, HS exaggerated contraction to NECA in $A_{2A}AR^{-/-}$ mice (Fig. 1a). Interestingly, we found no significant difference in contraction to NECA between NS-fed $A_{2A}AR^{-/-}$ mice and HS-fed $A_{2A}AR^{-/-}$ mice (Fig. 1a).

Role of $A_{2A}AR$ and cyp-epoxygenases in vascular relaxation

CGS 21680-induced aortic relaxation in HS/NS fed $A_{2A}AR^{+/+}$ mice were unaffected by cycloxygenase inhibitor, indomethacin and eNOS inhibitor, L-NAME (data not shown), indicating that HS-induced enhanced $A_{2A}AR$-mediated vascular response is independent of COX and NO. However, EETs antagonist 14,15-EEZE, significantly blocked HS/NS-induced relaxation to CGS 21680. This finding is in agreement with our previous published work (28-29) and others (6-7), and confirms the involvement of EETs in $A_{2A}AR$-mediated enhanced vasodilatation in mice on HS/NS diet. Supporting the vascular response data, we found that HS diet increased levels of cyp2c29, the enzyme responsible for the production of vasodilatory EETs, compared to NS diet in $A_{2A}AR^{+/+}$ mice (Fig. 7). On the contrary, we observed substantially reduced expression of cyp2c29 in HS-fed $A_{2A}AR^{-/-}$ mice compared to HS-fed $A_{2A}AR^{+/+}$ mice aortae, indicating that HS diet fails to up-regulate cy2c29 levels in the absence of $A_{2A}AR$. Our data suggest that diminished cyp2c29 expression in HS-fed $A_{2A}AR^{-/-}$ mice might account for the magnified contraction noted in these mice, possibly indicating an inability to efficiently adapt to HS in the absence of $A_{2A}AR$. Dahl salt-sensitive rats are also unable to increase cyp-epoxygenase enzymes in response to HS diet which parallels our findings in HS-fed $A_{2A}AR^{-/-}$ mice (21).

Role of A_1AR and cyp4a in vascular contraction

On the other hand, cyp4a enzyme catalyzes the metabolism of AA into 20-HETE, a potent vasoconstrictor. The NECA contraction response was blocked and transformed into relaxation by cyp4a inhibitor DDMS in NS-fed $A_{2A}AR^{+/+}$ mice (Fig. 3a), while it was significantly lowered in NS-fed $A_{2A}AR^{-/-}$ mice, suggesting the role of 20-HETE in mediating vascular contraction. Compared to NS-fed $A_{2A}AR^{+/+}$ mice, NS-fed $A_{2A}AR^{-/-}$ mice had substantially increased expression of cyp4a possibly leading to increased generation of 20-HETE. However, DDMS did not affect the contraction observed in HS-fed $A_{2A}AR^{-/-}$ mice, indicating that 20-HETE might not be involved in HS-induced contraction noted in $A_{2A}AR^{-/-}$ mice. We speculate that this discrepancy between the effects of DDMS in NS-fed $A_{2A}AR^{-/-}$ mice and HS-fed $A_{2A}AR^{-/-}$ mice could be due to different signaling mediators involved in these two groups and this finding needs to be further explored. Also, 20-HETE concentration-dependent vascular contraction was not blocked by DPCPX in NS/HS fed $A_{2A}AR^{-/-}$ and $A_{2A}AR^{+/+}$mice (Fig. 5a and 5b). This indicates that 20-HETE is downstream of A_1AR signaling. Western-blot analysis confirmed significantly suppressed levels of cyp4a in HS-fed $A_{2A}AR^{-/-}$ mice compared to NS-fed $A_{2A}AR^{-/-}$ mice possibly resulting in reduced production of 20-HETE with HS diet in $A_{2A}AR^{-/-}$ mice (Fig. 8). This may contribute to enhanced relaxation with HS as a result of decreased 20-HETE production. Our data indicate that levels of vasodilator (cyp-epoxygenase) and vasoconstrictor (ω-hydroxylase) agents appear to counterbalance each other thereby, maintaining vascular tone. Thus an imbalance in these vasoactive agents tends to disrupt this balance and lead to changes in vascular tone.

