Adenosine A2A receptor modulates vascular response in soluble epoxide hydrolase-null mice through CYP-epoxygenases and PPARγ

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Nayeem MA, Pradhan I, Mustafa SJ, Morisseau C, Falck JR, Zeldin DC. Adenosine A2A receptor modulates vascular response in soluble epoxide hydrolase-null mice through CYP-epoxygenases and PPARγ. Am J Physiol Regul Integr Comp Physiol 304: R23–R32, 2013. First published November 14, 2012; doi:10.1152/ajpregu.00213.2012.—The interaction between Adenosine A2A receptor and soluble epoxide hydrolase (sEH) enzyme is not known. Therefore, we hypothesized that lack of sEH in mice enhances adenosine-induced relaxation through A2A adenosine receptors (AR) via CYP-epoxygenases and peroxisome proliferator-activated receptor γ (PPARγ). sEH−/− showed an increase in A2A AR, CYP2J, and PPARγ by 31%, 65%, and 36%, respectively, and a decrease in A1AR and PPARα (30% and 27%, respectively) vs. sEH+/+. 5′-N-ethylcarboxamidoadenosine (NECA, an adenosine receptor agonist), CGS 21680 (A2A AR-agonist), and GW 7647 (PPARα-agonist)-induced responses were tested with nitro-L-arginine (NO-inhibitor; 10 M), 14,15-epoxyeicosatrienoic acid-antagonist (10 M), 12-(3-adamantan-1-yl-ureido) dodecanoic acid (AUDA; 10 μM) and trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB, sEH-inhibitors; 10 M), and T0070907 (PPARγ-agonist; 10 M). In sEH−/− mice, ACh response was not different from sEH+/+ (P > 0.05), and t-AUCB blocked ACh responses in both sEH−/− and sEH+/+ mice (P < 0.05). NECA (10 M)-induced relaxation was higher in sEH−/− (+12.94 ± 3.2%) vs. sEH+/+ mice (−5.35 ± 5.2%); however, it was blocked by ZM-241385 (−22.42 ± 1.9%) and SCH-58261 (−30.04 ± 4.2%). CGS 21680 (10 M)-induced relaxation was higher in sEH−/− (+37.4 ± 5.4%) vs. sEH+/+ (+2.14 ± 2.8%). t-AUCB (sEH−/−, +30.28 ± 4.8%, P > 0.05) did not block CGS 21680-induced response, whereas 14,15-EEZE (−7.1 ± 3.7%, P < 0.05) did also. AUDA and t-AUCB did not block CGS 21680-induced response in sEH−/− (P > 0.05), but reversed in sEH+/+ from +2.14 ± 2.8% to +45.33 ± 4.1%, and +63.37 ± 7.2%, respectively. PPARα-agonist did not relax as CGS 21680 (−2.48 ± 1.1 vs. +37.4 ± 5.4% in sEH−/−, and PPARγ-antagonist blocked (from +37.4 ± 5.4% to +9.40 ± 3.1) CGS 21680-induced relaxation in sEH−/−. Our data suggest that adenosine-induced relaxation in sEH−/− may depend on the upregulation of A2A AR, CYP2J, and PPARγ, and the downregulation of A1 AR and PPARα.

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Several cytchrome P-450 (CYP)-epoxygenases, including members of the CYP2C (CYP2C29) and CYP2J (CYP2J2, CYP2J5) subfamilies, have been identified in vascular endothelial cells (13, 41, 63, 64). Yang et al. (59) showed that overexpression of CYP2J2 protects endothelial cells against hypoxia-reoxygenation injury (59). Ma et al. (28) found abundant CYP2J5 in both the kidney and liver. The CYP-epoxygenases add an epoxide group across one of the four double bonds of arachidonic acid, forming four EET regioisomers, 5,6-, 8,9-, 11,12-, and 14,15-EET. Endothelial cells express CYP2C and CYP2J, which are the main source of EETs generation in the vascular system (13, 41, 46). The most potent biological effects of EETs occur in small-resistance vessels, as well as in the aorta (9, 36). For example, 14,15-EET has been observed to produce relaxation of isolated coronary microvessels at concentrations as low as 10 PM (9). This process occurs through hyperpolarization and suggests that the EETs function as an endothelium-derived hyperpolarizing factor (EDHF) in a number of vascular beds, including the coronary artery and aorta (3, 4, 13, 36, 37). The EDHF response in bovine coronary arteries and mouse aorta is inhibited by the EET antagonist, 14,15-epoxyeicosa-5(Z)-enoic acid (14, 36). The peroxisome proliferator-activated receptor (PPAR) transcription factors are members of the nuclear receptor superfamily that are activated by fatty acids and fatty acid derivatives (11). Both PPARα and PPARγ are expressed in blood vessels (5, 6), indicating that they have a role in vascular function. PPARγ is activated when bovine aortic endothelial cells are exposed to laminar flow through a process that is dependent on CYP-epoxygenases (26), and this leads to increases in the formation of 8,9-, 11,12-, and 14,15-EET in the endothelial cells within 15 min.

