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ORIGINAL ARTICLE

Involvement of NADPH oxidase in A_{2A} adenosine receptor-mediated increase in coronary flow in isolated mouse hearts

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Abstract Adenosine increases coronary flow mainly through the activation of A_{2A} and A_{2B} adenosine receptors (ARs). However, the mechanisms for the regulation of coronary flow are not fully understood. We previously demonstrated that adenosine-induced increase in coronary flow is in part through NADPH oxidase (Nox) activation, which is independent of activation of either A_1 or A_3ARs. In this study, we hypothesize that adenosine-mediated increase in coronary flow through Nox activation depends on A_{2A} but not A_{2B}ARs. Functional studies were conducted using isolated Langendorff-perfused mouse hearts. Hydrogen peroxide (H_2O_2) production was measured in isolated coronary arteries from WT, A_{2A}AR knockout (KO), and A_{2B}AR KO mice using dichlorofluorescein immunofluorescence. Adenosine-induced concentration-dependent increase in coronary flow was attenuated by the specific Nox2 inhibitor gp91 ds-tat or reactive oxygen species (ROS) scavenger EUK134 in both WT and A_{2B} but not A_{2A}AR KO isolated hearts. Similarly, the A_{2A}AR selective agonist CGS-21680-induced increase in coronary flow was significantly blunted by Nox2 inhibition in both WT and A_{2B}AR KO, while the A_{2B}AR selective agonist BAY 60-6583-induced increase in coronary flow was not affected by Nox2 inhibition in WT. In intact isolated coronary arteries, adenosine-induced (10 μM) increase in H_2O_2 formation in both WT and A_{2B}AR KO mice was attenuated by Nox2 inhibition, whereas adenosine failed to increase H_2O_2 production in A_{2A}AR KO mice. In conclusion, adenosine-induced increase in coronary flow is partially mediated by Nox2-derived H_2O_2, which critically depends upon the presence of A_{2A}AR.

Keywords Adenosine · A_{2A} receptor knockout · NADPH oxidase · Hydrogen peroxide · Coronary flow

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Introduction

Coronary flow is tightly regulated to maintain a consistently high level of myocardial oxygen extraction over a wide range of myocardial demands [1–3]. This tight regulation is dependent on numerous vasoconstrictor and vasodilator influences, exerted by the autonomic nervous system, endothelium, and myocardium [3–6]. Adenosine, a well-known locally released metabolite, has been postulated as one of the important agents responsible for coronary vascular tone regulation in various conditions [3, 5, 7–9], among which adenosine has also been thought to regulate resting coronary flow [10, 11]. However, the role of adenosine in regulating resting coronary flow remains controversial [3]. This discrepancy may be due to different animal models, differences in species, and/or different agonists and antagonists used in these studies. It is well established that the coronary effects of adenosine are mediated through the activation of its four subtypes of receptors, namely A_1, A_{2A}, A_{2B}, and A_3 receptors (ARs) [9, 12, 13]. The role of these receptors has been studied in

various species [12]. With an overall coronary vasodilation, A_{2A}AR predominantly and A_{2B}AR minimally contribute to dilating coronary vasculature [14–16], whereas A_1AR and A_3AR counteract the effects of A_{2A}/A_{2B}ARs resulting in a diminished coronary vasodilation [15, 17, 18]. At post-receptor levels, several effector pathways of adenosine-mediated coronary flow have been reported, such as activation of nitric oxide (NO) pathway [19, 20], cyclic adenosine 5′-monophosphate (cAMP)-dependent pathway [21], activation of potassium channels [9, 21], and the involvement of H_2O_2 [19]. However, the downstream effectors linked to the activation of ARs in this process are not completely understood.

H_2O_2, a molecule within the reactive oxygen species (ROS) family, has been proposed to serve as a pivotal vasodilator in coronary flow regulation in human, canine, porcine, and murine coronary vasculature [19, 22–26]. NADPH oxidases (Nox) are the major source of ROS in the vasculature that plays both physiological and pathophysiological roles in the control of vascular tone [27–29]. The Nox family consists of seven members, Nox1-5, Doux1, and Doux2, among which Nox2 (gp91 Phox) forms the major source of H_2O_2 in cells stimulated with growth factors or cytokines under normal circumstances [30, 31]. Indeed, in the coronary vasculature, Nox2 has been proposed to be a functionally relevant source of H_2O_2 that mediates agonist-induced vasodilation [32]. Several recent studies have shown the interaction between adenosine and ROS via the regulation of Nox activity. For instance, inhibition or deletion of Nox2 leads to the attenuation of adenosine vasoconstrictor responses in aortas [33] and renal arterioles [34] and adenosine vasodilator responses in cerebral arteries [35]. However, little is known about the involvement of Nox2-derived ROS in adenosine-mediated flow response in the coronary circulation. Although activation of A_1 and A_3AR has been shown to have vascular effects through ROS [33, 36], our recent studies have already excluded the involvement of A_1 and A_3ARs in ROS-mediated regulation of coronary flow [37]. Therefore, with the focus on the adenosine signaling, the first aim of the present study was to further investigate whether A_{2A} and/or A_{2B}AR are involved in Nox2-derived ROS-mediated coronary vasodilation. Given the previous findings that adenosine A_{2A}AR coupled to H_2O_2 contributes to coronary reactive hyperemia [38] and Nox2 acts as a functional source for H_2O_2 resulting in coronary vasodilation [32], the second aim of the present study was to examine whether Nox2-derived ROS production contributing to adenosine-mediated coronary flow is through H_2O_2 and which adenosine receptors are required in this process. We performed exogenous infusion of adenosine in Langendorff-perfused isolated hearts as well as immunohistochemistry in isolated coronary arteries from wild type (WT), A_{2A}AR knockout (KO), and A_{2B}AR KO mice for these studies.

Methods

Materials

EUK134 was purchased from Cayman Chemical (Ann Arbor, MI, USA), gp91 ds-tat was purchased from Anaspec (Fremont, CA, USA), and BAY 60-6583 was obtained as a gift from Bayer AG (Leverkusen, Germany). All other chemicals (adenosine, CGS-21680 and 2′,7′-dichloro-fluorescein diacetate (DCFH-DA)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of adenosine, BAY 60-6583, CGS-21680, EUK134, and DCFH-DA were made in dimethyl sulfoxide (DMSO), whereas gp91 ds-tat was dissolved in distilled water.

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee at West Virginia University School of Medicine. We followed guidelines set forth by National Institutes of Health regarding the care and use of laboratory animals. WT mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). A_{2A} and A_{2B}AR KO mice on an inbred C57BL/6 background were generously provided by Dr. Catherine Ledent (Universite Libre de Bruxelles, Belgium) and Dr. Stephen Tilley (University of North Carolina, Chapel Hill, NC, USA), respectively. Mice were kept in cages with 12:12-h light-dark cycles and maintained on a standard laboratory diet with access to water ad libitum. The absence of A_{2A} and A_{2B}ARs at mRNA level in A_{2A}AR KO and A_{2B}AR KO mice has been confirmed by our previous studies using PCR in isolated aortas, mesenteric arteries, and coronary arteries [16, 20, 39–41]. With difficulties in collecting large amount of tissues for Western blot, one of our recent studies has confirmed the absence of A_{2A}AR protein level in A_{2A}AR KO mice in isolated coronary arteries using immunohistochemistry [40]. Our functional data also confirmed the lack of CGS-21680 (a selective A_{2A}AR agonist)-induced coronary flow response in isolated hearts of A_{2A}AR KO mice and the lack of BAY 60-6583 (a selective A_{2B} AR agonist)-induced coronary flow response in isolated hearts of A_{2B}AR KO mice [16, 20].

