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Up-regulation of A_{2B} Adenosine Receptor in A_{2A} Adenosine Receptor Knockout Mouse Coronary Artery

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Abstract

In this study, we looked into possible compensatory changes of other adenosine receptors (AR) in A_{2A} genetic knockout mice (A2AKO) as well as the functional role of nitric oxide (NO) in A_{2A} AR-mediated vasodilation. Gene expression of ARs from coronary arteries of A_{2A} AR wild type mice (A2AWT) and A2AKO were studied using real time-PCR. Functional studies were carried out in isolated heart and isolated coronary artery preparations. A_{2B} AR was found to be 4.5 fold higher in A2AKO than in A2AWT, while A_{2A} AR expression was absent in A2AKO. There was no difference in A₁ and A₃ ARs between WT and KO animals. The concentration-relaxation curve for adenosine-5'-N-ethylcarboxamide (NECA, non-selective AR agonist) in isolated coronary arterial rings in A2AKO was shifted to the left when compared to A2AWT. The concentration-response curve for A_{2B} selective agonist (Bay 60-6583) was also shifted to the left in A2AKO hearts. L-NAME, a non-specific NO synthase inhibitor, did not affect baseline coronary flow (CF) until the concentration reached 10 μ M in A_{2A}WT (76.32 $\pm$ 11.35% from baseline, n=5). In A_{2A}KO, the CF decreased significantly by L-NAME only at a higher concentration (100 μ M, 93.32 $\pm$ 5.8% from baseline, n=5). L-NMA (1 μ M, n=4), another non-specific NO synthase inhibitor, also demonstrated similar results in decreasing CF (59.66 $\pm$ 3.23% from baseline in A2AWT, while 81.76 $\pm$ 8.91% in A2AKO). It was further demonstrated that the increase in CF by 100 μ M NECA was significantly blunted with 10 μ M L-NAME (377.08 $\pm$ 25.23% to 305.41 $\pm$ 30.73%, n=9) in A_{2A}WT but not in A_{2A}KO (153.66 $\pm$ 22.7% to 143.88 $\pm$ 36.65%, n=5). Similar results were also found using 50 nM of CGS-21680 instead of NECA in A_{2A}WT (346 $\pm$ 22.85 to 277 $\pm$ 31.39, n=6). No change in CF to CGS-21680 was noted in A_{2A}KO. Our data demonstrate, for the first time, that coronary A_{2B} AR was up-regulated in mice deficient in A_{2A} AR. We also provide direct evidence supporting a role for NO in A_{2A}AR-mediated coronary vasodilation. The data further support the role for A_{2A}AR in the regulation of basal coronary tone through the release of NO.

Keywords

coronary artery; endothelium; Nitric oxide; A_{2A} adenosine receptor; A_{2B} adenosine receptor

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Introduction

Adenosine is an autocrine that plays a critical role in regulating coronary circulation. An imbalance between oxygen supply and demand (ischemia) leads to alterations in cellular release of adenosine. Once adenosine is produced by the action of ecto-5'-nucleotidase, it is released from the parenchymal tissue (including endothelium) and interacts with specific extracellular receptors located on the smooth muscle and endothelial cells of the coronary artery to produce relaxation. Currently, there are four known adenosine receptor (AR) subtypes namely, A₁, A_{2A}, A_{2B}, and A₃. Previous studies from our group demonstrated that both A_{2A} AR and A_{2B} AR mediated endogenous and exogenous adenosine-induced dilation in mouse coronary circulation [1,2]. Cell culture studies also demonstrated the involvement of A_{2A} AR and A_{2B} AR mediated NO release in porcine and human coronary endothelial cells [3,4]. However, there are very few functional studies demonstrating whether NO release is responsible for A_{2A} AR mediated coronary vasodilation. Previously, a study from our group found that NG-methyl-L-arginine (L-NMA, 30 μ M), a NO synthase inhibitor, attenuated the relaxations of endothelium-intact but not -denuded rings to adenosine-5'-N-ethylcarboxamide (NECA) and CGS-21680 in porcine coronary arterial rings [5]. Beyond these reports in isolated tissues, there is no other evidence of how significant a role NO plays in A_{2A} AR mediated coronary vasodilation.

There is speculation that endogenously released adenosine and prostanoids activate NO-and/or K_{ATP} channel-dependent dilation to modulate basal coronary tone [6–9]. Inhibition of NO synthase has been found to limit basal coronary flow (CF) in different species including mice [6,9]. Furthermore, A_{2A} AR was also shown to contribute significantly to basal tone in mouse coronary circulation [6,9]. However, it is unclear whether NO plays a significant role in A_{2A} AR-mediated modulation of coronary vascular basal tone.

