Metabolic hyperemia requires ATP-sensitive $K^+$ channels and $H_2O_2$ but not adenosine in isolated mouse hearts

Xueping Zhou,1,2 Bunyen Teng,1,2 Stephen Tilley,3 Catherine Ledent,4 and S. Jamal Mustafa1,2

1Department of Physiology and Pharmacology, West Virginia University, Morgantown, West Virginia; 2Center for Cardiovascular and Respiratory Sciences, West Virginia University, Morgantown, West Virginia; 3Department of Medicine, University of North Carolina, Chapel Hill, North Carolina; and 4Universite Libre de Bruxelles, Brussels, Belgium

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Zhou X, Teng B, Tilley S, Ledent C, Mustafa SJ. 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: H1046–H1055, 2014. First published August 8, 2014; doi:10.1152/ajpheart.00421.2014.—We have previously demonstrated that adenosine-mediated $H_2O_2$ production and opening of ATP-sensitive $K^+$ (KATP) channels contributes to coronary reactive hyperemia. The present study aimed to investigate the roles of adenosine, $H_2O_2$, and KATP channels in coronary metabolic hyperemia (MH). Experiments were conducted on isolated Langendorff-perfused mouse hearts using combined pharmacological approaches with adenosine receptor (AR) knockout mice. MH was induced by electrical pacing at graded frequencies. Coronary flow increased linearly from 14.4 ± 1.2 to 20.6 ± 1.2 ml·min$^{-1}$·g$^{-1}$ with an increase in heart rate from 400 to 650 beats/min in wild-type mice. Neither non-selective blockade of ARs by 8-(p-sulfophenyl)theophylline (8-SPT; 50 μM) nor selective A2AAR blockade by SCH-58261 (1 μM) or deletion affected MH, although resting flow and left ventricular developed pressure were reduced. Combined A2AAR and A2BAR blockade or deletion showed similar effects as 8-SPT. Inhibition of nitric oxide synthesis by N-nitro-l-arginine methyl ester (100 μM) or combined 8-SPT administration failed to reduce MH, although resting flows were reduced by ~20%. However, glibenclamide (KATP channel blocker, 5 μM) decreased not only resting flow (by ~4%) and left ventricular developed pressure (by ~36%) but also markedly reduced MH by ~94%, resulting in cardiac contractile dysfunction. Scavenging of $H_2O_2$ by catalase (2,500 U/min) also decreased resting flow (by ~16%) and MH (by ~24%) but to a lesser extent than glibenclamide. Our results suggest that while adenosine modulates coronary flow under both resting and ischemic conditions, it is not required for MH. However, $H_2O_2$ and KATP channels are important local control mechanisms responsible for both coronary ischemic and metabolic vasodilation.

Coronary metabolic hyperemia; adenosine receptor knockouts; A2A adenosine receptor; A2B adenosine receptor; hydrogen peroxide; ATP-sensitive $K^+$ channels

Myocardial function is dependent on a constant $O_2$ supply from the coronary circulation to myocytes. Myocardial $O_2$ consumption (mVO2) increases whenever there is enhanced cardiac work. Because the myocardium has a very limited anerobic capacity with a high $O_2$ extraction (75%) (46) under resting conditions, the increased $O_2$ delivery during exercise mostly relies on increased coronary blood flow, termed as metabolic hyperemia. Numerous mechanisms are responsible for coronary metabolic hyperemia, allowing a balance between myocardial $O_2$ demand and supply. Disturbance of these mechanisms results in decreased cardiac output, hypotension, arrhythmia, heart failure, and death.

Metabolic coronary vasodilation has been found to be regulated by both local metabolic feedback and adrenergic feed-forward mechanisms (46). For decades, adenosine, a locally released metabolite from the myocardium, has been postulated as one of the important mechanisms responsible for metabolic coronary hyperemia (3, 4, 6, 16, 17, 19, 28). However, recent evidence suggests that adenosine is not required for metabolic coronary vasodilation (4, 14, 47–49), and its role in regulating resting coronary flow (CF) still remains controversial (14, 25–27, 49). The discrepancy may be due to different animal models, differences in species, and/or the different agonists and antagonists applied in these studies. It is well established that adenosine exerts its effect through activation of four receptor subtypes, namely, A1 and A2 adenosine receptors (ARs), which exert constrictive effects, and A2A and A2B ARs, which have dilatory effects (31). The majority of these studies to characterize the functional role of adenosine in coronary hyperemia have relied heavily on adenosine-related pharmacological compounds, which may lead to misinterpretation of the data due to the mixed specificity of the agonists and antagonists for the four different ARs. In addition, the complexity of the combined intrinsic (e.g., myogenic, shear-mediated, metabolic, and endothelium-originated mediators) with extrinsic (neural hormonal regulatory mechanism) blood flow control mechanisms make whole animal study a nonoptimal model for the mechanistic exploration in local metabolic coronary blood flow regulation. Therefore, there is a need to reexamine the functional role of adenosine in coronary metabolic hyperemia using combined AR knockout (KO) mice with traditional pharmacological agents in isolated Langendorff-perfused hearts.