Langendorff-perfused mouse heart preparation

Mice (14–18 weeks) of either sex were anesthetized with pentobarbital sodium (50 mg kg^{-1}, i.p.). Mice were weighed before hearts were rapidly removed into heparinized (5 U ml^{-1}) ice-cold Krebs-Henseleit buffer containing (in mM) 119 NaCl, 11 glucose, 22 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 2 pyruvate, and 0.5 EDTA. After removal of the surrounding tissue, the aorta

was rapidly cannulated with a 20-gauge, blunt-ended needle; then, the heart was continuously perfused with 37 °C buffer bubbled with 95 % O_2/5 % CO_2 at a constant perfusion pressure of 80 mmHg [20, 40]. Subsequently, through an opening in the left atrium, a fluid-filled balloon made of plastic wrap was inserted into the left ventricle across the mitral valve. The balloon was connected to a pressure transducer for continuous measurement of left ventricular developed pressure (LVDP) and heart rate. The heart was then immersed in a water-jacketed perfusate bath maintained at 37 °C and beats spontaneously. Coronary flow was continuously measured with an ultrasonic flow probe (Transonic Systems, Ithaca, NY, USA) placed in the aortic perfusion line. A PowerLab Chart data acquisition system (AD Instruments, Colorado Springs, CO, USA) was used for data acquisition. Hearts were allowed to equilibrate for 30 to 45 min before starting experimental protocols. Hearts with persistent arrhythmias were excluded from the study. As a positive control, a 15-s flow occlusion resulted in a twofold increase in coronary flow over baseline [19, 38], indicating that buffer-perfused hearts have minimum hypoxia and coronary vessels have the capacity to further dilate.

Langendorff experimental protocols

After equilibrium, baseline coronary flow, heart rate (HR), and LVDP were measured. Adenosine concentration response curves (10^{-8}–10^{-5} M) were acquired in perfused hearts from WT, A_{2A}AR KO, and A_{2B}AR KO mice. Each concentration of adenosine was infused for 5 min followed by a minimum of 5 min of perfusion for drug washout [16, 37]. In separate experiments, the specific Nox2 inhibitor gp91 ds-tat (1 μM) [42] or the superoxide dismutase and catalase-mimicking drug EUK134 (50 μM) was perfused for 20 min before acquiring adenosine concentration response curves (10^{-8}–10^{-5} M) in perfused hearts from those mice [37].

In another two experimental groups, the selective A_{2A}AR agonist CGS-21680 concentration response curves (10^{-10}–10^{-6} M) were acquired in perfused hearts from WT, A_{2A}AR KO, and A_{2B}AR KO mice, while the selective A_{2B}AR agonist BAY 60-658 concentration response curves (10^{-10}–10^{-6} M) were acquired in perfused hearts only from WT mice. In separate experiments, the specific Nox2 inhibitor gp91 ds-tat (1 μM) was perfused for 20 min before acquiring CGS-21680 and BAY 60-658 concentration response curves (10^{-10}–10^{-6} M), respectively [16, 37]. All compounds were infused at a rate of 1/100 ml min^{-1} of the coronary flow through an injection port directly proximal to the aortic cannula using a microinjection pump (Harvard Apparatus, Holliston, MA, USA) [16, 20, 37].

Fluorescence detection of H_2O_2 in mouse coronary arteries

Left coronary arteries (with diameter of 50–120 μm) were isolated from WT, A_{2A}AR KO, and A_{2B}AR KO mice. Then, coronary arteries were incubated in DCFH-DA (10 μM) prepared in DMEM (ATCC, Manassas, VA, USA) for 30 min at 37 °C followed by 10-min wash [40]. Vessels were then pinned on a layer of silicone gel lying on a plastic culture dish and incubated with DMEM buffer maintained at 37 °C. Baseline control images were obtained by optical *xyz* sectioning with a vertical depth of 1 μm using a Zeiss water immersion objective (W N-AchroplanX40/0.75 numerical aperture) on a confocal microscope (LSM 510, Zeiss, Heidelberg, Germany). Once the baseline control images were acquired, adenosine (10 μM) was added into the culture dish and images were taken at 5 min (in some vessels at both 5 and 10 min) after adenosine incubation. We previously showed that a maximal fluorescence intensity of DCFH-DA was reached after 15 min of adenosine incubation [40], and there was no difference in fluorescence intensity between 5 and 10 min in the present study (data not shown). The data at 5 min after adenosine incubation was used for analysis. In separate sets of experiments, the arteries were treated with the Nox2 inhibitor gp91 ds-tat (1 μM) for 20 min and baseline control images were obtained before the addition of adenosine. After 5 min of adenosine incubation, additional images were taken. Hydrogen peroxide (200 μM) served as the positive control at the end of experiments [37, 40]. Only the bottom half of vessels (more proximal to the scanner) were scanned by *xyz* sectioning, in order to avoid the quenching as much as possible. ImageJ software was used for fluorescence image analysis. Stacks of regions of interest (ROIs) were selected on the basis of the outline of the vessel indicated by the fluorescence.

Statistical analysis

Langendorff baseline data for WT, A_{2A}AR KO, and A_{2B}AR KO groups were compared using one-way ANOVA followed by post hoc analysis using Bonferroni's test. The effects of drug treatment on concentration response curves of adenosine, CGS-21680, and BAY 60-6583 were analyzed using two-way ANOVA followed by post hoc analysis using Bonferroni's test. Since the absolute coronary flow changes proportionally with heart mass, the coronary flow was presented as ml min^{-1} g^{-1} wet heart weight [16, 37, 43]. With regard to imaging, the mean fluorescence intensity of each ROI (including those on both endothelial cells and smooth muscle cells) was calculated by subtraction of the background signal and changes in fluorescence intensity were presented as a ratio normalized to control [40]. Image data were analyzed using paired *t* test. All the data are presented as mean±SEM; *n*

represents the number of animals. Statistical significance was accepted when $P<0.05$.

Results

Baseline function in isolated WT, A_{2A}AR KO, and A_{2B}AR KO mouse hearts

Table 1 summarizes the baseline functional parameters for heart rate, LVDP, and coronary flow in WT, A_{2A}AR KO, and A_{2B}AR KO mice after 30 min of equilibration of isolated hearts. Average heart weight to body weight ratio was significantly different between A_{2A}AR KO and A_{2B}AR KO mice, which is due to a heavier body weight in A_{2B}AR KO mice as compared to A_{2A}AR KO mice (27±0.7 g in A_{2B}AR KO mice vs. 24±0.5 g in A_{2A}AR KO mice), while the heart weight between the groups was comparable. However, no significant differences were observed in heart rate, LVDP, and baseline coronary flow from those mice ($P>0.05$, by one-way ANOVA) (Table 1).

Effects of Nox2 inhibition and A_{2A}AR/A_{2B}AR deletion on adenosine-induced increase in coronary flow

Adenosine produced a concentration-dependent increase in coronary flow in WT mice (E_{max}: 38.13±0.78 ml min^{-1} g^{-1}, data obtained from both Figs. 1a and 2a), which was significantly attenuated by Nox2 inhibition with gp91 ds-tat (Fig. 1a). In agreement with our previous studies [37], these findings indicate that adenosine-induced increase in coronary flow in WT mice is mediated in part through Nox2 activation.