Similar to our previous finding (29-30), we found HS diet decreased the expression of A_1AR protein compared to NS diet fed $A_{2A}AR^{+/+}$ mice (Fig. 9). Lower levels of vasoconstrictor A_1AR could probably aid in increased NECA-induced vascular relaxation in HS diet fed $A_{2A}AR^{+/+}$ mice. Since the role of A_1AR in vascular contraction, and that of $A_{2A}AR$ in vascular relaxation are well established (28, 36), we examined the involvement of A_1AR in NECA-induced vascular response. Selective A_1AR antagonist DPCPX blocked NECA-dependent contraction in NS-fed $A_{2A}AR^{+/+}$ mice and changed into a relaxation, suggesting the role of A_1AR. Furthermore, NECA-induced contraction in both NS and HS fed $A_{2A}AR^{-/-}$ mice were mitigated by the selective A_1 receptor antagonist, DPCPX, suggesting the contribution of A_1AR in vascular contraction. In addition, Western-blot data showed higher expression of A_1AR in both NS/HS diet fed $A_{2A}AR^{-/-}$ mice compared to respective $A_{2A}AR^{+/+}$ mice. Nontheless, compared to NS-fed $A_{2A}AR^{-/-}$ mice, HS-fed $A_{2A}AR^{-/-}$ mice displayed reduced A_1AR levels. This is an interesting observation which implies that HS diet has a tendency to mitigate the level of A_1AR but to a lesser degree in $A_{2A}AR^{-/-}$ mice comapred to $A_{2A}AR^{+/+}$ mice.

A_1AR-mediated vascular response in HS/NS-fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice

To further examine the A_1AR-mediated response in all the four groups, we used selective A_1AR agonist CCPA and found that CCPA-dependent response produced significantly greater contraction in both the HS and NS-fed $A_{2A}AR^{-/-}$ mice compared to their respective $A_{2A}AR^{+/+}$ mice. Corresponding to the A_1AR levels, NS-fed $A_{2A}AR^{-/-}$ mice demonstrated highest contraction to CCPA than HS-fed $A_{2A}AR^{-/-}$ mice, suggesting that A_1AR-mediated

contraction is decreased with HS diet. Also, HS-fed $A_{2A}AR^{+/+}$ mice had the least contraction to CCPA compared to any other group which corresponds well with the lowest observed level of A_1AR in these mice compared to all groups. Overall, these results suggest that besides $A_{2A}AR$ up-regulation, lowering A_1AR expression might be another protective mechanism against salt loading.

Conclusion

In conclusion, presence of $A_{2A}AR$ appears to be critical for an adaptive aortic relaxation response to HS intake (4-5 weeks). When the $A_{2A}AR$ is absent, HS diet fails to achieve relaxation response as observed in wild-type counterparts and produces amplified contraction to NECA, possibly through diminished expression and activation of vasodilatory cyp-epoxygenases pathway and enhancing the level and activity of vasoconstrictor factors like A_1AR.

Summary

Up-regulation of $A_{2A}AR$ and enhanced vascular relaxation through $A_{2A}AR$ in response to salt loading is most likely an adaptive response as shown in HS fed rat pre-glomerular vessels (21). This adaptive response is lost in the $A_{2A}AR$ null mice fed HS diet confirming a critical role of $A_{2A}AR$ in HS-induced vasodilation. NS and HS diet fed $A_{2A}AR^{-/-}$ mice showed contraction to NECA. Although, the degree of contraction observed in $A_{2A}AR^{-/-}$ mice fed NS diet is similar to $A_{2A}AR^{-/-}$ mice fed HS diet, the mediators responsible for generating the vascular resistance seem to be different. For the most part, cyp4a and A_1AR appear to be contributing to the contraction response in NS-fed $A_{2A}AR^{-/-}$ mice. Conversely, it turns out that reduced cyp-epoxygenase expression and activity along with increased A_1AR levels might be playing an important role in the contraction response observed in HS-fed $A_{2A}AR^{-/-}$ mice.