Increasing evidence suggests that $H_2O_2$, an endogenous metabolite, acts as an important mediator responsible for metabolic coronary vasodilation (37, 42, 53, 54). We (42, 57) have previously demonstrated that A2AAR-mediated $H_2O_2$ production opens ATP-sensitive $K^+$ (KATP) channels in coronary smooth muscle cells, contributing to the coronary ischemic vasodilation (42, 57). While the KATP channel is known to be important for coronary reactive hyperemia (1, 2, 10, 11, 42), it remains controversial regarding its role in metabolic hyperemia (13, 15, 16, 32, 34, 35, 47, 51). Therefore, the present study further examined whether $H_2O_2$ and/or KATP channels play an important role in coronary metabolic hyperemia using isolated Langendorff-perfused mouse hearts in the presence of catalase (an enzyme that decomposes $H_2O_2$ to water) or glibenclamide (a selective KATP channel blocker).
ADENOSINE IN CORONARY FLOW REGULATION

MATERIALS AND METHODS

**Animals.** The Institutional Animal Care and Use Committee of West Virginia University School of Medicine approved all experimental protocols. We followed guidelines set forth by the American Physiological Society and National Institutes of Health regarding the care and use of laboratory animals. A2AAR and A2BAR single KO mice (A2A KO and A2B KO mice, respectively) were generously provided by Dr. C. Ledent (Université Libre de Bruxelles, Brussels, Belgium) and Stephen Tilley (University of North Carolina, Chapel Hill, NC), respectively. A2A and A2B KO mice, both backcrossed 12 generations to the wild-type (WT) C57BL/6 background (Jackson Laboratory, Bar Harbor, ME), were bred to generate A2A/A2B double-KO (DKO) heterozygotes. Double heterozygotes were intercrossed, and 1/16 of the offspring were A2A/A2B DKO mice. A2A/A2B DKO (DKO) heterozygotes. Double heterozygotes were intercrossed, and 1/16 of the offspring were A2A/A2B DKO mice. A2A/A2B DKO breeding pairs were then established. Mice were caged in a 12:12-h light-dark cycle with free access to standard chow and water. The absence of A2AAR and A2BAR at both mRNA and protein levels in A2A KO, A2B KO, and A2A/2B DKO mice has been previously reported by our previous studies using PCR, Western blot analysis, and immunohistochemistry in the aorta, mesenteric arteries, and coronary arteries (30, 41, 43–45, 57). Our functional data also confirmed the lack of an adenosine response in A2A/2B DKO mice (41, 45).