Table 1 Baseline data for WT, A_{2A}AR KO, and A_{2B}AR KO mouse hearts

	WT (n=21)	A_{2A}AR KO (n=15)	A_{2B}AR KO (n=17)
Age, week	16±0.3	15±0.3	16±0.4
No. of mice	21	15	17
BW, g	25±0.8	24±0.5	27±0.7
HW, mg	96.2±2.6	99.1±2.6	98.4±2.4
HW/BW, %	0.39±0.01	0.42±0.01	0.37±0.02*
CF, ml min^{-1} g^{-1}	18±0.6	18±0.9	20±0.4
HR, beats min^{-1}	437±10	391±8	446±10
LVDP, mmHg	90±4	90±5	80±4

All parameters were collected after 30 min of equilibration in a Langendorff preparation. Values are means±SEM

WT wild type, *KO* knockout mice, *BW* body weight, *HW* heart weight, *CF* coronary flow, *HR* heart rate, *LVDP* left ventricular developed pressure

*$P<0.05$ compared to A_{2A}AR KO

Notably, inhibition of Nox2 by gp91 ds-tat in A_{2A}AR KO mice had no effect on adenosine-induced increase in coronary

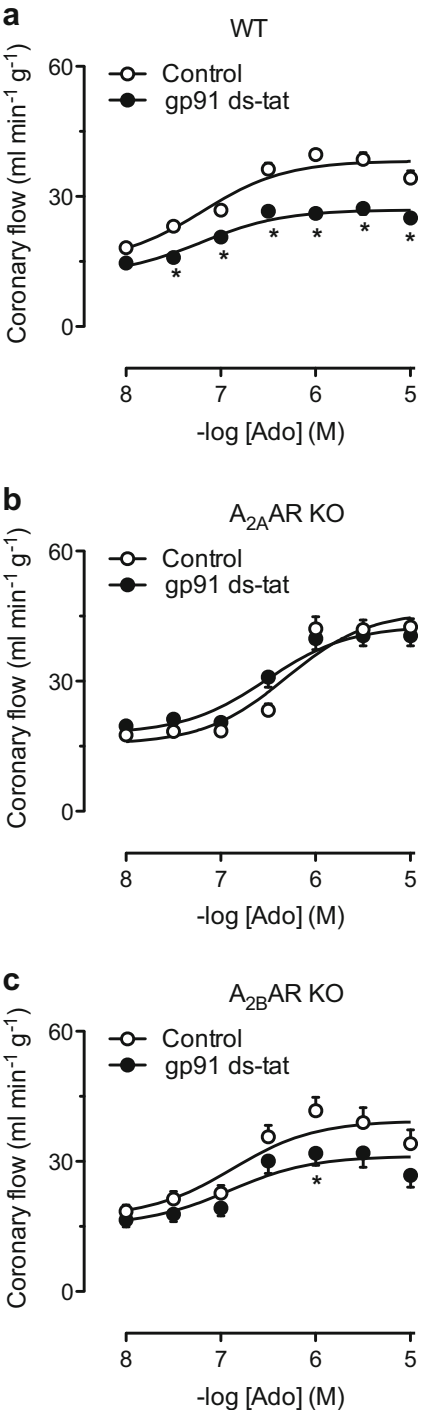

Fig. 1 Effects of NADPH oxidase (Nox2) inhibition on adenosine-induced increase in coronary flow. Shown are the effects of gp91 ds-tat (1 μM) on adenosine (Ado) concentration response curves (10^{-8} to 10^{-5} M) in isolated hearts of wild type (WT) (**a**, n=6), A_{2A} receptor (AR) knockout (KO) (**b**, n=6), and A_{2B}AR KO mice (**c**, n=6). Values are mean±SEM. *Significant difference vs. corresponding control points using two-way ANOVA followed by post hoc analysis using Bonferroni's test ($P<0.05$)

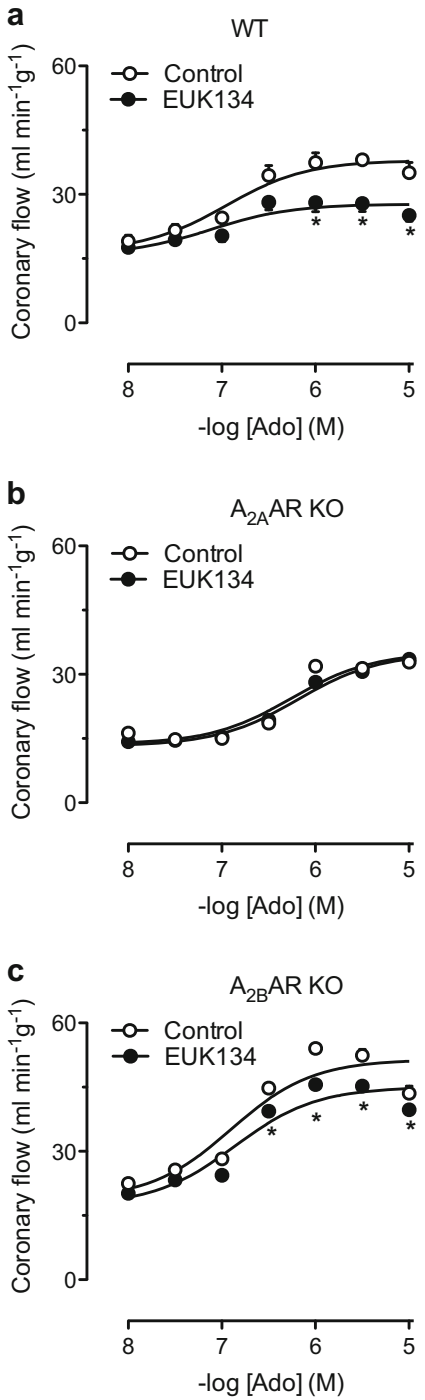

Fig. 2 Effects of ROS scavenging on adenosine-induced increase in coronary flow. Shown are the effects of EUK134 (50 μM) on adenosine (Ado) concentration response curves (10^{-8} to 10^{-5} M) in isolated hearts of WT (**a**, n=5), A_{2A}AR KO (**b**, n=4), and A_{2B}AR KO mice (**c**, n=6). Values are mean±SEM. *Significant difference vs. corresponding control points using two-way ANOVA followed by post hoc analysis using Bonferroni's test (P<0.05)

flow (Fig. 1b) but significantly attenuated the increased coronary flow in A_{2B}AR KO mice (Fig. 1c). All together, these findings indicate that adenosine-induced increase in coronary flow is partially mediated via activation of Nox2 and likely depends on A_{2A} but not A_{2B}ARs.

Effects of ROS scavenging and A_{2A}/A_{2B}AR deletion on adenosine-induced increase in coronary flow

In accordance with previous studies from our laboratory [37], ROS scavenging with EUK134 markedly attenuated the adenosine-induced increase in coronary flow in WT mice (Fig. 2a). Similar to the effects of Nox2 inhibition, ROS scavenging with EUK134 had no effect on adenosine-induced increase in coronary flow in A_{2A}AR KO mice (Fig. 2b) but significantly attenuated the increased coronary flow in A_{2B}AR KO mice (Fig. 2c). All together, these findings indicate that ROS production is involved in adenosine-induced increase in coronary flow, which depends on A_{2A} but not A_{2B}AR activation.