The soluble epoxide hydrolase (sEH) enzyme is detected in a variety of mammalian tissues, including the liver, kidney,
intestine, and blood vessels (40, 56, 60). Within these tissues, sEH metabolizes epoxide-containing compounds to their corresponding diols (31, 40). Conversion of arachidonic acid epoxides to diols by sEH diminishes the beneficial cardiovascular properties of these epoxygenoids. Inhibition of sEH causes EETs to accumulate and be retained for longer periods after they are formed (9). Some reports provide further evidence that sEH inhibition may be an effective approach for the treatment of hypertension and diseases associated with vascular inflammation (7, 8, 18, 47, 61). Also, the targeted disruption of the sEH gene in male mice lowers systolic blood pressure (51). Recently, we found that there is a possible link between the upregulation of A2AAR, CYP-epoxygenases, and downregulation of sEH with adenosine-induced relaxation in mouse aorta (38). Also, we found that there is a possible link between the downregulation of A2AAR, CYP-epoxygenases, and upregulation of sEH with 5′-N-ethylcarboxamidoadenosine (NECA)-induced contraction in mouse aorta (38, 39). Therefore, we hypothesized that the lack of sEH enhances adenosine-induced vascular relaxation through A2AAR via CYP-epoxygenases and PPARγ.

MATERIALS AND METHODS

The generation of sEH−/− mice was described by Sinal et al. (51). sEH−/− and sEH+/+ mice were provided by Dr. Zeldin, National Institute of Environmental Health Sciences/National Institutes of Health (NIH). All animal care and experimentation protocols were approved and carried out in accordance with the West Virginia University Institutional Animal Care and Use Committee and were in accordance with the principles and guidelines of the NIH’s Guide for the Care and Use of Laboratory Animals. Both male and female mice (14–18 wk old) in equal ratio were used in our study.

Protein extraction, gel electrophoresis, and Western blot analysis. Mice were killed with pentobarbital sodium (100 mg/kg ip). According to our previously described protocol (36–39), after thoracotomy, the aorta was gently removed, cleaned of fat and connective tissue. In brief, aortas from both sEH−/− and sEH+/+ mice were treated with 1 ml of lysis buffer for protein extraction. Gel electrophoresis and Western blot analysis were done according to the protocol described by us (36–39). Following blocking with nonfat dry milk, the nitrocellulose membranes were incubated with polyclonal primary antibodies for CYP2J5 (Dr. Zeldin, NIEHS/NIH), A1AR (Sigma Chemicals), A2AAR (Alpha Diagnostic), CYP4A, PPARα, PPARγ, and β-actin (Santa Cruz Biotechnology). The secondary antibody, horse-radish peroxidase-conjugated anti-rabbit IgG, was used. The membranes were developed using enhanced chemiluminescence (Amer sham Biosciences) and exposed to X-ray film for appropriate time.

sEH−/− and sEH+/+ aortic rings. After thoracotomy the aorta was gently removed, cleaned of fat and connective tissue, and cut transversely into rings of 3–4 mm in length. Care was taken not to damage the endothelium. The rings were hung vertically between two wire hooks. Two rings were suspended in organ baths containing 10 ml of modified Krebs-Henseleit buffer (36–39). After the equilibration period (60 min), tissues were contracted with KCl (50 mM) to assess the viability of the tissue. Rings were then constricted with phenylephrine (60 min), tissues were contracted with KCl (50 mM) to assess viability and to obtain the internal diameter of the rings. Rings were then constricted with PE (10−5 M) to obtain the external diameter of the rings. The rings were precontracted with 50 mM KCl to 80% of the initial length.

Effect of nitric oxide inhibitor (L-NAME) on ACh-induced response in sEH−/− and sEH+/+ mice. Concentration-response curves (CRCs) were obtained by cumulative addition of drugs in 1-log increments as described by us (36–39). A single CRC was constructed for each ring in parallel in pairs of rings from either sEH−/− or sEH+/+ in the same organ bath. L-NAME (100 μM) was added 30 min before the PE contraction and was present throughout the ACh CRC.

Effect of A2AAR antagonists (ZM 241385 or SCH 58261) on NECA-induced CRC in sEH−/− and sEH+/+ mice. Adenosine analog NECA-induced CRC was obtained as described above. ZM 241385 or SCH 58261 (1 μM) was added 30 min before PE contraction and was present throughout the experiment as described above.

Effect of nitric oxide (L-NAME)/epoxyeicosatrienoic acids (EETs) receptor antagonist 14,15-EEZE on CGS 21680-induced CRC in sEH−/− and sEH+/+ mice. CGS 21680 (A2AAR agonist)-CRC was obtained with and without L-NAME (100 μM) or 14,15-epoxyeicosatrienoic acid (14,15-EEZE; 10 μM), as described above.