**Langendorff-perfused mouse heart preparations.** Mice (14–17 wk) of either sex (equal ratio) were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), and hearts were excised into heparinized (50 mg/kg body wt ip) ice-cold Krebs-Henseleit buffer containing (in mM) 119 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.5 glucose, 22 NaHCO3, 5.0 CaCl2, 2 pyruvate, and 0.5 EDTA. After removal of the lung and surrounding tissue, the heart was rapidly cannulated with a 20-gauge, blunt-ended needle and continuously perfused with 37°C buffer bubbled with 95% O2 and 5% CO2 at a constant perfusion pressure of 80 mmHg. The left atrium was removed, and a fluid-filled balloon made of plastic wrap was inserted into the left ventricle (LV) across the mitral valve. The balloon was connected to a pressure transducer for the continuous measurement of LV developed pressure (LVDP). The heart was then immersed in a water-jacketed perfusate bath maintained at 37°C and allowed to beat spontaneously. LV diastolic pressure was adjusted to 2–5 mmHg. CF was continuously measured along with an ultrasonic flow probe (Transonic Systems, Ithaca, NY) placed in the aortic perfusion line. A Power Lab Chart data-acquisition system (AD Instruments, Colorado Springs, CO) was used for data acquisition. After stabilization for 20 min, hearts were then paced at 400 beats/min by two platinum wires connected to the stainless steel line (8-SPT; nonselective adenosine receptor antagonist, 50 μM, Sigma), N-nitro-L-arginine methyl ester (l-NNAME; nitric oxide (NO) synthase inhibitor, 100 μM, Sigma, based on our previous study (45) showing that l-NNAME at ≥10−4 reached its maximum inhibition on resting CF and 5′-(N-ethylcarboxamido)adenosine (nonselective adenosine agonist)-induced CF responses], SCH-58261 (selective A1AAR antagonist, Ki = 1.3 nM, 1 μM, Toecis Bioscience), CVT-6883 (selective A2B AR antagonist, Ki = 22 nM, 1 μM, Toecis Bioscience), caffeine (the enzyme that decompenses H2O2 to water, 2,500 μM, Sigma), or glibenclamide (KATP channel blocker, 5 μM, Sigma) were delivered into the aortic perfusion line for 15 min using a microinjection pump (Harvard Apparatus, Holliston, MA) at 1% of CF. In the presence of each pharmacological reagent, a second pacing-induced functional hyperemia was elicited. Time-matched control experiments with two consecutive inductions of functional hyperemia showed no differences in baseline function as well as the magnitude of pacing-induced changes in CF (n = 3 animals).

**Data analysis and statistics.** All data collected were analyzed by LabChart 7.0 software. Baseline CF, LVDP, and dP/dt were calculated as mean values after each heart was paced at 400 beats/min for 10 min. Mean CF was calculated during the 2-min pacing at each frequency. Because absolute CF rates changed proportionally with heart mass, CF was normalized by heart mass and presented as milliliters per gram of wet heart weight per minute. Because mvO2 is a function of HR, cardiac contractility, ventricular wall tension, and muscle shortening (9, 12, 20), it is represented by the rate-pressure product (RPP). To examine the relationship between mvO2 and RPP, Po2 of the inflow (P(O2)); an indication of arterial O2 tension) and outflow perfusate (P(O2)); an indication of venous O2 tension collected through a tube inserted in the right atrium close to the coronary sinus) were measured using a GEM Premier 4000 gas analyzer (n = 2). Because of the absence of hemoglobin in the perfusion buffer, O2 content is dependent solely on the O2 solubility in the buffer at 37°C, which was 0.023 ml O2/ml buffer−1-atm−1 (39). mvO2 was calculated using the following equation: mvO2 = CF × (P(O2) − Po2(O2)) × 0.023/760, as previously described (39). As expected, mvO2 linearly correlated with RPP as hearts were paced at graded frequencies (mvO2 = 1.49 × RPP + 101.5, R2 = 0.9596, P < 0.01), indicating that mvO2 is a function of the HR and LVDP product.

Least-square linear regression analysis was conducted between HR or RPP, and CF to calculate the slope of the relationship. The slope of each curve was compared before and after drug treatment or between groups. All data are presented as means ± SE; n represents the number of animals unless otherwise indicated. Paired t-test was used for paired data analysis. One-way ANOVA was used to compare the data between groups. The effect of treatment on the relationship between two variables was analyzed by analysis of covariance using GraphPad Prism5. P values of <0.05 were considered as statistically significant.

**RESULTS**

**Baseline function from WT, A2A KO, A2B KO, and A2A2B DKO mouse hearts.** Baseline functional parameters of isolated mouse hearts were recorded at 10 min of pacing (400 beats/min) after 20-min stabilization. There were no statistically significant differences in the heart-to-body weight ratio and maximum −dP/dt among all animal strains, although +dP/dt had a trend to be less in A2A KO, A2B KO, or A2A2B DKO mice compared with WT mice. Baseline CF as well as LVDP were significantly lower in KO mice compared with WT mice (P < 0.05 vs. WT mice; Table 1).