Effects of Nox2 inhibition and A_{2A}AR/A_{2B}AR deletion on A_{2A}AR/A_{2B}AR agonist-mediated increase in coronary flow

To further investigate the role of A_{2A}AR in this process, the selective A_{2A}AR agonist CGS-21680 concentration response curves were acquired. CGS-21680 produced a concentration-dependent increase in coronary flow in WT, which was significantly blunted by Nox2 inhibition with gp91 ds-tat (Fig. 3a). A similar finding was also observed in A_{2B}AR KO mice in which CGS-21680-induced increase in coronary flow was significantly attenuated by the Nox2 inhibitor gp91 ds-tat (Fig. 3c). As expected, CGS-21680 failed to increase coronary flow in A_{2A}AR KO mice (Fig. 3b). To further exclude the involvement of A_{2B}AR, the selective A_{2B}AR agonist BAY 60-6583 concentration response curves were acquired in WT mice. BAY 60-6583 produced a concentration-dependent increase in coronary flow, which was not affected by the Nox2 inhibitor gp91 ds-tat (Fig. 4). Taken together, these findings indicate that adenosine-induced increase in coronary flow is partially mediated by Nox2-derived ROS, which requires A_{2A}ARs.

Effects of Nox2 inhibition and A_{2A}AR/A_{2B}AR deletion on H_2O_2 production by adenosine in isolated coronary arteries

To assess the potential involvement of vasodilator ROS (H_2O_2) in adenosine-induced increase in coronary flow, DCFH-DA immunofluorescence was performed on intact isolated coronary arteries. As shown in Fig. 5, adenosine (10 μM) increased H_2O_2 generation in both WT and A_{2B}AR KO mice (Fig. 5a), as evidenced by increases in fluorescence intensity ratio in adenosine-treated tissues (1.36±0.04 and 1.24±0.02 times corresponding baselines, respectively,

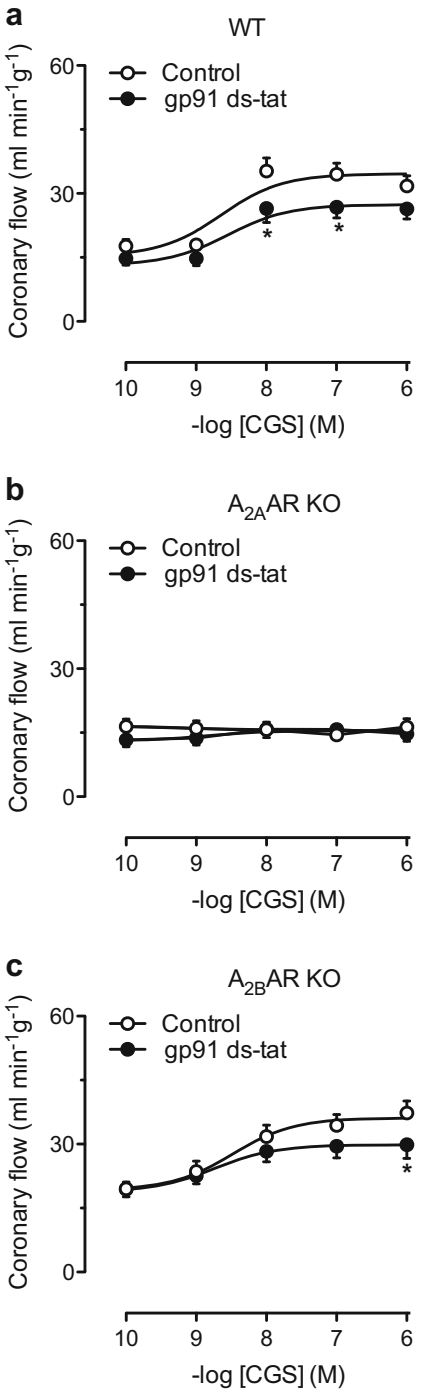

Fig. 3 Effects of Nox2 inhibition on adenosine A_{2A}AR agonist-induced increase in coronary flow. Shown are the effects of gp91 ds-tat (1 μM) on CGS-21680 (CGS, the A_{2A}AR selective agonist) concentration response curves (10^{-10} to 10^{-6} M) in isolated hearts of WT (**a**, n=6), A_{2A}AR KO (**b**, n=5), and A_{2B}AR KO mice (**c**, n=5). Values are mean±SEM. *Significant difference vs. corresponding control points using two-way ANOVA followed by post hoc analysis using Bonferroni's test (P<0.05)

P<0.05) as compared to corresponding baseline (Fig. 5b). H_2O_2 production was observed in both endothelial cells (ECs, arrow head) as well as smooth muscle cells (SMCs, arrow) (Fig. 1a). Of note, adenosine-increased H_2O_2

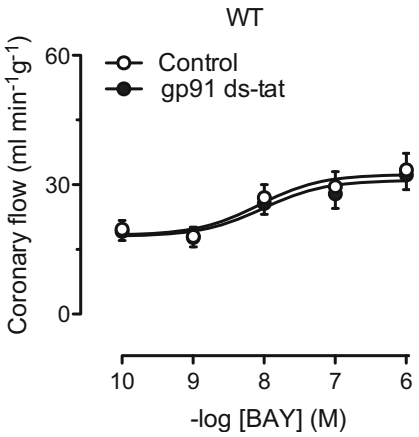

Fig. 4 Effects of Nox2 inhibition on adenosine A_{2B}AR agonist-induced increase in coronary flow. Shown are the effects of gp91 ds-tat (1 μM) on BAY 60-6583 (BAY, the A_{2B}AR selective agonist) concentration response curves (10^{-10} to 10^{-6} M) in isolated hearts of WT mice (n=4). Values are mean±SEM

production was inhibited by the Nox2 inhibitor gp91 ds-tat in both WT and A_{2B}AR KO mice to baseline levels (Fig. 5a, b). More importantly, adenosine failed to increase H_2O_2 production from A_{2A}AR KO mice either in the presence or absence of Nox2 inhibition (Fig. 5a, b). Hydrogen peroxide (200 μM) added at the end of experiments further increased fluorescence (Fig. 5a), indicating that the dye did not reach the saturation point [40]. Taken together, these data further suggest that Nox2 is an important source for H_2O_2 production, which depends on the activation of A_{2A}ARs by adenosine.

Discussion

This study examined the hypothesis that A_{2A} and/or A_{2B}AR-mediated coronary vasodilation involves Nox2-derived ROS. With the focus on the adenosine signaling pathway, we measured changes in coronary flow in isolated hearts from WT, A_{2A}AR, and A_{2B} AR KO mice in response to Nox2 inhibition (gp91 ds-tat) or ROS scavenging (EUK134) during exogenous adenosine infusion. In addition, the downstream effector (H_2O_2) of ROS in this process was also assessed in isolated coronary arteries from WT, A_{2A}AR, and A_{2B} AR KO mice. The main findings of the present study are as follows: (1) either Nox2 inhibition with gp91 ds-tat or ROS scavenging with EUK134 attenuated the adenosine-induced increase in coronary flow in WT and A_{2B}AR KO mice but not in A_{2A}AR KO mice; (2) Nox2 inhibition blunted the A_{2A}AR agonist-increased coronary flow in WT and A_{2B}AR KO mice, whereas the A_{2B}AR agonist responses were unaltered by Nox2 inhibition in WT mice; (3) furthermore, adenosine-mediated increase in H_2O_2 formation in intact isolated coronary arteries of WT and A_{2B}AR KO mice was inhibited by Nox2 inhibition, whereas the H_2O_2 formation was not affected