Effect of sEH inhibitors on CGS 21680-induced CRC in sEH−/− and sEH+/+ mice. CGS 21680 CRC was obtained with and without 12-(3-adamantan-1-yl-ureido) dodecanoic acid (AUDA, a sEH inhibitor; 10 μM) or trans-4-[3-(adamantan-1-yl-ureido)-cyclohexyloxy]benzoic acid (t-AUCB, a sEH inhibitor; 10 μM) as described above.

Western blot analysis for A2AAR, A1AR, CYP2J5, CYP4A, PPARα, and PPARγ (Santa Cruz Biotechnology), and A2AAR (Alpha Diagnostic) antibodies were obtained and used for Western blot experiments.

Statistical analysis. Statistical data were reported as means ± SE. One-way ANOVA was used to compare difference among groups, and two-way ANOVA was used for repeated measures, followed by Tukey post hoc test to compare the NECA, CGS 21680, and GW 7647-induced vascular responses to antagonist (L-NAME, 14,15-EEZE, T0070907, ZM 241385, SCH 58261, AUDA, and t-AUCB). Differences were considered significant when P < 0.05.

RESULTS

Expression of A2AAR, A1AR, CYP2J5, CYP4A, PPARα, and PPARγ proteins in aortas from sEH−/− and sEH+/+ mice. Western blot analysis for A2AAR (45 kDa) protein showed 31% more in sEH−/− than sEH+/+ mouse aorta (P < 0.05, Fig. 1A) whereas, A1AR (~37 kDa) protein showed 30% less in sEH−/− than sEH+/+ mice (P < 0.05, Fig. 1B). The amount of CYP2J5 (~58 kDa) protein in sEH−/− was increased by 65% compared with sEH+/+ mouse aorta (P < 0.05, Fig. 2A). Further, the level of CYP4A (~50 kDa) protein in sEH−/− was increased by 60% compared with sEH+/+ mouse aorta (P < 0.05, Fig. 2B). Western blot analysis for PPARγ (~58 kDa)Western blots. Western blot analysis for A2AAR (45 kDa) and A1AR (~37 kDa) proteins was performed using mouse aorta (n = 4) and sEH−/− and sEH+/+ mouse aorta (n = 3) as described above. The Western blots were performed on 12% SDS-PAGE gels and antibody incubations were performed according to the manufacturer’s instructions. The proteins were visualized using chemiluminescence and the grayscale intensity was quantified using ImageJ software. The Western blots were normalized to the loading control, β-actin, and the resulting data were expressed as a percentage of the control group.
protein showed 36% more in sEH-/− than sEH+/+ mouse aorta (P < 0.05, Fig. 3A), whereas PPARα (~52 kDa) protein showed 27% less in sEH-/− than sEH+/+ mice (P < 0.05, Fig. 3B).

CRC for ACh and the effect of nitric oxide inhibitor in sEH-/− and sEH+/+ mice. ACh caused a concentration (10−7−10−5 M)-dependent relaxation in both sEH-/− and sEH+/+, but the response was not significantly different (P > 0.05) between aortas from sEH-/− and sEH+/+ (Fig. 4). Also, l-NAME (100 μM) had altered vascular response significantly (P < 0.05) in both sEH-/− (+3.32 ± 6.0% at 10−6 ACh) and sEH+/+ (−3.4 ± 2.9% at 10−6 M ACh) compared with untreated sEH-/− and sEH+/+ mouse aortas (P < 0.05, Fig. 4). But, no significant difference was observed in concentration response curves between sEH-/− and sEH+/+ (P > 0.05, Fig. 4).

CRC for NECA with and without ZM 241385 or SCH 58261 in sEH-/− and sEH+/+ mice. NECA produced a concentration-dependent relaxation in sEH-/− as opposed to contraction in sEH+/+ (Fig. 5, A and B). For example, the response to 10−6 M NECA in sEH-/− aorta was +12.94 ± 3.2% relaxation, while in sEH+/+ had −5.35 ± 5.2% contraction (P < 0.05, Fig. 5, A and B). CRC in sEH-/− vs. sEH+/+ for NECA (10−7−10−5 M, P < 0.05) were significantly different. ZM 241385 (1 μM), an A2A AR antagonist produced a change from NECA-induced relaxation to contraction in sEH-/− (from +12.94 ± 3.2% to −22.42 ± 1.9 at 10−6 NECA, P < 0.05, Fig. 5A). No significant difference was found with ZM 241385 treatment between sEH-/− and sEH+/+ mice (P > 0.05, Fig. 5A). Another A2A AR antagonist, SCH 58261 produced a similar change from NECA-induced relaxation to contraction in sEH-/− (from +12.94 ± 3.2% to −30.04 ± 4.2 at 10−6 NECA, P < 0.05, Fig. 5B). No significant difference was found between SCH 58261 treated sEH-/− and SCH 58261 treated sEH+/+ mice (P > 0.05, Fig. 5B).