Acknowledgments

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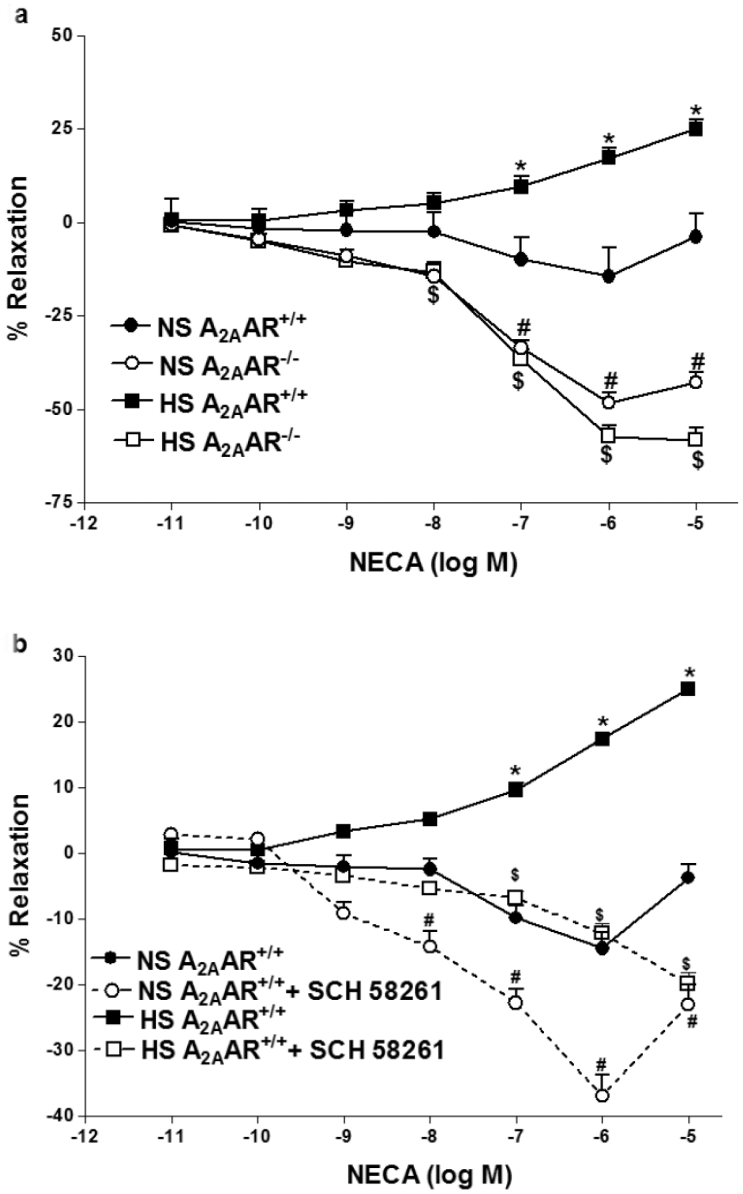


Fig. 1.

a: NECA-induced vascular responses in aortic rings of $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice fed NS and HS diet. Values are means ± SE. *$p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, $^{\$}p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{-/-}$, and $^{\#}p<0.05$ between NS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{-/-}$, $n = 6$. On the y-axis, postive and negative values represent relaxation and contraction, respectively. **b**: Effects of SCH 58261 (10^{-6} M) on NECA-induced vascular responses in aortic rings isolated from HS and NS fed mice. Values are means ± SE. *$p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, $^{\$}p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{+/+}$ with SCH 58261, and $^{\#}p<0.05$ between NS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$ with SCH 58261, $n = 6$.