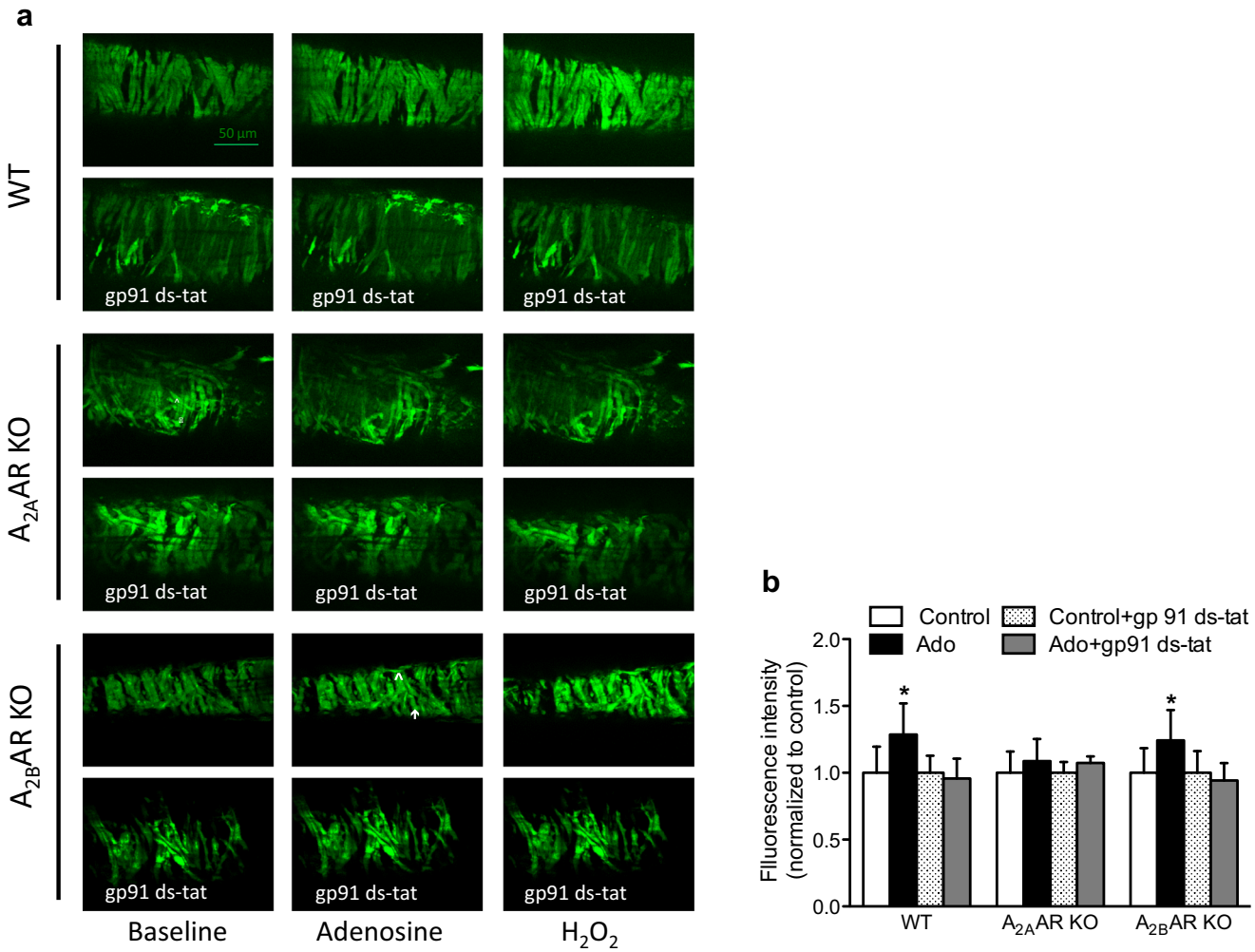

Fig. 5 Effects of Nox2 inhibition on adenosine-induced H_2O_2 production in isolated coronary arteries. **a** Representative confocal fluorescence images showing changes in DCFH-DA (dichlorofluorescin) fluorescence intensity before (baseline) and after 5 min of adenosine (Ado) (10 μM) or 10 min of H_2O_2 (200 μM) stimulation in the presence and absence of gp91 ds-tat (1 μM) from WT, A_{2A}AR KO, and A_{2B}AR KO mice. *Scale bar*: 50 μm. **b** Adenosine-induced increase in H_2O_2 in isolated coronary arteries of WT (n=8), A_{2A}AR KO (n=5), and A_{2B}AR KO mice (n=5). Values are mean± SEM. *P<0.05 vs. corresponding control (by paired t test)

by adenosine either with or without Nox2 inhibition in A_{2A}AR KO mice. The implications of these findings are discussed below.

Adenosine-induced increase in coronary flow is mediated in part through Nox2-derived ROS

Adenosine is known to regulate coronary vascular tone in various species. We [20, 44] and others [10, 11, 45] previously demonstrated that adenosine also plays a physiological role in regulating coronary resting flow in human [11], swine [10], dogs [45], guinea pigs [46], and mice [20, 44], while some other groups failed to observe an endogenous role of adenosine [3, 47, 48], which leaves this scientific topic controversial. This discrepancy may be due to different animal models, differences in species, and/or different agonists and antagonists applied in these studies [19]. However, the application of exogenous adenosine to increase coronary flow for the investigation of adenosine receptor-mediated signaling pathways has been well established [37, 44]. Thus, in agreement with previous studies from our laboratory [37, 44, 49] and others [14], adenosine produced a large increase in coronary flow in isolated mouse hearts, supporting the overall effect of vasodilation by adenosine in the coronary circulation [14, 15, 37, 50, 51].

Several recent studies showed the interaction between adenosine and ROS via the activation of Nox, which has been reported to be the major source of ROS in the vasculature and involved in vascular tone regulation [27, 28]. Among Nox isoforms, Nox2 has been extensively studied in various vascular beds. Nox2 inhibition or deletion attenuated the adenosine-mediated vasoconstriction in aortas [33] and renal arterioles [34]. In contrast, Nox2 inhibition or ROS scavenging blunted the adenosine-induced vasodilation in cerebral

arteries [35] and coronary circulation [37], both of which suggest that adenosine is capable of generating ROS in these vasculatures. Consistent with our previous findings in the coronary circulation but in contrast to those in other vascular beds [33, 34, 37], Nox2 inhibition or ROS scavenging significantly attenuated the adenosine-mediated increase in coronary flow, suggesting that adenosine-mediated coronary vasodilation is in part attributed to Nox2-derived ROS. This heterogeneity might be due to vasoconstrictor ROS vs. vasodilator ROS [29, 31] generated from different vascular beds (coronary vs. renal artery) [34, 37]. Furthermore, in the coronary vasculature, the varying effects of ROS may depend on which source ROS are generated from (NADPH vs. uncoupled NO synthase) [31, 52, 53] or the pathophysiological conditions [32, 52, 53], e.g., diabetes, and the right ventricular hypertrophy [54–56]. In accordance with our previous studies [37], the effect of ROS scavenging with EUK134 was comparable with the effect of Nox2 inhibition with gp91 ds-tat in WT isolated hearts (P=0.24), suggesting that the majority of ROS generated by adenosine is likely from Nox2, which leads to increases in coronary flow. Although EUK134 is capable of scavenging both vasoconstrictor and vasodilator ROS [57], the observation that EUK134 significantly attenuated the adenosine-mediated increase in coronary flow in one of our previous studies [37] as well as in the present study suggest that vasodilator ROS are likely generated by adenosine.