The use of genomic knockout mice, such as A_{2A} AR knockout mice (A2AKO) in our study, provides a valuable tool in exploring physiological role of ARs. Similar to pharmacological approach (i.e. antagonist), increases in heart rate and blood pressure were found in A2AKO, which supported a significant role for this receptor in cardiovascular function [10]. However, since the gene deletion was done during embryogenesis, some adaptive "compensatory" mechanisms may develop and hinder interpretation as suggested by others [11]. For instance, serotonin 5-HT_{1B} antagonists have no effect on aggressive behavior while 5-HT_{1B} knockout mice are hyper-aggressive [12]. These authors proposed that other than producing a change of phenotype like in a pharmacological manipulation, a genomic knockout may also invoke compensatory changes in mice that may show a different phenotype, which makes the interpretation difficult. So far, such discrepancy in A2AKO has not been reported in cardiovascular study. One of the reason may be the compensatory mechanism is "making up" for the loss of A_{2A} AR. The prime candidate is the possibly the enhancement of A_{2B} AR.

The cardiovascular effects of A_{2B} AR were found to be similar to A_{2A} AR but it is a low affinity AR [13–15]. It has been speculated that under pathological conditions, such as ischemia, A_{2B} AR may be up-regulated to compensate for the down-regulation of A_{2A} AR-mediated responses. Indeed, an up-regulation of A_{2B} AR gene expression has been found in ischemic mouse hearts [16,17]. We suspect that this kind of adaptive mechanism may also happen in the knockout animals.

Using non-specific adenosine agonist (NECA), and an A_{2A} AR specific agonist (CGS-21680), our previous functional study in A2AKO suggested the role of A_{2B} AR in CF regulation [1]. Recently, a A_{2B} AR specific agonist, BAY 60-6583, showed 1000x more specificity to A_{2B} AR than to A₁ and A_{2A} AR [18,19], hence provided a valuable tool for our further studies on A_{2B} AR function.

Furthermore, there are no reports of any adenosine gene expression in mouse coronary artery and therefore, one of the objectives of our studies were to explore the changes in AR gene expression and characterize their functional role in relation to endogenous NO (including eNOS expression) in A2AKO mice.

In summary, we intended to address whether other ARs in coronary arteries were modulated in A2AKO, assess the role of A_{2A} AR in background NO release, and further define the role of NO in exogenous adenosine-induced increase in coronary flow.

Materials and Methods

Animals

All animals were cared for in accordance with protocol approved by the Animal Care and Use Committee of the Health Science Center at West Virginia University. A2AKO and WT mice were obtained from the Institute of Experimental Medicine, Universite Libre de Bruxelles [10]. Breeding and selection of the mice were described previously [1]. All animals used in a given experiment originated from the same breeding pairs and were matched for age and weight. Standard laboratory food and water were available ad libitum. Temperature was held constant at $23 \pm 2^\circ\text{C}$ and humidity was $60 \pm 10\%$. An inverted light-dark cycle of 12:12 h was used (lights off at 1700 h). Experiments were conducted in accordance with national legislation and with the Declaration of Helsinki regarding the use of experimental animals.

Langendorff Experiments

Isolated heart experiments were performed in accordance with the methods previously described [17,20,21]. Animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), a thoracotomy was performed, and the hearts were excised into ice-cold heparinized (5 U/mL) modified Krebs-Henseleit buffer containing (in mM): NaCl 120, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, glucose 15, and EDTA 0.05. After removal of lung and surrounding tissue, the aorta were rapidly cannulated with a 20-gauge, blunt-ended needle, and coronary perfusion was initiated at constant pressure of 80 mmHg with the Krebs-Henseleit buffer. The perfusate was equilibrated with 95% O₂-5% CO₂ at 37°C, giving a pH of 7.4 and pO₂ of ~550 mmHg. The left atrium was removed, and the left ventricle was vented with a small polyethylene apical drain. A fluid-filled balloon constructed of plastic film was inserted into the left ventricle across the mitral valve and connected to a pressure transducer permitting continuous measurement of left ventricular pressure. Balloon volume was modified through a stopcock attached to the ventricular pressure transducer using a 500-μL glass syringe (Hamilton Co.; Reno, NV) to maintain a left ventricular diastolic pressure of 2–5 mmHg. Hearts were immersed in a water-jacketed perfusate bath maintained at 37°C, and the bath was rinsed once at the onset of perfusion to clear blood from the bathing environment. CF was continuously monitored via a Doppler flow probe (Transonic Systems; Ithaca, NY) located in the aortic perfusion line. CF, aortic pressure and left ventricular pressure were recorded on a PowerLab multi-channel data acquisition system (ADInstruments; Castle Hill, Australia) connected to a computer. Data were expressed as percentage of change from baseline for each parameter before the application of drugs.