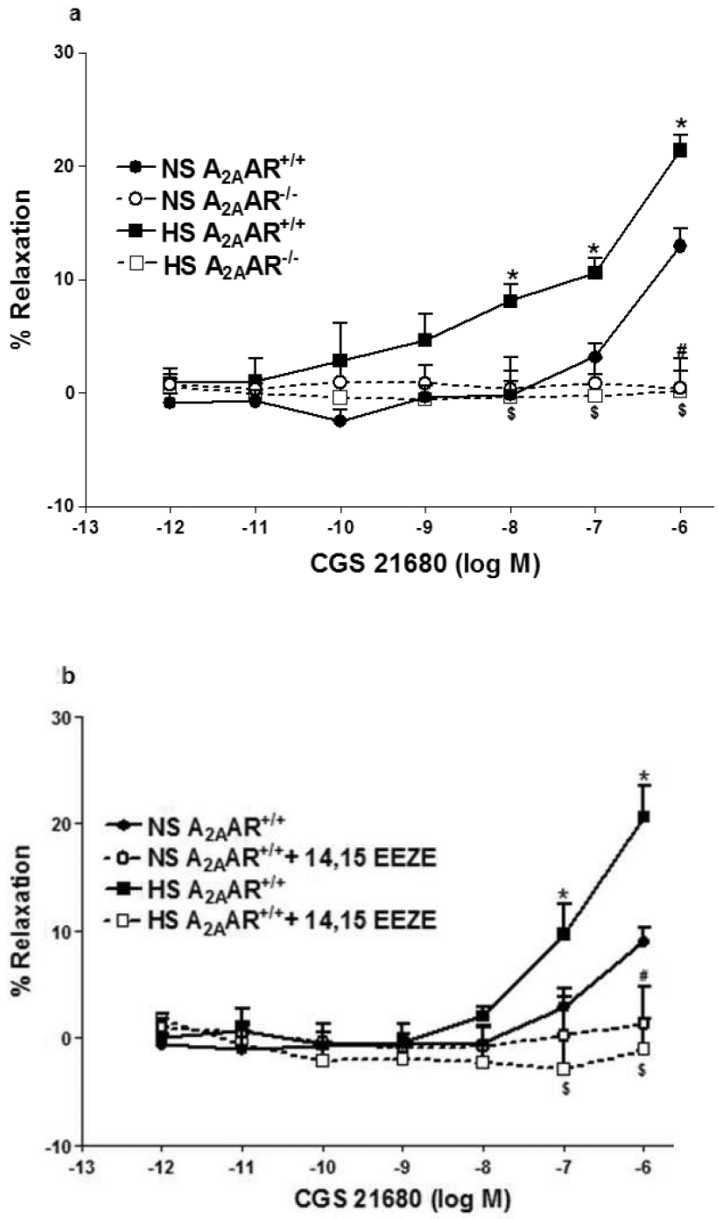


Fig. 2.

a: CGS 21680-induced vascular responses in aortic rings of $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice fed NS and HS diet. Values are means ± SE. *p<0.05 between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, $^{\$}$p<0.05 between HS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{-/-}$, and $^{\#}$p<0.05 between NS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{-/-}$, *n* = 6. **b:** Effects of 14,15-EEZE (10^{-5} M) on CGS 21680-induced vascular response in NS and HS fed $A_{2A}AR^{+/+}$ aortic rings. Values are mean ± SE. *p<0.05 between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, and $^{\$}$p<0.05 between HS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{+/+}$ with 14,15-EEZE, *n* = 6.