In the healthy coronary vasculature, several recent studies addressed a crucial role of NADPH oxidase in coronary vasodilation in response to various agonists. Indeed, downregulation of Nox activity by cytosolic p47phox knockdown or inhibition of Nox2 attenuated the coronary vasodilation to vascular endothelial growth factor (VEGF) [52] and bradykinin [32], respectively. In addition, Nox2 overexpression enhanced the coronary vasodilation in response to both VEGF and acetylcholine [53], further confirming that the vasodilator ROS is derived from Nox in this process. More importantly, Larsen and colleagues proposed that Nox2 is a functionally relevant source of H_2O_2 that mediates agonist-induced coronary vasodilation [32]. In accordance with this concept, we found in the present study as well as one of our previous studies [40] that adenosine-increased H_2O_2 formation in intact isolated coronary arteries was attenuated by not only Nox2 inhibition but also catalase [40]. The production of H_2O_2 induced by adenosine might stem from the intermediate ROS product namely, superoxide anion [27, 28, 31], as we previously reported that adenosine is also capable of producing superoxide [37]. Thus, it is likely that H_2O_2 acts as a downstream effector of superoxide contributing to adenosine-induced increase in coronary flow. Regarding the source of H_2O_2, we believe that H_2O_2 produced from EC and SMC of coronary arteries may play a major role in adenosine-mediated coronary flow. This idea is based on our previous [40] as well as present findings that the increased H_2O_2 was observed in both EC (arrow head) and SMC (arrow) (Fig. 5a). In addition, functional A_{2A}AR is reported to be expressed on both EC and SMC [9], which is in agreement with our previous data showing positive A_{2A}AR staining in both cell types of isolated mouse coronary arteries [40]. Therefore, it is reasonable to speculate that adenosine may act through A_{2A}AR in both cell types producing H_2O_2, and activation of A_{2A}AR-H_2O_2 pathway may result in different downstream vasodilator mechanisms, as activation of A_{2A}AR in different cell types of coronary artery stimulates either cAMP-PKA or cGMP-PKG signaling [9, 12, 58, 59]. On the other hand, the cross talk of these vasodilator mechanisms exists. For instance, there is a possibility that endothelium-derived H_2O_2 may elicit further H_2O_2 production in SMC through activation of Nox [60], independent of A_{2A}AR activation on SMC to activate PKG pathway, as H_2O_2 has been shown to be able to regulate both PKA and PKG pathways [61]. Taken together, these findings support Nox2 as a relevant source for H_2O_2 in the coronary vasculature [32] and suggest that the involvement of Nox2-derived ROS in adenosine-mediated coronary vasodilation is through H_2O_2 production.

Involvement of Nox2-derived ROS in adenosine-induced increase in coronary flow requires the presence of A_{2A}ARs

We [9, 16] and others [14, 15] have demonstrated that adenosine-mediated coronary vasodilation is predominantly attributed to A_{2A}AR as compared to vasodilator A_{2B}AR. In contrast, adenosine A_1 and A_3ARs counteract the adenosine-mediated coronary vasodilation [15, 17, 18]. Despite the findings that all four adenosine receptor subtypes are expressed and functional in the coronary vasculature [13], their roles in regulating coronary vascular tone through generation of ROS remain unclear. Given the fact that Nox has been suggested to be the major source of ROS in coronary vasculature [32, 52], it is not clear whether this leads to the generation of ROS by adenosine. We recently found that activation of A_3AR enhances Nox2-derived ROS contributing to vascular contraction in mouse aorta [33], suggesting a role of A_3AR in relation to ROS production. In contrast, in the coronary circulation, we have already excluded the involvement of A_1 and A_3ARs in increased flow that is coupled to the activation of Nox2 [37].

In the present study, we further investigated the role of adenosine A_{2A} and A_{2B}ARs in this process. We found that adenosine A_{2A} but not A_{2B}ARs are required for Nox2-mediated coronary vasodilation, as evidenced by the attenuation of adenosine-increased coronary flow by Nox2 inhibition (gp91 ds-tat) or ROS scavenging (EUK134) in both WT and A_{2B}AR KO mice but not in A_{2A}AR KO mice. Furthermore, the selective A_{2A}AR stimulation-induced increase in coronary flow was significantly attenuated by Nox2 inhibition in both

WT and A_{2B}AR KO mice, whereas Nox2 inhibition failed to blunt the increased coronary flow upon the selective A_{2B}AR stimulation in WT mice. Taken together, these findings suggest that A_{2A}AR could be a potential mediator of adenosine-induced ROS generation contributing to increases in coronary flow. Moreover, in the isolated coronary arteries, adenosine-increased H_2O_2 formation was inhibited by Nox2 blockade in both WT and A_{2B}AR KO mice but not in A_{2A}AR KO mice, further suggesting that the involvement of Nox2-derived ROS in adenosine-induced increase in coronary flow requires the presence of A_{2A}AR. In contrast to coronary circulation, both A_{2A} and A_{2B}ARs have been observed to generate ROS accounting for the vasodilation in cerebral circulation [35]. This discrepancy may be due to the different distribution, expression, and the downstream effectors of A_{2A} and A_{2B}ARs between these vascular beds [12, 13].

A_{2A}AR-mediated coronary vasodilation through Nox2-derived ROS observed in the present study may bolster the functional influences of A_{2A}AR by integrating the vascular actions of the generated ROS to the previously known A_{2A}AR-mediated releases of other vasodilators (such as NO [20] and prostacyclin [62]). Furthermore, pertinent to this effect, it may also be possible that adenosine-generated ROS is involved in mediating reactive or functional hyperemia, despite the role of adenosine in functional hyperemia remaining controversial [19]. Indeed, the involvement of adenosine in either reactive or functional hyperemia has been shown to be mediated by endogenously released vasodilators, including NO, prostacyclin, and endothelium-derived hyperpolarizing factor [38, 40, 63, 64]. The influence and interaction of these vasoactive factors in generation of ROS by A_{2A}AR in the coronary circulation remain unclear. Although we previously reported that NO and H_2O_2 function in a parallel manner contributing to the reactive hyperemia [40], future studies focusing on this aspect will provide greater insights into the interaction of these vasoactive factors.

Conclusions and implications

The present study shows that adenosine induces generation of ROS (likely H_2O_2) through the activation of Nox2 resulting in coronary vasodilation. This whole process requires the presence of A_{2A}AR. These findings further support NADPH oxidase as a functionally relevant source of ROS for regulating coronary vascular tone. As both adenosine and ROS are generated and released under pathophysiological conditions [9, 65] and generated ROS mainly exerts a detrimental influence on the coronary vasculature [54–56], future studies addressing the role of A_{2A}AR in ROS-mediated coronary vascular tone regulation in pathophysiological conditions will bring us greater insights into mechanisms underlying ischemic heart disease.