Coronary Wire Myograph Experiments

The hearts from 8 A2AWT and 6 A2AKO mice were excised as described in Langendorff Experiments and placed in oxygenated (5% CO₂ and 95% O₂) modified Krebs-Henseleit buffer (in mM: NaCl 120, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 1.8, MgSO₄ 1.2, glucose 15, and EDTA 0.05) at 37 °C, pH 7.4. Left anterior descending coronary arteries (LAD) were isolated and clean of surrounding tissue. The arterial rings (3–5 mm long, 50–100 μm inside diameter) were mounted on an isometric myograph (Danish Myo Technology A/S, Aarhus,

Denmark) based on the work of Mulvany [22]. Each vascular ring was stretched to a resting tension (100 mg) that consisted mainly of passive tension and was allowed to equilibrate for at least 30 min. The optimal resting tension was determined by measuring the tension that produced the greatest contractile response after the addition of 50 mM KCl (determined separately). Viability of the vascular ring was verified by recording contraction after the addition of 50 mM KCl to the tissue bath. The integrity of endothelium was confirmed by the addition of the endothelium-dependent vasodilator acetylcholine (ACh, 10^{-6} M) during the plateau phase of 5 μ M of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contraction, a submaximal contraction that is predetermined in separate experiments (data not shown). Vascular rings that did not contract after the addition of KCl or that relaxed after the addition of ACh were eliminated from further study.

Real-Time RT PCR for Mouse Coronary Arteries

Total RNA was isolated from the mouse coronary arteries (left anterior descending and septal branches) using RNeasy total RNA isolation kit from Qiagen. This was followed by conversion of 0.5 μ g of total RNA into cDNA using High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer in a total volume of 100 μ L. Because of the relatively low expression of ARs, PCR PreAmplification kit from ABI was used. Real Time-PCR was performed using ABI PRISM 7300 Detection System (Applied Biosystems) using Taqman Universal Mastermix (Applied Biosystems, Branchburg, New Jersey) according to the instructions of the manufacturer. The reaction volume (25 μ L) included 12.5 μ L of 2X Taqman Universal Mastermix, 1 μ L of cDNA, and 1.25 μ L of 20X FAM-labeled Taqman gene expression assay master mix solution. For the real-time PCR for ARs and eNOS genes, the Taqman inventoried gene expression product was purchased from Applied Biosystems (Foster City, CA). 18S rRNA (Ribosomal RNA) was used as an endogenous control. The fold difference in expression of target cDNA was determined using the comparative CT method. The Δ CT value was determined in each experiment by subtracting the average 18S CT value from the corresponding average CT for A₁, A_{2A}, A_{2B}, A₃ AR and eNOS in coronary arteries. The standard deviation was calculated using the formula

$s = \sqrt{(s_1^2 + s_2^2)}$. To set the relative unit to 1, $\Delta\Delta$ CT was calculated by subtraction of the Δ CT calibrator value (A₁ AR Δ CT values in A_{2A}WT). The fold difference in gene expression of the target was calculated as the average value from $2^{-\Delta\Delta CT+s}$ and $2^{-\Delta\Delta CT-s}$ [17,23].

Experimental Protocol

Role of A_{2A}AR in basal release of NO—After 30 minutes of equilibration period, CF, heart rate, and developed pressure (systolic pressure minus diastolic pressure) were examined in WT and A_{2A}KO hearts, and baseline data for these parameters were sampled at the end of equilibration. Dose-response relationships were constructed for N-nitro-L-arginine methylester (L-NAME, 10^{-7} – 10^{-4} M) and N^G-methyl-L-arginine (L-NMA, 10^{-11} – 10^{-5} M), both non-specific NO synthase inhibitors, by infusing the inhibitors into the coronary perfusate through an injection port directly proximal to the aortic cannula. Infusion rate was controlled to a maximum of 1% of CF with a microinjection infusion pump (Harvard Apparatus; Holliston, MA). After baseline data were acquired, each heart was exposed to progressively increasing concentrations of L-NAME or L-NMA to develop a dose-response relationship. Each concentration of L-NAME or L-NMA was infused for 5 min, and data were sampled at the end of the 5-min infusion period. After the infusion of each concentration of L-NAME, a minimum of 5 min of perfusion was allowed for drug washout. Readings for each concentration were normalized to the original baseline.