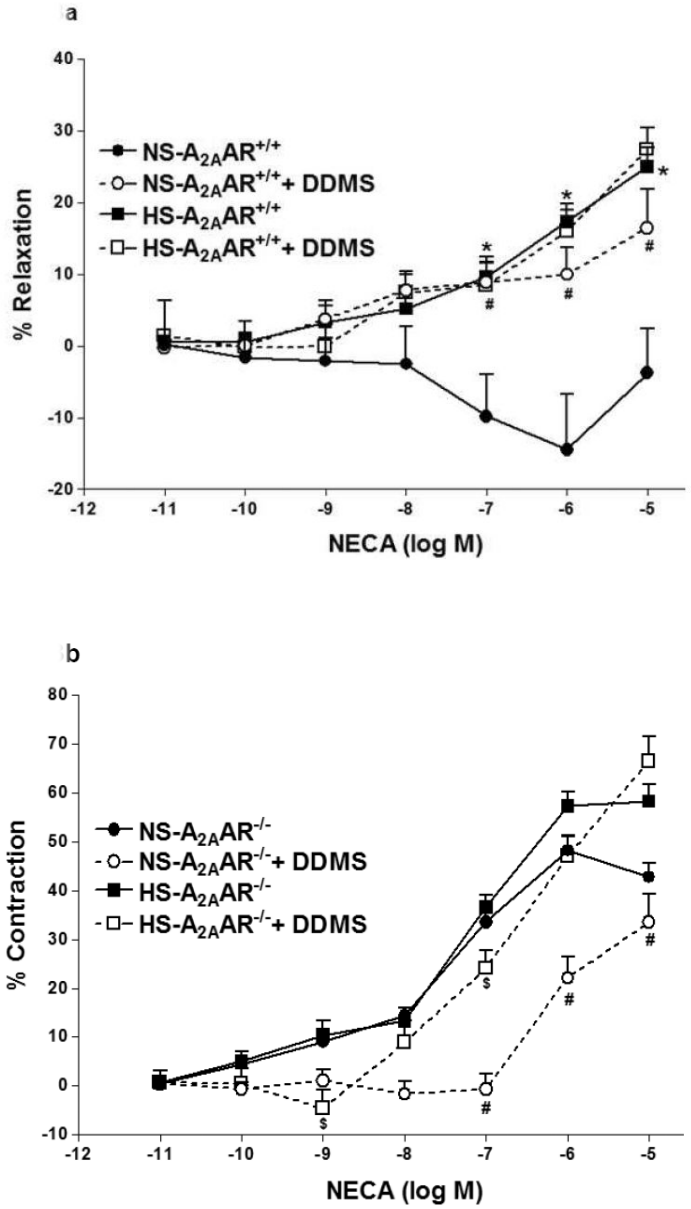
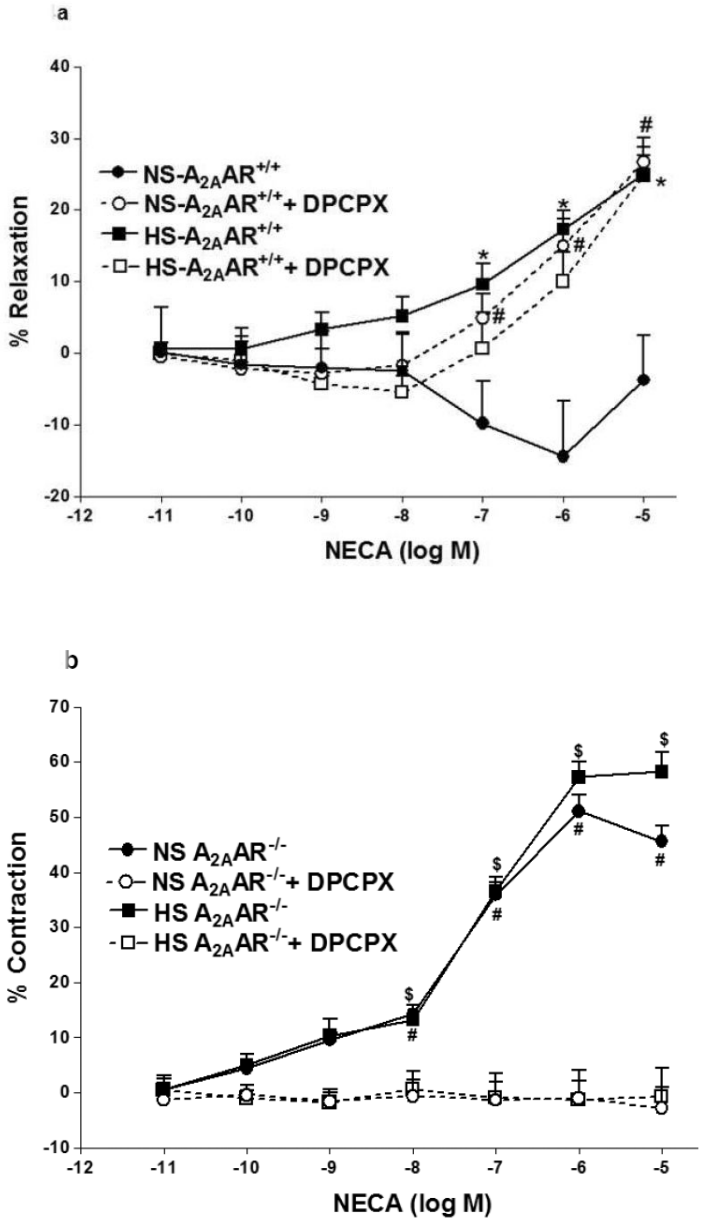


Fig. 3.

a: Effects of DDMS (10^{-5} M) on NECA-induced vascular response in HS and NS fed A_{2A} $AR^{+/+}$ aortic rings. Values are mean ± SE. *p<0.05 between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, and #p<0.05 between NS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$ with DDMS, *n* = 4-6. **b:** Effects of DDMS (10^{-5} M) on NECA-induced vascular response in NS and HS fed $A_{2A}AR^{-/-}$ aortic rings. Values are mean ± SE. #p<0.05 between NS-$A_{2A}AR^{-/-}$ vs. NS-$A_{2A}AR^{-/-}$ with DDMS, and $p<0.05 between HS-$A_{2A}AR^{-/-}$ vs. HS-$A_{2A}AR^{-/-}$ with DDMS, *n* = 4-6.