CRC for CGS 21680 and the effects of nitric oxide/epoxyc sắtenoic acid receptor-antagonist in sEH-/− and sEH+/+ mice. CGS 21680 produced a concentration-dependent relaxation (P < 0.05) in sEH-/− compared with the contraction in sEH+/+ mouse (P < 0.05; Fig. 6). For example, at 10−6 M CGS 21680, the relaxation response was +37.4 ± 5.4% in sEH-/− compared with +2.1 ± 2.8% in sEH+/+ mouse (P < 0.05; Fig. 6A). l-NAME (100 μM) did not significantly alter vascular responses in both the treated sEH-/− (±30.28 ± 4.8% at 10−6 CGS 21680) and the control sEH-/− (±37.4 ± 5.4%, P > 0.05, Fig. 6A) tissues. Also, no significant difference was observed in CRC between treated (l-NAME) and control

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**Fig. 1.** Representative Western blots and densitometric analysis for A2A AR (~45 kDa; A) and A1AR (~37 kDa; B) proteins in aortas of sEH-/− and sEH+/+ mice. Values are expressed as means ± SE. *P < 0.05, sEH+/+ compared with sEH-/− aortas; n = 6.

**Fig. 2.** Representative Western blots and densitometric analysis for CYP2J5 (~58 kDa; A) and CYP4A (~50 kDa; B) proteins in aortas of sEH-/− and sEH+/+ mice. Values are expressed as means ± SE. *P < 0.05, sEH+/+ compared with sEH-/− aortas; n = 6.
Effects of sEH inhibitors on CGS 21680 CRC in sEH<sup>+/−</sup> mice. AUDA (10 μM), a soluble epoxide hydrolase (sEH) inhibitor, reversed and produced a significantly higher relaxation (+63.37 ± 7.2%, P < 0.05, Fig. 7B) at 10<sup>−6</sup> M CGS 21680 in sEH<sup>+/+</sup> as opposed to its control (+2.1 ± 2.8%; Fig. 7B). In contrast, t-AUCB produced no significant effect on CGS 21680 CRC in sEH<sup>+/−</sup> (P > 0.05, Fig. 7B).

CRCs for PPARα-agonist (GW 7647) and CGS 21680 and the effect of PPARγ-antagonist (T0070907) in sEH<sup>+/−</sup> and sEH<sup>+/+</sup> mice. GW 7647 produced a concentration-dependent contractions in both sEH<sup>−/−</sup> and sEH<sup>+/+</sup> mice (P < 0.05; Fig. 8A). For example, at 10<sup>−6</sup> M GW 7647, the contraction response was −2.48 ± 1.1% in sEH<sup>−/−</sup> compared with −6.52 ± 1.4% in sEH<sup>+/−</sup> (P < 0.05; Fig. 8A), while CGS 21680 produced a concentration-dependent enhanced relaxation in sEH<sup>−/−</sup> compared with less relaxation in sEH<sup>+/+</sup> mice (P < 0.05; Fig. 8B). For example, at 10<sup>−6</sup> M CGS 21680, the relaxation response was +37.4 ± 5.4% in sEH<sup>−/−</sup> compared with +2.1 ± 2.8% in sEH<sup>+/+</sup> (P < 0.05; Fig. 8B).

In comparison between CGS 21680 and GW 7647, the CGS 21680 (10<sup>−6</sup> M)-induced relaxation was +37.4 ± 5.4% in sEH<sup>−/−</sup> mice, whereas the GW 7647 (10<sup>−6</sup> M)-induced contraction response was −2.48 ± 1.1% in sEH<sup>−/−</sup> mice (P < 0.05; Fig. 8A). A significant blockade was found in the CRC of CGS 21680-induced relaxation with T0070907 (PPARγ-antagonist; 0.1 μM) compared with control in sEH<sup>−/−</sup> aortas. At 10<sup>−6</sup> M CGS 21680, T0070907 had changed the vascular response in sEH<sup>−/−</sup> (+9.40 ± 3.1%; Fig. 8C) aortas compared with controls (+37.46 ± 5.4%, P < 0.05; Fig. 8C).

**DISCUSSION**

The sEH<sup>−/−</sup> mice show an increase in aortic A<sub>2A</sub> AR, CYP2F1, and PPARα protein expressions, and a decrease in A<sub>1</sub> AR, PPARα proteins compared with sEH<sup>+/+</sup> mice (Figs. 1–3). The relationship between sEH and adenosine-induced vascular responses in mice is not known. Therefore, this study was designed to investigate the role of A<sub>2A</sub>, A<sub>1</sub> ARs, CYP-epoxygenases, PPARα, and PPARγ in sEH<sup>+/+</sup> and sEH<sup>−/−</sup> mice. Our data demonstrate that I) ACh-induced vascular relaxation
human population with risk of coronary heart disease, ischemic stroke, restenosis, diabetes heart, heart failure, ischemic stroke in white Europeans, Chinese populations, and in the African American population with hypertension (2, 10, 21, 24, 30, 66). As the earlier studies showed the differential renal sEH gene expression in prehypertensive, hypertensive, and spontaneously hypertensive rats (49), and mice lacking A2A AR have hypertensive characteristics (22). Therefore, there is a need to identify the possible targets and develop novel pharmacological agents to treat vascular deregulation in patients in the long run, who have allelic variants that may possibly act similar to our gene-manipulated mice (A2A AR−/−, sEH−/−), which may be involved in the regulation of blood pressure and vascular tone.