**Pacing-induced coronary hyperemia in the absence or presence of 8-SPT.** The CF response in isolated hearts of WT mice was measured before and after the addition of 8-SPT (50 μM, a nonselective adenosine antagonist, n = 6). Under control conditions, mean CF increased significantly from 14.4 ± 1.2 to 16.8 ± 1.1, 18.5 ± 1.3, 19.5 ± 0.9, and 20.6 ± 1.2 ml-min−1g−1 when hearts were paced from 400 to 500, 550, 600, and 650 beats/min, respectively (Fig. 1A). The increased CF linearly correlated with the enhanced mvO2, indicated as
Coronary flow, ml·min⁻¹·g⁻¹
Heart-to-body weight ratio, % 0.46

Ventricular developed pressure (LVDP; mmHg), rate-pressure product (RPP) and CF (A), and HR and left ventricular developed pressure (LVDP; C) before and after the addition of 8-SPT (n = 6). D: adenosine (10⁻⁹–10⁻⁵ M)-induced coronary response before and after the infusion of 8-SPT (50 μM, n = 3). *P < 0.05, significant difference between the curves.

To confirm the antagonistic ability of 8-SPT against adenosine, we examined the effect of 8-SPT at the same concentration (50 μM) on adenosine (10⁻⁹–10⁻⁵ M)-induced CF responses. 8-SPT significantly decreased both baseline CF (from 13.2 ± 0.55 to 10.8 ± 0.52 ml·min⁻¹·g⁻¹, n = 3, P < 0.05) as well as the adenosine-induced CF increase (maximal coronary flow was reduced from 35 ± 1.4 to 15.4 ± 1.5 ml·min⁻¹·g⁻¹, P < 0.05; Fig. 1D).

Combined effect of L-NAME and 8-SPT on pacing-induced coronary hyperemia. Baseline CF was significantly decreased by L-NAME (10⁻⁴ M) from 18.0 ± 0.86 to 14.8 ± 0.78 ml·min⁻¹·g⁻¹ (P < 0.05, n = 8; Fig. 2A). However, the pacing-induced CF increase was not affected by L-NAME (CF increased linearly from 14.8 ± 0.78 to 22.9 ± 1.53 ml·min⁻¹·g⁻¹ when hearts were paced from 400 to 650 beats/min; Fig. 2, A and B). The slope of the relationship between HR or RPP and CF was not significantly changed before or after the addition of L-NAME (P > 0.05), and the linear curve was shifted downward in a parallel manner. Concomitant with the decreased baseline flow, LVDP was significantly diminished by L-NAME from 110 ± 5.9, 130 ± 4.9, 136 ± 3.5, 135 ± 3.5,
and 134 ± 3.4 to 88 ± 6.1, 104 ± 6.4, 112 ± 44.8, 112 ± 4.8, and 116 ± 4.1 mmHg when hearts were paced at 400, 500, 550, 600, and 650 beats/min, respectively (P < 0.05; Fig. 2C).

In four of eight animals, 8-SPT (50 μM) was added in the presence of l-NAME. 8-SPT did not further decrease baseline CF, nor did it affect pacing-induced hyperemia (P > 0.05 vs. l-NAME; Fig. 2, A–C).

Effect of A2AAR blockade or deletion on pacing-induced coronary hyperemia. The role of A2AARs in coronary functional hyperemia was examined in WT mice (n = 6) in the presence of SCH-58261 (1 μM, a selective A2AAR antagonist). SCH-58261 significantly decreased baseline CF from 17.5 ± 1.54 to 11.3 ± 0.70 ml·min⁻¹·g⁻¹ (P < 0.05; Fig. 3, A and B) and LVDP from 101 ± 3.5 to 70 ± 5.8 mmHg, respectively.

Fig. 2. Effect of N-nitro-l-arginine methyl ester (l-NAME) on the pacing-induced CF response in the absence or presence of 8-SPT. A–C: relationship between HR and CF (A), RPP and CF (B), and HR and LVDP (C) under control conditions (n = 8), in the presence of l-NAME (nitric oxide synthase inhibitor, 100 μM, n = 4), and in the combined presence of 8-SPT (50 μM) and l-NAME (100 μM, n = 4). *P < 0.05, significant difference from the control curve (A and B) and versus control (C).

Fig. 3. Effect of A2A adenosine receptor (AR) blockade or deletion on pacing-induced coronary hyperemia. A–C: relationship between HR and CF (A), RPP and CF (B), and HR and LVDP (C) in control wild-type (WT) mice (n = 6), A2AAR knockout (A2A KO) mice (n = 9), and WT mice treated with SCH-58261 (selective A2AAR antagonist, 1 μM, n = 6). *P < 0.05, significant difference between the curves or significant difference versus control; #P < 0.05, significant difference versus A2A KO mice.
(P < 0.05; Fig. 3C). However, after A2AAR blockade, CF still increased from 11.3 ± 0.70 to 16.3 ± 0.54 ml-min⁻¹·g⁻¹ when hearts were paced from 400 to 650 beats/min. The slope of the relationship between HR or RPP and CF was not different before or after A2AAR blockade (P > 0.05; Fig. 3, A and B).