NO involvement in adenosine-induced coronary vasodilation—After baseline data for each heart were sampled at the end of the 30-min equilibration period, 100 nM NECA, (a

non-specific adenosine agonist) or 50 nM CGS-21680 (a A_{2A} AR specific agonist) was infused at 1% of CF for 5 min, and the plateau effect on CF, heart rate, and left ventricular developed pressure was recorded. The concentrations of these 2 agonists were chosen because they are close to the EC_{50} of the drug effect in CF, according to our previous study in the same strain of mice [1]. This initial infusion of NECA (or CGS-21680) was allowed to wash out for 10 min and then 10 μ M L-NAME was infused at 1% of CF for 15 min. Ten minutes into the L-NAME infusion, data were sampled and normalized as a new "baseline," and 100 nM NECA (or CGS-21680) was added to the coronary perfusate at 1% of CF for the remaining 5 min of the L-NAME infusion. Data were sampled at the end of this two-drug infusion for comparison with data resulting from infusion of NECA alone. After L-NAME and NECA (or CGS-21680) were allowed to wash out for 10 min, a repeat infusion of 100 nM NECA (or 50 nM CGS-21680) was performed for comparison with the first NECA (or CGS-21680) infusion.

Responses to A_{2B} agonist in A2AWT and A2AKO—The A_{2B} AR-selective adenosine agonist BAY 60-6583 was used. EC_{50} values for BAY 60-6583 are 3–10 nM for human A_{2B} and $>10 \mu$ M for A_1 and A_{2A} ARs [18,19]. Protocol for constructing the concentration-response relationship to BAY 60-6583 was the same as for L-NAME concentration-response curves, except the concentration range is from 10^{-11} to 10^{-5} M.

Coronary Wire Myograph Experiments—After equilibration and verification of arterial ring integrity, the concentration-response curves for NECA were obtained by cumulative addition of the agonists in $PGF_{2\alpha}$ (5 μ M)-contracted tissues. The agonists were added to yield the next higher concentration only when the response to the earlier dose reached a steady state (usually about 5 min). At the end of the highest concentration, all baths were washed 4 times. After baseline was established, 10 μ M of papaverine was added to ensure that the maximal relaxation was achieved. Relaxation responses were expressed as percent decrease in the contraction with respect to 5 μ M $PGF_{2\alpha}$ in response to each concentration of agonist used.

Materials

NECA, CGS-21680, L-NAME, and all the chemicals for the buffers were purchased from Sigma-Aldrich. BAY 60-6583 was a generous gift from Dr. Thomas Krahn of Bayer Healthcare, Germany.

Statistical Analysis

For Langendorff experiments, differences in responses to L-NAME at the same concentration between A2AWT and A2AKO hearts were analyzed using student t-test. CF, developed pressure, and heart rate responses to antagonist infusion for A2AWT and A2AKO hearts were compared with one-way ANOVA with Bonferroni's correction for multiple comparisons. For tissue bath experiments, the data are expressed as percentages of relaxation from the $PGF_{2\alpha}$ -induced maximal contraction and are presented as means $\pm$ SE. Student t-test was used to compare data between A2AWT and A2AKO at the same concentration.

Results

Role of A_{2A} AR in basal release of NO

L-NAME did not significantly reduce CF in A2AWT until it reached a concentration of 10^{-5} M ($76.32 \pm 11.35\%$ from baseline, Figure 1A). Higher dose (10^{-4} M) of L-NAME severely depressed cardiac function to a point that the heart ceased to function. In A2AKO, however, there is no significant decrease of CF until 10^{-4} M ($93.32 \pm 5.8\%$ from baseline, Figure 1A). On the other hand, there is no significant change in heart rate in both A2AWT and A2AKO at all doses (Figure 1B). Contractility decreased only in A2AWT at 10^{-5} M ($84.27 \pm 8.37\%$, from baseline, Figure 1C).