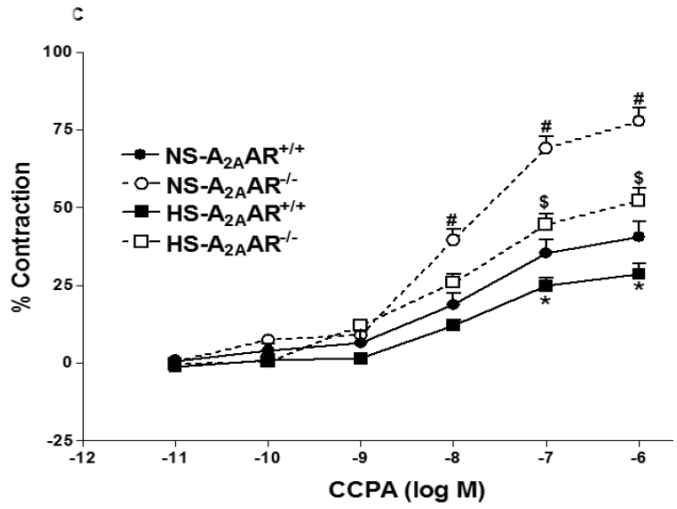


Fig. 4.

a: Effects of DPCPX (10^{-5} M) on NECA-induced vascular response in NS and HS fed $A_{2A}AR^{+/+}$ aortic rings. Values are mean ± SE. $^{\#}p<0.05$ between NS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$ with DPCPX, and $^{*}p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, n = 4-6. **b:** Effects of DPCPX (10^{-5} M) on NECA-induced vascular response in NS and HS fed $A_{2A}AR^{-/-}$ aortic rings. Values are mean ± SE. $^{\#}p<0.05$ between NS-$A_{2A}AR^{-/-}$ vs. NS-$A_{2A}AR^{-/-}$ with DDMS, and $^{\$}p<0.05$ between HS-$A_{2A}AR^{-/-}$ vs. HS-$A_{2A}AR^{-/-}$ with DDMS, n = 4-6. **c:** CCPA-induced vascular response in aortic rings isolated from A_{2A} $AR^{+/+}$ and A_{2A} $AR^{-/-}$ mice fed NS and HS containing diet. Values are means ± SE. $^{*}p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, and $^{\$}p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. HS-A_{2A} $AR^{-/-}$, and $^{\#}p<0.05$ between NS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{-/-}$, n = 4.