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References

1. Feigl EO (1983) Coronary physiology. Physiol Rev 63(1):1–205
2. Duncker DJ, Bache RJ, Merkus D (2012) Regulation of coronary resistance vessel tone in response to exercise. J Mol Cell Cardiol 52(4):802–813
3. Duncker DJ, Bache RJ (2008) Regulation of coronary blood flow during exercise. Physiol Rev 88(3):1009–1086
4. Durand MJ, Gutterman DD (2013) Diversity in mechanisms of endothelium-dependent vasodilation in health and disease. Microcirculation 20(3):239–247
5. Deussen A, Ohanyan V, Jannasch A, Yin L, Chilian W (2012) Mechanisms of metabolic coronary flow regulation. J Mol Cell Cardiol 52(4):794–801
6. Zhou Z, de Beer VJ, Bender SB, Jan Danser AH, Merkus D, Laughlin MH, Duncker DJ (2014) Phosphodiesterase-5 activity exerts a coronary vasoconstrictor influence in awake swine that is mediated in part via an increase in endothelin production. Am J Physiol Heart Circ Physiol 306(6):H918–927
7. Berne RM (1963) Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. Am J Physiol 204:317–322
8. Tune JD, Gorman MW (1985) Feigl EO (2004) Matching coronary blood flow to myocardial oxygen consumption. J Appl Physiol 97(1):404–415
9. Mustafa SJ, Morrison RR, Teng B, Pelleg A (2009) Adenosine receptors and the heart: role in regulation of coronary blood flow and cardiac electrophysiology. Handb Exp Pharmacol 193:161–188
10. Duncker DJ, Stubenitsky R, Verdouw PD (1998) Role of adenosine in the regulation of coronary blood flow in swine at rest and during treadmill exercise. Am J Physiol 275(5 Pt 2):H1663–1672
11. Edlund A, Conradsson T, Sollevi A (1995) A role for adenosine in coronary vasoregulation in man. Effects of theophylline and enprofylline. Clin Physiol 15(6):623–636
12. Burnstock G, Ralevic V (2014) Purinergic signaling and blood vessels in health and disease. Pharmacol Rev 66(1):102–192
13. Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN (2013) Cardiovascular adenosine receptors: expression, actions and interactions. Pharmacol Ther 140(1):92–111
14. Flood A, Headrick JP (2001) Functional characterization of coronary vascular adenosine receptors in the mouse. Br J Pharmacol 133(7):1063–1072
15. Sato A, Terata K, Miura H, Toyama K, Loberiza FR Jr, Hatoum OA, Saito T, Sakuma I, Gutterman DD (2005) Mechanism of vasodilation to adenosine in coronary arterioles from patients with heart disease. Am J Physiol Heart Circ Physiol 288(4):H1633–1640
16. Sanjani MS, Teng B, Krahn T, Tilley S, Ledent C, Mustafa SJ (2011) Contributions of A_{2A} and A_{2B} adenosine receptors in coronary flow responses in relation to the K_{ATP} channel using A_{2B} and $A_{2A/2B}$ double-knockout mice. Am J Physiol Heart Circ Physiol 301(6):H2322–2333
17. Talukder MA, Morrison RR, Jacobson MA, Jacobson KA, Ledent C, Mustafa SJ (2002) Targeted deletion of adenosine $A_{(3)}$ receptors augments adenosine-induced coronary flow in isolated mouse heart. Am J Physiol Heart Circ Physiol 282(6):H2183–2189
18. Tawfik HE, Teng B, Morrison RR, Schnermann J, Mustafa SJ (2006) Role of A_1 adenosine receptor in the regulation of coronary flow. Am J Physiol Heart Circ Physiol 291(1):H467–472