L-NMA also showed similar responses but at much lower concentration (Figure 1). L-NMA caused a decrease in CF starting at 10^{-10} M and above in A2AWT, while 10^{-8} M and above in A2AKO. The maximal decrease in flow is also greater in A2AWT than A2AKO ($59.66 \pm 3.23\%$ from baseline in A2AWT, while $81.76 \pm 8.91\%$ in A2AKO at 10^{-6} M, Figure 1D). L-NMA significantly decreased heart rate in both A2AWT and A2AKO, but only at concentration higher than 10^{-8} M (maximal effect, $85.39 \pm 11.08\%$ from baseline in A2AWT vs. $92.47 \pm 1.09\%$ in A2AKO, Figure 1E). Similarly, the effect of L-NMA on LVDP was seen only at concentrations above 10^{-8} M (maximal effect, $82.73 \pm 6.49\%$ from baseline in A2AWT vs. $82.88 \pm 48.4\%$ in A2AKO, Figure 1F). There is no significant difference between A2AWT and A2AKO with L-NMA on heart rate and LVDP.

NO involvement in adenosine-induced coronary vasodilation

The control responses to 100 nM of NECA and 50 nM of CGS-21680 in A2AWT hearts and KO hearts were similar to our previous studies [1]. One hundred nM of NECA induced $377 \pm 25.23\%$ increase of CF in A2AWT, while only $153.66 \pm 23.7\%$ in A2AKO (Figure 2). Fifty nM of CGS-21680 induced $346.67 \pm 22.85\%$ increase of CF in A2AWT, but no response in A2AKO. NECA also significantly slowed the heart rate in both A2AWT and KO ($81.7 \pm 13.94\%$ and $63.61 \pm 9.12\%$), though statistically insignificant. CGS-21680 had no effect on heart rate in both groups. NECA increased LVDP in A2AWT but not in A2AKO ($128.32 \pm 18.1\%$ vs $107.39 \pm 10.07\%$). Similar LVDP responses were found using CGS-21680 in A2AWT ($132.42 \pm 13.39\%$) and no effect in A2AKO.

The new baseline of CF after 10 Min of $10 \mu\text{M}$ infusion of L-NAME was decrease to $77.32 \pm 7.15\%$ from baseline (data not shown), while heart rate and LVDP did not change significantly ($95.71 \pm 6.2\%$ and $94.41 \pm 8.6\%$ from baseline, respectively). Additional infusion of 100 nM of NECA in the presence of $10 \mu\text{M}$ L-NAME increased CF to $305.41 \pm 30.73\%$ of baseline in A2AWT while $143.88 \pm 36.65\%$ in A2AKO (Figure 2A). After 10 min wash, the infusion of NECA returned to control level ($405.19 \pm 76.56\%$) in A2AWT. In A2AKO, there is no significant change between NECA infusion with or without L-NAME. Heart rate and LVDP did not change significantly over the course of NECA and L-NAME infusion in both A2AWT and A2AKO (Figure 2B and 2C).

CGS-21680 had no effect in A2AKO as was reported in our previous study [1]. In A2AWT, the increase in CF induced by CGS-21680 was significantly blunted by $10 \mu\text{M}$ L-NAME (from $346.67 \pm 22.85\%$ to $277.54 \pm 31.39\%$, Figure 3A). There was no significant change in heart rate by CGS-21680 (Figure 3B) similar to our previous study [1]. LVDP was higher in A2AWT ($130 \pm 13.37\%$) and was not affected by the addition of L-NAME (Figure 3C). However, CGS-21680-induced increase in contractility was enhanced at the final application of CGS-21680 ($170 \pm 17.22\%$).

Real Time RT PCR

A₁ AR and A_{2A} AR have the highest expression of ARs in major coronary arteries from A2AWT (no significant difference, Figure 4A). Lack of A_{2A} AR in A2AKO was also confirmed. A₃ AR was not significantly expressed in coronary arteries in both A2AWT and A2AKO. There was no significant difference between A2AWT and A2AKO in eNOS (Figure 4B), A₁ and A₃ AR. However, there was 4.5 times more A_{2B} AR expressed in A2AKO than in A2AWT (Figure 4A).