We further examined the effect of A2AAR deletion on pacing-induced coronary hyperemia using A2A KO mice (n = 9). Baseline CF and LVDP were 14.4 ± 0.56 ml-min⁻¹·g⁻¹ and 90 ± 3.9 mmHg, respectively, which were significantly lower than those in WT mice (Table 1 and Fig. 3) but were higher than those in WT mice treated with SCH-58261 (P < 0.05; Fig. 3, A and C). Consistent with the effect of SCH-58261, A2AAR deletion did not affect the pacing-induced CF increase, although receptor deletion resulted in a parallel downward shift of the relationship between RPP or HR and CF (Fig. 3, A and B).

Effect of double blockade or deletion of A2AAR and A2BAR on pacing-induced coronary hyperemia. Both A2AARs and A2BARs are involved in coronary vasodilation (8, 41, 45). Therefore, we examined the effect of double blockade or deletion of both ARs on pacing-induced coronary hyperemia.

In A2A KO mice (n = 4), CVT-6883 (1 μM) further decreased baseline CF from 13.5 ± 1.03 to 11.6 ± 0.75 ml-min⁻¹·g⁻¹ (P < 0.05) and LVDP from 91 ± 2.3 to 71 ± 5.7 mmHg (P < 0.05; Fig. 4D). Similarly, in A2B KO mice, which have lower baseline CF (14.1 ± 1.78 ml-min⁻¹·g⁻¹, n = 6) and LVDP (93 ± 5.3 mmHg) than WT mice (P < 0.05; Table 1 and Fig. 4D), combined blockade of A2AARs by SCH-58261 (1 μM) further decreased baseline CF and LVDP to 10.6 ± 0.65 ml-min⁻¹·g⁻¹ and 74 ± 3.3 mmHg, respectively (P < 0.05; Fig. 4D). However, in both cases, pacing-induced hyperemia was not affected (CF increased from 11.0 ± 0.58 and 10.6 ± 0.65 to 16.0 ± 0.81 and 15.7 ± 0.77 ml-min⁻¹·g⁻¹, respectively when hearts were paced from 400 to 650 beats/min; Fig. 4, A–C). The slope of the relationship between HR and/or RPP and CF under each experimental condition was not significantly different from WT mice (Fig. 4, A–C).

To confirm the role of A2AARs and A2BARs in coronary metabolic hyperemia, we further examined the hyperemic response in A2A2B DKO mice (n = 6). Baseline CF (13.4 ± 0.52 ml-min⁻¹·g⁻¹) and LVDP (88 ± 2.5 mmHg) were lower than those in WT mice (P < 0.05) and were less than those in either A2A KO or A2B KO mice, although they did not reach statistical significance (Table 1 and Fig. 4, E and F). However, the pacing-induced hyperemic response was not affected (CF increased from 13.3 ± 0.52 to 19.9 ± 0.73 ml-min⁻¹·g⁻¹, linearly correlated with the increased HRs from 400 to 650 beats/min; Fig. 4E). The slope of the relationship between HR and/or RPP and CF in A2A2B DKO mice was not significantly different from that in WT mice, suggesting that neither A2AARs nor A2BARs are required for the pacing-induced increase in CF.

Effect of catalase on pacing-induced coronary hyperemia. The role of H₂O₂ in pacing-induced hyperemia was examined in WT mice (n = 6) before and after the addition of catalase (2,500 U/ml). Catalase significantly decreased CF from 16.5 ± 1.22 to 13.8 ± 1.40 ml-min⁻¹·g⁻¹ (Fig. 5A) and significantly decreased LVDP from 96 ± 7.4 to 88 ± 7.1 mmHg (Fig. 5C), respectively (P < 0.05 by paired t-test). Additionally, catalase significantly attenuated the pacing-induced increase in CF by ~37% (the net increase in CF of hearts paced from 400 to 650 beats/min was decreased from 7.4 ± 0.12 to 4.6 ± 0.49 ml-min⁻¹·g⁻¹, P < 0.05). The slope of the relationship between HR and/or RPP and CF in the presence of catalase was significantly lower than that under control conditions (P < 0.05 by paired t-test; Fig. 5, A and B).