19. Zhou X, Teng B, Tilley S, Ledent C, Mustafa SJ (2014) Metabolic hyperemia requires ATP-sensitive K^+ channels and H_2O_2 but not adenosine in isolated mouse hearts. Am J Physiol Heart Circ Physiol 307(7):H1046–1055
20. Teng B, Ledent C, Mustafa SJ (2008) Up-regulation of A_{2B} adenosine receptor in A_{2A} adenosine receptor knockout mouse coronary artery. J Mol Cell Cardiol 44(5):905–914
21. Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W (2000) Structure and function of adenosine receptors and their genes. Naunyn Schmiedebergs Arch Pharmacol 362(4-5):364–374
22. Liu Y, Bubolz AH, Mendoza S, Zhang DX, Gutterman DD (2011) H_2O_2 is the transferrable factor mediating flow-induced dilation in human coronary arterioles. Circ Res 108(5):566–573
23. Rogers PA, Dick GM, Knudson JD, Focardi M, Bratz IN, Swafford AN Jr, Saitoh S, Tune JD, Chilian WM (2006) H_2O_2-induced redox-sensitive coronary vasodilation is mediated by 4-aminopyridine-sensitive K^+ channels. Am J Physiol Heart Circ Physiol 291(5):H2473–2482
24. Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Goto M, Ogasawara Y, Kajiya F (2003) Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. Circulation 107(7):1040–1045
25. Owen MK, Witzmann FA, McKenney ML, Lai X, Berwick ZC, Moberly SP, Alloosh M, Sturek M, Tune JD (2013) Perivascular adipose tissue potentiates contraction of coronary vascular smooth muscle: influence of obesity. Circulation 128(1):9–18
26. Yada T, Shimokawa H, Hiramatsu O, Shinozaki Y, Mori H, Goto M, Ogasawara Y, Kajiya F (2007) Important role of endogenous hydrogen peroxide in pacing-induced metabolic coronary vasodilation in dogs in vivo. J Am Coll Cardiol 50(13):1272–1278
27. Takac I, Schroder K, Brandes RP (2012) The Nox family of NADP H oxidases: friend or foe of the vascular system? Curr Hypertens Rep 14(1):70–78
28. Amanso AM, Griendling KK (2012) Differential roles of NADPH oxidases in vascular physiology and pathophysiology. Front Biosci (Schol Ed) 4:1044–1064
29. Frazziano G, Champion HC, Pagano PJ (2012) NADPH oxidase-derived ROS and the regulation of pulmonary vessel tone. Am J Physiol Heart Circ Physiol 302(11):H2166–2177
30. Schroder K (2010) Isoform specific functions of Nox protein-derived reactive oxygen species in the vasculature. Curr Opin Pharmacol 10(2):122–126
31. Taverne YJ, Bogers AJ, Duncker DJ, Merkus D (2013) Reactive oxygen species and the cardiovascular system. Oxid Med Cell Longev 2013:862423
32. Larsen BT, Bubolz AH, Mendoza SA, Pritchard KA Jr, Gutterman DD (2009) Bradykinin-induced dilation of human coronary arterioles requires NADPH oxidase-derived reactive oxygen species. Arterioscler Thromb Vasc Biol 29(5):739–745
33. El-Awady MS, Ansari HR, Fil D, Tilley SL, Mustafa SJ (2011) NADPH oxidase pathway is involved in aortic contraction induced by A_3 adenosine receptor in mice. J Pharmacol Exp Ther 338(2): 711–717
34. Carlstrom M, Lai EY, Ma Z, Patzak A, Brown RD, Persson AE (2009) Role of NOX2 in the regulation of afferent arteriole responsiveness. Am J Physiol Regul Integr Comp Physiol 296(1):R72–79
35. Gebremedhin D, Weinberger B, Lourim D, Harder DR (2010) Adenosine can mediate its actions through generation of reactive oxygen species. J Cereb Blood Flow Metab 30(10):1777–1790
36. Narayan P, Mentzer RM Jr, Lasley RD (2001) Adenosine A_1 receptor activation reduces reactive oxygen species and attenuates stunning in ventricular myocytes. J Mol Cell Cardiol 33(1):121–129
37. El-Awady MS, Rajamani U, Teng B, Tilley SL, Mustafa SJ (2013) Evidence for the involvement of NADPH oxidase in adenosine receptors-mediated control of coronary flow using A and A knockout mice. Physiol Rep 1(3), e00070
38. Sharifi-Sanjani M, Zhou X, Asano S, Tilley S, Ledent C, Teng B, Dick GM, Mustafa SJ (2013) Interactions between $A_{(2A)}$ adenosine receptors, hydrogen peroxide, and K_{ATP} channels in coronary reactive hyperemia. Am J Physiol Heart Circ Physiol 304(10): H1294–1301
39. Teng B, Fil D, Tilley SL, Ledent C, Krahn T, Mustafa SJ (2013) Functional and RNA expression profile of adenosine receptor subtypes in mouse mesenteric arteries. J Cardiovasc Pharmacol 61(1): 70–76
40. Zhou X, Teng B, Tilley S, Mustafa SJ (2013) A_1 adenosine receptor negatively modulates coronary reactive hyperemia via counteracting A_{2A}-mediated H_2O_2 production and K_{ATP} opening in isolated mouse hearts. Am J Physiol Heart Circ Physiol 305(11):H1668–1679
41. Ponnoth DS, Sanjani MS, Ledent C, Roush K, Krahn T, Mustafa SJ (2009) Absence of adenosine-mediated aortic relaxation in $A_{(2A)}$ adenosine receptor knockout mice. Am J Physiol Heart Circ Physiol 297(5):H1655–1660
42. Rey FE, Cifuentes ME, Kiarash A, Quinn MT, Pagano PJ (2001) Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular $O_{(2)}$(-) and systolic blood pressure in mice. Circ Res 89(5):408–414
43. van Nierop BJ, Coolen BF, Bax NA, Dijk WJ, van Deel ED, Duncker DJ, Nicolay K, Strijkers GJ (2014) Myocardial perfusion MRI shows impaired perfusion of the mouse hypertrophic left ventricle. Int J Cardiovasc Imaging 30(3):619–628
44. Talukder MA, Morrison RR, Ledent C, Mustafa SJ (2003) Endogenous adenosine increases coronary flow by activation of both A_{2A} and A_{2B} receptors in mice. J Cardiovasc Pharmacol 41(4):562–570
45. Tune JD, Richmond KN, Gorman MW, Olsson RA, Feigl EO (2000) Adenosine is not responsible for local metabolic control of coronary blood flow in dogs during exercise. Am J Physiol Heart Circ Physiol 278(1):H74–84
46. Headrick JP, Ely SW, Matherne GP, Berne RM (1993) Myocardial adenosine, flow, and metabolism during adenosine antagonism and adrenergic stimulation. Am J Physiol 264(1 Pt 2):H61–70
47. Dole WP, Yamada N, Bishop VS, Olsson RA (1985) Role of adenosine in coronary blood flow regulation after reductions in perfusion pressure. Circ Res 56(4):517–524
48. Saito D, Steinhart CR, Nixon DG, Olsson RA (1981) Intracoronary adenosine deaminase reduces canine myocardial reactive hyperemia. Circ Res 49(6):1262–1267
49. Morrison RR, Talukder MA, Ledent C, Mustafa SJ (2002) Cardiac effects of adenosine in $A_{(2A)}$ receptor knockout hearts: uncovering $A_{(2B)}$ receptors. Am J Physiol Heart Circ Physiol 282(2):H437–444
50. Berwick ZC, Payne GA, Lynch B, Dick GM, Sturek M, Tune JD (2010) Contribution of adenosine $A_{(2A)}$ and $A_{(2B)}$ receptors to ischemic coronary dilation: role of $K_{(V)}$ and $K_{(ATP)}$ channels. Microcirculation 17(8):600–607
51. Zhou Z, Merkus D, Cheng C, Duckers HJ, Jan Danser AH, Duncker DJ (2013) Uridine adenosine tetraphosphate is a novel vasodilator in the coronary microcirculation which acts through purinergic P1 but not P2 receptors. Pharmacol Res 67(1):10–17
52. Feng J, Damrauer SM, Lee M, Sellke FW, Ferran C, Abid MR (2010) Endothelium-dependent coronary vasodilatation requires NADPH oxidase-derived reactive oxygen species. Arterioscler Thromb Vasc Biol 30(9):1703–1710
53. Shafique E, Choy WC, Liu Y, Feng J, Cordeiro B, Lyra A, Arafah M, Yassin-Kassab A, Zanetti AV, Clements RT, Bianchi C, Benjamin LE, Sellke FW, Abid MR (2013) Oxidative stress improves coronary endothelial function through activation of the pro-survival kinase AMPK. Aging (Albany NY) 5(7):515–530

54. Lu T, Chai Q, Yu L, d'Uscio LV, Katusic ZS, He T, Lee HC (2012) Reactive oxygen species signaling facilitates FOXO-3a/FBXO-dependent vascular BK channel beta1 subunit degradation in diabetic mice. Diabetes 61(7):1860–1868
55. Lu X, Dang CQ, Guo X, Molloi S, Wassall CD, Kemple MD, Kassab GS (2011) Elevated oxidative stress and endothelial dysfunction in right coronary artery of right ventricular hypertrophy. J Appl Physiol (1985) 110(6):1674–1681
56. Kassan M, Choi SK, Galan M, Lee YH, Trebak M, Matrougui K (2014) Enhanced p22phox expression impairs vascular function through p38 and ERK1/2 MAP kinase-dependent mechanisms in type 2 diabetic mice. Am J Physiol Heart Circ Physiol 306(7): H972–980
57. Lawler JM, Kim JH, Kwak HB, Barnes WS (2010) Redox modulation of diaphragm contractility: Interaction between DHPR and RyR channels. Free Radic Biol Med 49(12):1969–1977
58. Rekik M, Mustafa JS (2003) Modulation of A_{2A} adenosine receptors and associated Galphas proteins by ZM 241385 treatment of porcine coronary artery. J Cardiovasc Pharmacol 42(6):736–744
59. Olanrewaju HA, Mustafa SJ (2000) Adenosine $A_{(2A)}$ and $A_{(2B)}$ receptors mediated nitric oxide production in coronary artery endothelial cells. Gen Pharmacol 35(3):171–177
60. Cai H (2005) Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. Cardiovasc Res 68(1):26–36
61. Burgoyne JR, Eaton P (2013) Detecting disulfide-bound complexes and the oxidative regulation of cyclic nucleotide-dependent protein kinases by H_2O_2. Methods Enzymol 528: 111–128
62. Maddock HL, Broadley KJ, Bril A, Khandoudi N (2002) Effects of adenosine receptor agonists on guinea-pig isolated working hearts and the role of endothelium and NO. J Pharm Pharmacol 54(6): 859–867
63. Otomo J, Nozaki N, Tomoike H (1997) Roles of nitric oxide and adenosine in the regulation of coronary conductance in the basal state and during reactive hyperemia. Jpn Circ J 61(5): 441–449
64. Duffy SJ, Castle SF, Harper RW, Meredith IT (1999) Contribution of vasodilator prostanoids and nitric oxide to resting flow, metabolic vasodilation, and flow-mediated dilation in human coronary circulation. Circulation 100(19):1951–1957
65. Cai H, Griendling KK, Harrison DG (2003) The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. Trends Pharmacol Sci 24(9):471–478