Coronary Wire Myograph

NECA induced biphasic responses in A2AWT and A2AKO coronary arterial rings (Figure 5). From 10^{-11} M to 10^{-8} M, NECA induced a concentration dependent constriction. When compared to baseline, significant differences were found in concentration at 10^{-8} and 10^{-7} M

in A2AWT, while only at 10^{-9} M in A2AKO (Figure 5). From 10^{-8} M to 10^{-5} M, NECA induced a concentration dependent relaxation with significant differences at 10^{-5} M in A2AWT, while 10^{-6} and 10^{-5} M in A2AKO. When compared to A2AWT, the differences in relaxations were found at 10^{-7} M and higher concentrations, with the curve being shifted to the left in A2AKO (Figure 5). The EC₅₀ value for NECA-induced relaxation in A2AKO ($4.18 \pm 2.04 \mu\text{M}$) was significantly lower than that for A2AWT ($8.17 \pm 1.25 \mu\text{M}$, $p < 0.05$)

Selective A_{2B} agonist (BAY 60-6583) concentration-response curve

Selective A_{2B} agonist, BAY 60-6583 induced concentration dependent increase in CF both in A2AWT and A2AKO. However, there was a significant leftward shift in A2AKO (EC₅₀ = 8.7 ± 1.44 nM in A2AKO vs 24.1 ± 14.4 nM in A2AWT, $p < 0.05$). Statistically significant differences were found at 5×10^{-9} M and 10^{-8} M (Figure 6A). Bay 60-6583 had no effect on heart rate (Figure 6B), while LVDP was increased in both A2AWT and A2AKO. In regard to LVDP changes in response to Bay 60-6583, there was no significant difference between A2AKO and A2AWT (Figure 6C).

Discussion

This study demonstrates for the first time that a compensatory A_{2B} AR up-regulation in A_{2A} AR knockout mice, both in function and gene expression. In addition, A_{2A} AR contributes significantly to basal nitric oxide release in coronary circulation. Our data also provide evidence that nitric oxide release also plays a significant role in exogenous adenosine-induced vasodilation in mouse coronary circulation.

The most important finding in this study was the up-regulation of A_{2B} AR in A2AKO mouse coronary artery. Alteration of gene expression and responsiveness of other receptors and their effectors have been reported in numerous genetic knock out mice. For instance, mice deficient in both β_1 - and β_2 -adrenergic receptors show an exaggerated response to a β_3 agonist [24] as well as enhanced dopamine function was found in 5-HT_{1B} receptor knockout mice [25]. Also, in the absence of nNOS, eNOS was able to somehow change its expression to compensate for the loss of nNOS [26]. Generally, in coronary circulation, A_{2B} AR was found to play a minor role, compared to A_{2A} AR, in regulating coronary blood flow and/or myocardial contraction [1,2,27]. In this study, we found that NECA (a non-specific adenosine agonist known to activate low affinity A_{2B} AR)-induced relaxation at high concentration in wire myograph experiments and the concentration-response relationship was shifted to the left in A2AKO (Figure 5). The concentration-response curve for BAY 60-6583-induced CF increase was also shifted to the left (Figure 6A). In separate experiments, BAY 60-6583 was found to have no effect in A_{2B} AR knockout mouse using isolated heart preparation (personal communication from Maryam Sharifi Sanjani), supporting A_{2B} AR specificity for BAY 60-6583. In addition, real time RT PCR of the isolated coronary artery showed a 4.5 fold increase in the expression of A_{2B} AR in A2AKO compared to A2AWT (Figure 4A). Taken together, we demonstrate, for the first time, that A_{2B} AR is compensating for the deletion of A_{2A} AR, both in gene expression and functional response in the coronary artery. Furthermore, our finding of A_{2B} AR up-regulation in A2AKO mice also supports the notion that genetic knock out mice not only serves to understand the specific phenotype of the knockout gene, but also serves as a tool to understand the regulation of other related genes [24].

Genetic knockout mice have become valuable tools in studying physiological, pathological, and pharmacological mechanisms. Typically, the effect of the gene knockout is interpreted like antagonist application. However, questions have been raised about how one gene deletion will affect other genes, especially when the results from knockout mice are in conflict with the results from the traditional antagonistic experiments, as with 5-HT_{1B} knockout mice

mentioned earlier in introduction [12]. We found a cooperative compensatory mechanism i.e. the up-regulation of A_{2B} AR to compensate for the deletion of A_{2A} AR.

The conclusion of this finding is two fold. First, compensatory mechanism does occur in A2AKO, but may only happen in selected organs. For instance, we do not found any changes of other AR expression in the brain of the same animal (data not shown), but the A_{2B} AR was found to be up-regulated in coronary arteries, suggesting that the compensation is not universal. Therefore, we have to take this up-regulation into account when interpreting the CF data in A2AKO. For example, the A_{2B} AR responses found in our previous study of A2AKO may include the over expression of A_{2B} AR [1]. Hence the role of A_{2B} AR in physiological sense may not be as significant as indicated. Second, what is intriguing is that this finding is very similar to the adaptive mechanism found in pathophysiological condition, such as cardiac ischemia. Previous studies from our group and others found that A_{2B} AR was up-regulated in ischemic mouse hearts [16,17]. A_{2B} AR has recently been shown to have cardio-protective properties [18,19] However, A_{2B} AR-mediated CF regulation under ischemic conditions has not been studied. A comparative study of cardiac ischemia-reperfusion in A2AWT and A2AKO may give us more information about the role of A_{2B} AR in CF regulation under ischemia, i.e. A2AKO may also be able to serve as a pathophysiological model.