ACh data between sEH+/+ and sEH−/− suggest that there is no relationship between ACh and the presence or absence of

was not different between sEH−/− and sEH+/+ mice, and L-NAME was able to block ACh-induced vascular relaxation equally in both sEH−/− and sEH+/+ mice; 2) A2A AR modulates nitric oxide (NO)-independent vascular relaxation in sEH−/− mice compared with sEH+/+; 3) A2A AR-mediated relaxation was blocked by EET receptor antagonist, but not with NO inhibitor; 4) sEH inhibitors (AUDA and t-AUCB) reversed the CGS 21680-mediated vascular response to enhanced relaxation in sEH+/+, but ineffective in sEH−/− mice; 5) CGS 21680 enhanced dose-dependent relaxation in sEH−/− mice, whereas GW 7647 (PPARα-agonist) reduced relaxation significantly, and finally 6) T0070907 (PPARγ-antagonist) significantly inhibited the CGS 21680-enhanced vascular relaxation in sEH−/− mice.

Genetic polymorphisms in CYP-epoxygenases have been observed in different populations, which affect cardiovascular function, including hypertension (10, 12, 20, 23, 54, 55, 57, 66). Genetic variation in sEH and CYP4A was also found in

Fig. 5. Effect of ZM 241385 (1 μM; A) and SCH 58261 (1 μM; B) on NECA-induced vascular response in aortic rings of sEH+/+ and sEH−/− mice. @ P < 0.05 between sEH+/+ vs. sEH−/− mice. #P < 0.05, sEH+/+ vs. sEH−/− mice treated with ZM 241385. *P < 0.05, sEH+/+ vs. sEH−/− mice treated with ZM 241385, n = 8 (A). @ P < 0.05, between sEH+/+ vs. sEH−/− mice. *P < 0.05, sEH+/+ vs. sEH+/+ mice with SCH 58261. #P < 0.05, sEH+/+ vs. sEH+/+ mice with SCH 58261; n = 8 (B). Values are expressed as means ± SE.

Fig. 6. Effect of L-NAME (100 μM; A) and 14,15-EEZE (10 μM; B) on CGS 21680-induced vascular response in aortic rings of sEH+/+ and sEH−/− mice. *P < 0.05, sEH+/+ vs. sEH−/− mice. #P < 0.05, sEH+/+ vs. sEH−/− mice treated with L-NAME, and sEH+/+ treated with L-NAME vs. sEH+/+ treated with L-NAME; n = 8 (A). *P < 0.05, between sEH+/+ vs. sEH−/− mice. #P < 0.05, sEH+/+ treated with L-NAME vs. sEH+/+ treated with 14,15-EEZE. @ P < 0.05, sEH+/+ vs. sEH−/− mice treated with 14,15-EEZE vs. sEH−/−; n = 8 (B). #P < 0.05, sEH+/+ vs. sEH−/− mice treated with 14,15-EEZE. Values are expressed as means ± SE.
fibers and acts on A₂A AR to evoke vasodilatation independent during exercise in humans, rats, and rabbits (43, 44, 50, 58).

sEH (Fig. 4). Also, L-NAME, a nitric oxide synthase inhibitor completely blocked ACh-induced vascular response equally in both sEH+/+ and sEH−/− mice (Fig. 4). These data also suggest that the presence or absence of sEH does not matter in ACh-induced vascular response in both sEH+/+ and sEH−/− mice (Fig. 4). Similarly, Zhang et al. (65) reported that the inhibition of sEH ameliorates endothelial dysfunction and effects in the db/db mice, which is independent of NO, but dependent on CYP-epoxygenase-derived metabolites. Other studies have also shown that nitric oxide synthase inhibition does not affect the increase of blood flow in forearm or leg during exercise in humans, rats, and rabbits (43, 44, 50, 58). But, during exercise, adenosine originates from skeletal muscle fibers and acts on A₂A AR to evoke vasodilatation independent of NO (45). Our own studies have shown that adenosine-

Fig. 7. Effect of AUDA (10 μM) on CGS 21680 induced vascular response in aortic rings of sEH+/+ and sEH−/− mice (A). The control curve is the same as in Fig. 6. *P < 0.05, sEH+/+ vs. sEH−/− mice. **P < 0.05, sEH+/+ vs. sEH−/− mice treated with AUDA. t-P < 0.05, sEH+/+ vs. sEH−/− treated with AUDA; n = 8 (A). Effect of t-AUCB (10 μM) on CGS 21680-induced vascular response in aortic rings of sEH+/+ and sEH−/− mice (B). The control curve is the same as in Fig. 6. *P < 0.05, sEH+/+ vs. sEH−/− mice. **P < 0.05, sEH+/+ vs. sEH−/− mice treated with t-AUCB. *P < 0.05, sEH+/+ vs. sEH−/− mice treated with t-AUCB; n = 8 (B). Values are expressed as means ± SE.