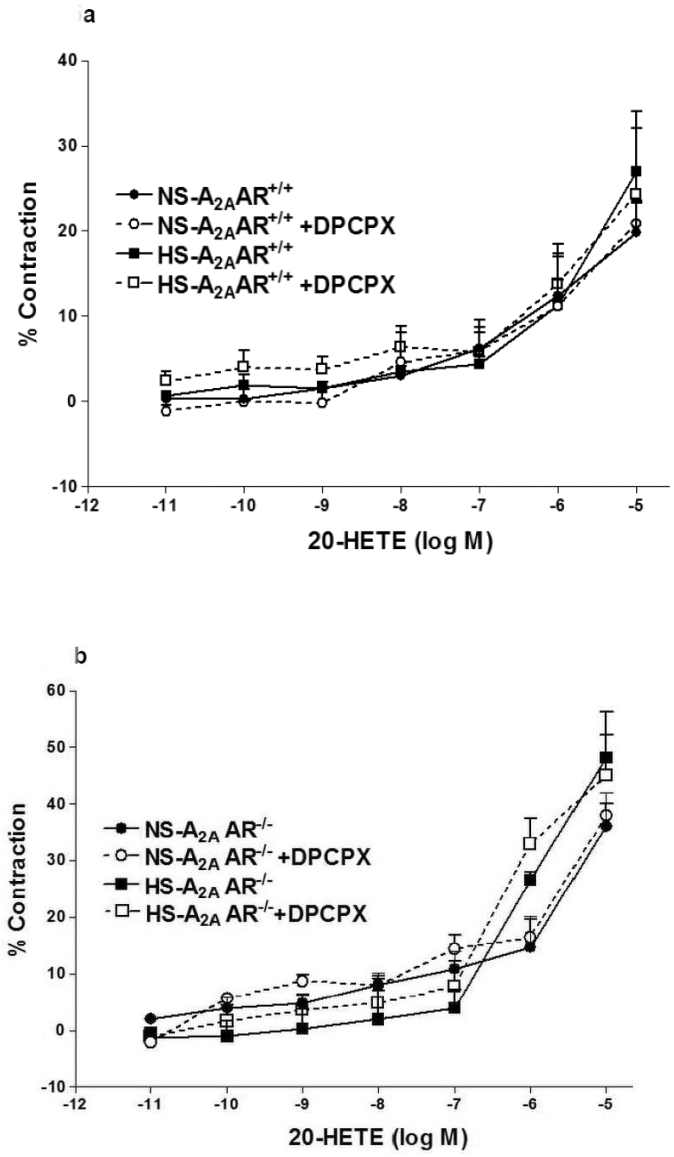


Fig. 5.

a: Effects of DPCPX (10^{-7} M) on 20-HETE- induced vascular response in HS and NS fed A_{2A} $AR^{+/+}$ aortic rings. Values are mean ± SE, n = 3. **b:** Effects of DPCPX (10^{-7} M) on 20-HETE-induced vascular response in NS and HS fed $A_{2A}AR^{-/-}$ aortic rings. Values are mean ± SE, n = 3.

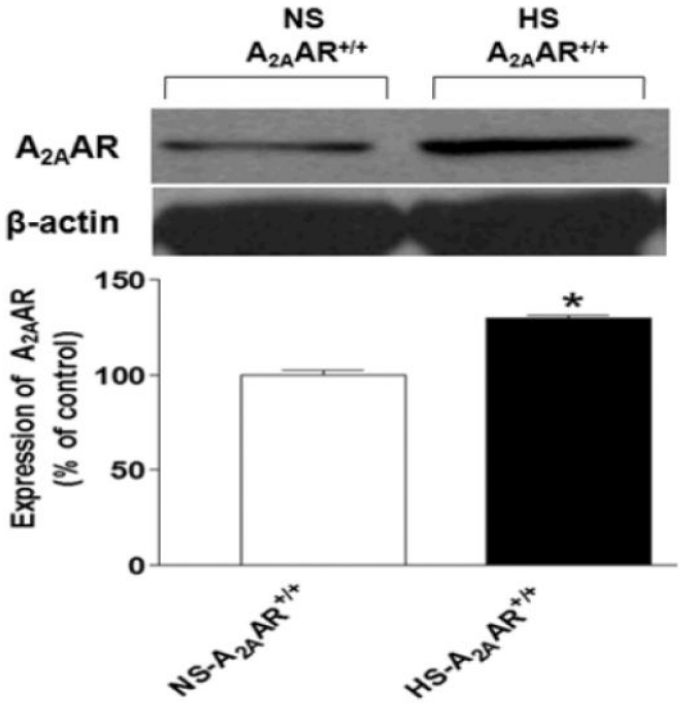


Fig. 6.

Representative Western-blot and densitometric analysis for $A_{2A}AR$ (~45 kDa) protein in aortae of HS and NS fed $A_{2A}AR^{+/+}$ mice. Values are mean ± SE, *$p<0.05$ between NS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{+/+}$ mouse aortae, n=4.