To assess the role of NO in A_{2A} AR-mediated CF regulation, we used the NO synthase inhibitors, L-NAME and L-NMA, in this investigation. Numerous studies have shown that NO synthase inhibition limits basal CF in various species including mice [6,9]. Studies have also shown that A_{2A} AR contributes significantly to coronary basal tone [2,9]. In this study, L-NMA was found to be more potent in decreasing CF than L-NAME. The reason may be the mode of transport of L-NMA into the cell being different from L-NAME. In another study, L-NAME was found to be a weak inhibitor of NO synthase than L-NMA in porcine aortic endothelial cells [28]. The authors suggested that it may be that L-NMAE is transported into the cell using either diffusion or a transport system other than cationic amino acid transporter used by L-NMA [28,29]. In addition, NO is a part of the complex redox system that are known to be heavily involved in multiple cardiovascular signal transduction systems, which regulate contractility, blood flow, and heart rate [30–32]. Nevertheless, both inhibitors demonstrated similar results in this study and their effect on heart rate is minimal. Furthermore, the decrease in contractility is corresponded to the decrease in CF and there is no difference between A2AWT and A2AKO (Figure 1), strongly indicating that the CF changes are due to vascular NO inhibition.. However, because of the complexity of the redox system, we cannot completely rule out that the effect is not part of the redox system imbalance caused by NO synthase inhibitors. This needs to be investigated further. In summary, our study is the first to demonstrate that at least part of A_{2A} AR effect in modulating coronary basal tone by endogenous release of adenosine is due to NO, as both L-NAME and L-NMA significantly inhibited CF in A2AWT but less in A2AKO (Figure 1A).

A_{2A} AR involvement with exogenous application of adenosine mediated coronary vasodilation is well established [2,27,33]. A_{2A} AR mediated NO release is also well recognized in various endothelial cell culture studies, including human, rat and porcine [4,34,35]. In another study [36], the authors stated that L-NAME abrogates 1 nM CGS-21680 dilatory response in Langendorff mouse heart. However, actual data from this study were not presented. In this study, L-NAME significantly blocked the NECA and CGS-21680-induced coronary vasodilation in A2AWT but not in A2AKO, which provided supporting evidence that NO is partially responsible for exogenous adenosine-induced A_{2A} AR-mediated coronary vasodilation.

Previous studies from our group have demonstrated that selective A_{2A} AR antagonist significantly inhibited CGS-21680-induced NO production but only partially inhibited the

effect induced by NECA [4] using porcine coronary endothelial cell cultures, suggesting that A_{2B} AR may also play a role in NO production. In a study using mouse thoracic aorta [37], CGS-21680, an A_{2A} specific agonist, had minimal effects on the relaxation responses suggesting A_{2A} AR may only play a limited role in this tissue. Alloxazine, a relatively selective A_{2B} AR antagonist, inhibited the relaxation to NECA. Removal of functional endothelium also attenuated NECA-induced relaxation. Taken together, these studies strongly suggest that A_{2B} AR is involved in mediating the vasorelaxing effect via endothelium.

In the current study, both L-NMA and L-NAME inhibited CF in A2AKO but significantly less when compared to A2AWT (Figure 1), suggesting that A_{2B} AR may also play a role in baseline NO release. However, the small NECA-induced CF increase (Figure 2A) in A2AKO (presumably A_{2B} effect) and the possible confounding up-regulation of A_{2B} AR we found in the coronary circulation makes the interpretation difficult. Further studies are needed to define the role of NO in A_{2B} AR-mediated CF regulation.

Previous study from our group [1] demonstrated that NECA and CGS-21680 increase cardiac contractility in a concentration dependent manner in A2AWT, while only NECA in A2AKO, supporting a major role for A_{2A} AR in increasing inotropy. Using single concentration of NECA (10⁻⁷ M), we also confirmed this finding. The reason for the enhanced contractility on 3rd application of CGS-21680 (Figure 3C) is not known. However, it is worth noting that the baseline CF never recovered to the baseline before L-NAME was applied while the maximal LVDP recovered to pre-L-NAME level (data not shown), suggesting that L-NAME may have a lasting effect on basal ventricular function, even after washing.