Fig. 8. GW 7647 (PPARα-agonist)-induced vascular response in aortic rings of sEH+/+ and sEH−/− mice. *P > 0.05 for GW 7647-sEH+/+ vs. GW 7647-sEH−/−; n = 8 (A). CGS 21680-induced vascular response in aortic rings of sEH+/+ and sEH−/− mice. **P < 0.05, CGS 21680-sEH+/+ vs. CGS 21680-sEH−/− mice; n = 8 (B). Effect of PPARγ-agonist (0.1 μM; B) on CGS 21680-induced vascular response in aortic rings of sEH+/+ and sEH−/− mice. *P < 0.05, CGS 21680-sEH+/+ vs. CGS 21680-sEH−/− mice; n = 8 (B). Effect of PPARγ-agonist (0.1 μM; B) on CGS 21680-induced vascular response in aortic rings of sEH+/+ and sEH−/− mice. **P < 0.05, LP 867−/− vs. LP 867−/− mice. *P < 0.05, LP 867−/− treated with PPARγ-agonist vs. LP 867−/− mice alone. **P < 0.05, LP 867−/− treated with PPARγ-agonist vs. LP 867−/− mice alone; n = 8 (C). Values are expressed as means ± SE.
induced mouse aortic relaxation through $A_2\alpha$ AR is independent of NO and COX (36–38).

The sEH enzyme metabolizes EET that serve as substrates for the sEH (31, 40). The conversion of EETs in the presence of sEH into the corresponding dihydroxyeicosatetraenoic acids (DHETs) results in loss of beneficial effects (29, 52, 62). The conversion of epoxides to diols by sEH diminishes the beneficial cardiovascular properties of these epoxyeicosanoids. Inhibition of sEH causes EETs to accumulate and be retained for longer periods after they are formed (9). Some reports provide further evidence that sEH inhibition may be an effective approach for the treatment of hypertension and diseases associated with vascular inflammation (7, 8, 18, 47, 61). Also, the targeted disruption of the sEH gene in male mice lowers systolic blood pressure (51). Recently, we found that there is a possible link between the upregulation of $A_2\alpha$ AR, CYP-epoxygenases, and downregulation of sEH with adenosine-induced relaxation in high-salt diet-fed WT mice (C57BL/6J) and eNOS$^{-/-}$ (C57BL/6J background) compared with low-salt diet-fed mice (38, 39). Also, we found that there is a possible link between the downregulation of $A_2\alpha$ AR, CYP-epoxygenases and upregulation of sEH with NECA-induced contraction in low-salt diet-fed WT mice and eNOS$^{-/-}$ (C57BL/6J background) compared with high-salt diet-fed mice (38, 39). The current study shows that in the absence of sEH, there is an increase in aortic $A_2\alpha$ AR, CYP2J5, PPAR$\gamma$, and a decrease in $A_1$ AR, PPAR$\alpha$s proteins compared with WT mice. Therefore, we hypothesize that, in the absence of sEH, adenosine induces vascular relaxation through $A_2\alpha$ AR via CYP-epoxygenases and PPAR$\gamma$, whereas in the presence of sEH, adenosine induces vascular contraction through PPAR$\alpha$.

NECA (nonselective adenosine agonist) induces significant relaxation in sEH$^{+/+}$ compared to sEH$^{-/}$ mice (Fig. 5), suggesting that sEH deletion enhances NECA-induced vascular relaxation.