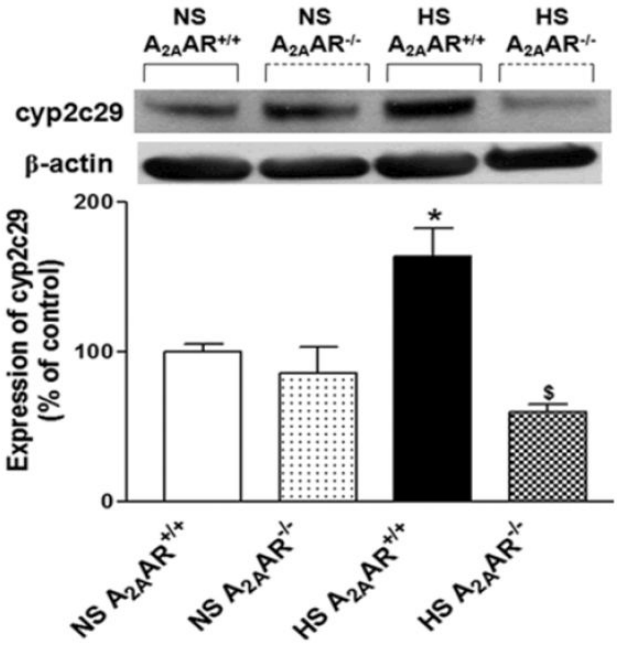


Fig. 7.

Representative Western-blot and densitometric analysis for cyp2c29 (~50 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-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, and $^{\$}$p<0.05 between HS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{-/-}$, *n*=4.

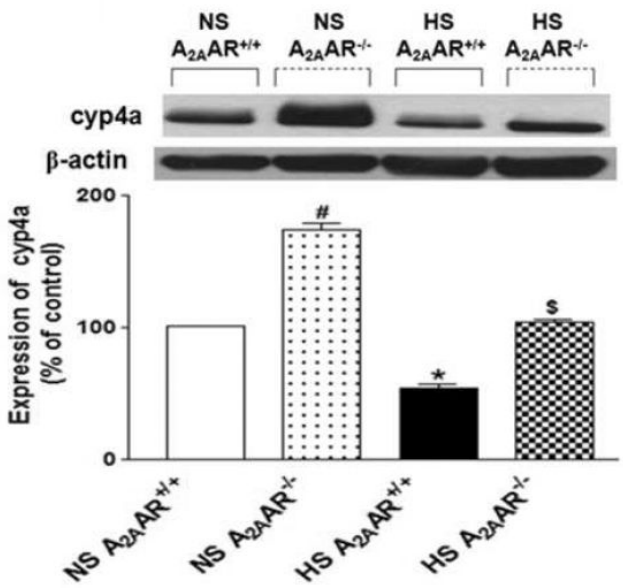


Fig. 8.

Representative Western-blot and densitometric analysis for cyp4a (~50 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-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, $^{\$}$p<0.05 between HS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{-/-}$, and $^{\#}$p<0.05 between NS-$A_{2A}AR^{+/+}$ vs. NS -$A_{2A}AR^{-/-}$, *n*=4.

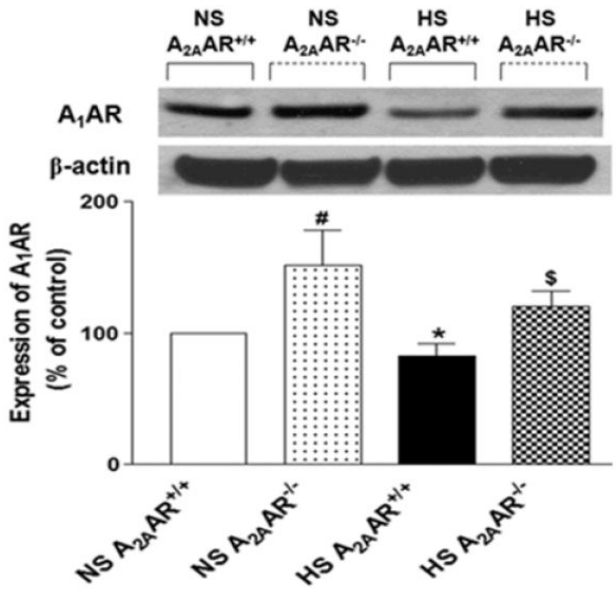


Fig. 9.

Representative Western-blot and densitometric analysis for A_1AR (~37 kDa) protein in aortae of HS and NS fed $A_{2A}AR^{+/+}$ and $A_{2A}AR^{-/-}$ mice. Values are mean ± SE, *$p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{+/+}$, $^{\$}p<0.05$ between HS-$A_{2A}AR^{+/+}$ vs. HS-$A_{2A}AR^{-/-}$, and $^{\#}p<0.05$ between NS-$A_{2A}AR^{+/+}$ vs. NS-$A_{2A}AR^{-/-}$, n=4.