In addition, BAY 60-6583 was also found to increase LVDP in both A2AWT and A2AKO, supporting our earlier notion that A_{2B} AR may also play a role in cardiac contractility [1]. Our current study also confirmed the previous finding that A_{2A} AR may not be involved in heart rate regulation because CGS-21680, an A_{2A} AR specific agonist, has no effect on heart rate in both A2AWT and A2AKO, while NECA, a non-specific agonist, reduces heart rate in both.

Another interesting finding is that eNOS expression is not different between A2AWT and A2AKO, while responses to L-NAME are. The reason for this is not clear. Although the majority of NO production in vascular tissue is produced by eNOS, numerous factors, such as NADPH oxidase, BH₄, Zinc, and the availability of L-arginine, also affect the bioavailability of NO (for details please see review [38]). To complicate the situation further, compensatory or adaptive mechanisms, which we found in this case with A_{2B} AR, may also play a significant role. For instance, as mentioned previously, we found a small but significant decrease of CF by L-NAME in A2AKO only at 10⁻⁴ M (Figure 1A). With the finding of A_{2B} AR up regulation, we cannot be sure if that is due to the effect on A_{2B} AR or other possible mechanisms. Therefore, further clarification of the role of A_{2B} AR-NO in coronary circulation is needed.

In conclusion, we found for the first time that coronary A_{2B} AR is significantly up-regulated in mice that are deficient in A_{2A} AR, both in gene expression and functional responses. We also provided functional evidences that A_{2A} AR is involved in basal NO release and NO is partially responsible for exogenous adenosine-induced A_{2A} AR-mediated coronary vasodilation. Finally, the mechanisms behind the bioavailability of NO in A2AKO need further investigation.

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Figure 1.

Concentration–response curves for coronary flow (A and D), heart rate (B and E), and left ventricular developed pressure (LVDP, C and F) with L-NAME (A, B, and C) and L-NMA (D, E, and F) in isolated perfused hearts from A2AWT (squares, n=5) and A2AKO (triangles, n=5). Values are mean $\pm$ S.E.M. * represents significant difference from baseline (p<0.05). # represents significant difference from A2AWT. Data were expressed as percent changes from the baseline reading before L-NAME or L-NMA was applied.

Figure 2.

Effect of L-NAME on NECA-induced changes in coronary flow (A), heart rate (B), and LVDP (C) in isolated perfused hearts from A2AWT (n=9) and A2AKO (n=5). The 3rd set of the bars labeled NECA were a repeat treatment of NECA after washout of L-NAME and NECA co-treatment. Values are means $\pm$ S.E.M. * represents significant difference from the first NECA response in the same animal. # represents significant different from L-NAME treated group in the same animal. $p < 0.05$ was considered significant.

Figure 3.

Effect of L-NAME on CGS-21680-induced changes in coronary flow (A), heart rate (B), and LVDP (C) in isolated perfused hearts from A2AWT (n=6). CGS-21680 has no effect on A2AKO (not shown). The 3rd set of the bars labeled CGS were a repeat treatment of CGS-21680 after washout of L-NAME and CGS co-treatment. Values are means $\pm$ S.E.M. * represents significant difference from the first NECA treatment in the same animal. # represents significant different from L-NAME treated group in the same animal. $p < 0.05$ was considered significant.

Figure 4.

Relative adenosine receptor and eNOS gene expression by real-time PCR in mouse coronary artery from A2AWT and A2AKO. Each experiment required coronary arteries from 8 mice. The experiments were repeated 3 times. All the gene expressions were normalized to 18s rRNA and calibrated to A₁ AR expression in A2AWT, i.e. expressed as ratio to the A₁ AR in A2AWT (mean $\pm$ S.E.M.). * represents significant difference from A2AWT at $p < 0.05$.

Figure 5. Concentration-response curve for NECA in coronary arteries (LAD, size 50 –100 μ m inside diameter) from A2AWT (n=6) and A2AKO (n=4). Data are presented as percent relaxation. * represents significant difference from zero baseline. # represents significant difference from A2AWT at $p<0.05$.

Figure 6. Concentration–response curve for coronary flow (A), heart rate (B), and LVDP (C) for BAY 60-6583 in isolated perfused hearts from A2AWT (n=6) and A2AKO (n=6). Values are mean $\pm$ S.E.M. * represents significant difference from A2AWT at $p < 0.05$.