Our data show that NECA-induced vascular response in aortas of sEH$^{+/+}$ mice is similar to that from our earlier reports in which we found that high-salt diet-fed WT (C57BL/6J) mice had an enhanced NECA-induced relaxation with upregulation of $A_2\alpha$ AR, CYP2J-epoxygenase and downregulation of sEH, whereas, NECA-induced vascular response in aortas of sEH$^{-/}$ mice is similar to that found in our earlier reports in which low-salt diet-fed WT (C57BL/6J) mice had an enhanced NECA-induced contraction with downregulation of $A_2\alpha$ AR, CYP2J-epoxygenase and upregulation of sEH (37, 38). Therefore, in the present study, we used specific $A_2\alpha$ AR antagonists (ZM-241385 and SCH-58261) to rule out the involvement of other adenosine receptors ($A_1$, $A_2B$, $A_3$). Both ZM 241385 and SCH-58261 ($A_2\alpha$ AR-antagonists) blocked completely the NECA-induced vascular relaxation in sEH$^{+/+}$ compared with untreated mice (Fig. 5, A and B). We also confirmed the involvement of $A_2\alpha$ AR with the use of a highly selective $A_2\alpha$ AR agonist (CGS 21680) in sEH$^{+/+}$ and sEH$^{-/}$ mouse aortas. A highly robust CGS 21680-induced vascular relaxation was observed in sEH$^{+/+}$ compared with sEH$^{-/}$ mice (Fig. 6). Also, in the present study, we found a significant upregulation of $A_2\alpha$ AR protein in sEH$^{+/+}$ compared with sEH$^{-/}$ mice (Fig. 1A), whereas a significant downregulation of $A_1$ AR protein in sEH$^{+/+}$ compared with sEH$^{-/}$ mice (Fig. 1B). These data suggest that $A_2\alpha$ AR is the only adenosine receptor involved in enhancing adenosine-induced relaxation in sEH$^{+/+}$ compared with sEH$^{-/}$ mouse aortas.

In the present study, we also tested whether $A_2\alpha$ AR-induced vascular relaxation is NO-dependent or not. No significant changes were found between l-NAME-treated and untreated sEH$^{+/+}$ and sEH$^{+/+}$ mouse aortas with CGS 21680-induced vascular response (Fig. 6A). These data suggest that there is no role for NO in adenosine-induced vascular response through $A_2\alpha$ AR in sEH$^{-/}$ and sEH$^{+/+}$ mice. These data confirm our earlier reports from this laboratory in which l-NAME and indomethacin (COX inhibitor) were unable to block aortic relaxation in $A_2\alpha$ AR$^{+/+}$ compared with $A_2\alpha$ AR$^{-/}$ mice, and in high-salt diet-fed compared with low-salt diet-fed C57BL/6J mice (36–39). Also, Ray and Marshall (45) reported that during exercise, skeletal muscle fibers release adenosine, which is independent of NO.

In this study, NECA- and CGS 21680-induced relaxation in sEH$^{-/}$ mouse aortas (Figs. 5–8) suggests that this relaxation depends on $A_2\alpha$ AR via CYP-epoxygenases leading to EET formations. Therefore, we were able to block CGS 21680-induced vascular relaxation with 14,15-EEZE (EET receptor antagonist) in sEH$^{-/}$ mouse aortas (Fig. 6B). Similarly, the EDHF response in bovine coronary arteries was inhibited by the EET antagonist, 14,15-epoxyeicosatrienic acid (44).

Recently, a report from our laboratory also showed that in $A_2\alpha$ AR$^{-/}$ mouse aorta, the adenosine-induced vascular relaxation was inhibited by the EET antagonist, 14,15-epoxyeicosatrienic acid (36). In the present study, we also found an upregulation of CYP2J5 proteins in sEH$^{-/}$ compared with sEH$^{+/+}$ mouse aortas (Fig. 2A). Our data suggest that the endothelium-dependent NECA- and CGS-21680-induced vascular relaxation is mediated through EETs possibly by increased in CYP2J-epoxygenase in sEH$^{-/}$ compared sEH$^{+/+}$ mouse aortas.

The current study further confirmed that the adenosine-induced vascular relaxation through $A_2\alpha$ AR is dependent on CYP-epoxygenases, including CYP2J-epoxygenase, but not NO or COX.

We also demonstrated that the treatment with 12-(3-adaman-t-1-yl-ureido) dodecanoic acid (AUDa) or trans-4-[4-(3-adaman-t-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB), inhibitors of the sEH enzyme have reversed the CGS 21680-induced weak vascular response into a strong vascular relaxation in sEH$^{+/+}$ compared with controls (Fig. 7, A and B), while, no significant change was observed with AUDa or t-AUCB-treated and untreated sEH$^{-/}$ mice (Fig. 7, A and B). These data suggest that, in the presence of sEH, AUDa or t-AUCB effectively block the endogenous sEH enzyme activity. Therefore, we observed complete reversal of CGS 21680-induced vascular response in AUDa- or t-AUCB-treated sEH$^{+/+}$ mouse aortas compared with controls. Also, it is obvious that, no significant change was observed with CGS 21680-induced vascular response between AUDa or t-AUCB-treated and untreated sEH$^{-/}$ mice, because these mice do not have endogenous sEH enzyme activity. Use of sEH enzyme inhibitors or deleting the sEH gene increases endogenous EETs by decreasing the metabolic conversion of EETs into less active DHETs. If the mechanism by which sEH inhibitors (AUDa or t-AUCB) or deletion of sEH gene enhance CGS 21680-induced vascular relaxation occurs by increasing endogenous EET levels, then it would be expected that inhibiting EET synthesis would attenuate the CGS 21680-induced vascular response in sEH$^{-/}$ mice. As expected, pretreatment of sEH$^{-/}$ mouse aorta with the EETs antagonist 14,15-EEZE did...
alter significantly CGS 21680-induced vascular response from relaxation to contraction (Fig. 6B). These data suggest that in absence or blocking of sEH, enhanced CGS 21680-induced vascular relaxation is mostly likely due to an increase EET levels. Liu et al. (26) suggests that selective sEH inhibitors will potentiate the anti-inflammatory effect in the endothelial cells, presumably by increasing the retention of 11,12- and 14,15-EET so that PPARγ activation is prolonged. Acute activation of PPARγ leads to endothelium-dependent aortic relaxation in nondiabetic (+db/+/m) mice (48). Therefore, we found upregulation of PPARγ and down regulation of PPARαs with A2A AR enhanced relaxation in sEH−/− compared with sEH+/+ mice (Fig. 3, A and B). The PPAR expression data are also supported by the functional data, in which PPARα agonist dose-dependent vascular response in sEH−/− was not significantly different compared with sEH+/+ mice (Fig. 8A), whereas CGS 21680 dose-dependent vascular response in sEH−/− was significantly different compared with sEH+/+ mice (Fig. 8B). There was no significant difference found between CGS 21680-induced vascular response in sEH+/+ mice compared with PPARα-agonist-induced response in both sEH−/− and sEH+/+ mice (Fig. 8, A and B), suggesting that the vascular contraction in sEH+/+ mouse aorta is possible due to PPARα activity (Figs. 3B and 8A). Moreover, PPARγ antagonist was able to block significantly the CGS 21680-induced vascular relaxation (Fig. 8C), and there was no significant difference found between CGS 21680-induced vascular response in sEH+/+ mice compared with CGS 21680 + PPARγ antagonist response in both sEH−/− and sEH+/−-treated mice (Fig. 8C). These data suggest a possible role for PPARγ in CGS 21680-induced vascular relaxation in the absence of sEH compared with the presence of sEH in mice (Figs. 3A and 8C). Also, the data suggest a possible role of PPARα in vascular contraction (Figs. 3B and 8A).

Surprisingly, we found an upregulation of CYP4A (Fig. 2B), a vasoconstrictor enzyme in sEH−/− mouse aorta compared with sEH+/+. The reason behind the upregulation of CYP4A in the absence of sEH may be due to a compensatory response to maintain vascular tone, as well as the blood pressure in sEH knockout mouse. Similar upregulation of CYP4A in kidneys of Ephx2 gene-disrupted mice has been reported as a compensatory mechanism to maintain blood pressure regulation (27).

The present data suggest that there is a relationship between A2A AR-enhanced vascular relaxation and lack of sEH, and there is relationship between the presence of sEH and adenosine-induced vasoconstriction or less relaxation through upregulation of A1 AR. The signaling mechanism may be involved by the activation of A2A AR, functional endothelium to activate CYP2J-epoxygenases to generate more EETs than DHETs, downregulation of A1 AR, PPARα, and upregulation of A2A AR, PPARγ, leading to NECA or CGS 21680-enhanced vascular relaxation in sEH−/− mouse aorta. In contrast, the contraction or less relaxation with NECA or CGS 21680 in sEH+/+ mice provides evidence that there is a possible link among the less availability of A2A AR, less functional endothelium to activate CYP2J-epoxygenases, less generation of EETs than DHETs, and upregulation of A1 AR and PPARα. Therefore, we conclude that upregulation of CYP-epoxygenases, A2A AR, and PPARγ and downregulation of A1 AR and PPARα protein expression contribute to NECA- or CGS 21680-induced dilation in sEH−/− mouse aorta, while the upregulation of A1 AR and PPARα and downregulation of CYP-epoxygenases, A2A AR, and PPARγ protein expression contribute to NECA or CGS 21680-induced vasoconstriction or less relaxation in sEH+/+ mouse aorta.

Perspectives and Significances

These findings suggest that the relationship among A2A AR, CYP-epoxygenases, arachidonic acid-derived metabolites, and PPARγ in vasodilation, while a relationship among A1 AR, sEH, and PPARα in vasoconstriction may have clinical implication in the regulation of vascular tone and regulation of blood pressure. Any deregulation in these pathways or slight allelic variations may possibly lead to hypertension and coronary artery disease in humans. Future studies are necessary to identify the possible targets and develop novel pharmacological agents to treat vascular deregulation in patients in the long run, who have allelic variants that may possibly act similar to our gene-manipulated mice (A2A AR−/−, sEH−/−), which may play a role in the regulation of vascular tone and ultimately blood pressure.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.A.N. conception and design of research; M.A.N. and I.P. performed experiments; M.A.N. analyzed data; M.A.N. interpreted results of experiments; M.A.N. prepared figures; M.A.N. drafted manuscript; M.A.N., D.C., C.M., J.R.F., and D.C.Z. approved final version of manuscript.

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