Effect of glibenclamide on pacing-induced coronary hyperemia. The role of K_ATP channels in pacing-induced coronary hyperemia was determined in isolated WT mouse hearts (n = 6) in the absence or presence of glibenclamide (K_ATP channel blocker, 5 μM). In the absence of glibenclamide, CF increased by 6.2 ml-min⁻¹·g⁻¹ after hearts were paced from 400 to 650 beats/min (Fig. 6, A and B). After glibenclamide infusion, CF decreased significantly by ~45% (from 20 ± 1.6 to 11 ± 0.9 ml-min⁻¹·g⁻¹, P < 0.05; Fig. 6, A and B) and LVDP was reduced by ~36% (from 112 ± 13.3 to 72 ± 7.0 mmHg, P < 0.05; Fig. 6C). Moreover, pacing-induced hyperemia was dramatically decreased by ~94% (the net CF increase before and after glibenclamide was 6.1 and 0.39 ml-min⁻¹·g⁻¹, respectively; Fig. 6, A and B). The dramatic reduction in functional hyperemia was concomitant with a significant decrease in LVDP during pacing (LVDP decreased from 72 ± 7.0 to 63 ± 9.0, 57 ± 4.8, and 59 ± 9.2 mmHg when hearts were paced at 550, 600, and 650 beats/min, respectively; Fig. 6C), suggesting compromised heart function as a result of an imbalance between O₂ supply and demand after K_ATP channel blockade.

DISCUSSION

In the present study, using targeted AR KO mice combined with pharmacological approaches, we further examined the role of adenosine, H₂O₂, and K_ATP channels in coronary metabolic hyperemia. In isolated Langendorff-perfused mouse hearts from A2A KO, A2B KO, and A2A2B DKO mice, we demonstrated that adenosine and its receptor subtypes (A2A and A2B) are not required for pacing-induced coronary metabolic hyperemia, although they exert important roles in maintaining CF under resting conditions. Similarly, broad pharmacological blockade of ARs by 8-SPT in the absence or presence of a NO synthase inhibitor or selective A2AAR and A2BAR blockade did not affect pacing-induced increase in CF, although baseline flow was reduced. However, K_ATP channel blockade by glibenclamide remarkably reduced both resting flow and pacing-induced hyperemia, resulting in a dramatic decrease in LVDP. Scavenging of H₂O₂ by catalase also decreased resting flow and pacing-induced coronary hyperemia but to a lesser extent than glibenclamide. Our results suggest that while adenosine modulates coronary vascular tone under both resting and ischemic conditions, it is not required for metabolic hyperemia even when the NO pathway is inhibited. However, H₂O₂ and K_ATP channels are important local control mechanisms responsible for both coronary ischemic and metabolic vasodilation.

Since Berne (6) proposed that adenosine is an important metabolite that links the changes in CF to mvO₂, there have been numerous studies that have examined the adenosine hypothesis in coronary metabolic hyperemia (3, 4, 7, 14, 21, 47–49). Early studies supported this hypothesis in that the augmentation in cardiac interstitial adenosine concentration was observed to be correlated with increased mvO₂ in chronically instrumented dogs (3, 19, 21) and pharmacological block-
Adenosine in coronary flow regulation

The role of adenosine in coronary flow regulation is under investigation. A recent study showed that adenosine decreases coronary metabolic hyperemia in isolated guinea pig hearts (21). However, more recent studies in awake dogs have reported that there was no significant increase in cardiac interstitial adenosine levels that was sufficient to cause coronary vasodilation (49, 50, 52). AR blockade (49, 50) or triple blockade of adenosine, KATP channels, and NO (47, 48) did not affect exercise or norepinephrine-induced coronary hyperemia, suggesting that adenosine is not required in coronary functional hyperemia. Interestingly, Duncker and colleagues (16, 17) suggested that adenosine is an important mediator for coronary metabolic hyperemia only when the KATP channel is blocked or under conditions where coronary perfusion pressure is reduced. The discrepancies among these studies may be due to the mixed drug specificity or to the complexity of mixed local and neuronal control mechanisms responsible for coronary blood flow regulation under in vivo conditions. Using targeted AR KO mice combined with an isolated heart preparation to solve the specificity.
issue as well as to exclude the neurohormonal effect on CF regulation, the present study showed that A2AARs and A2BARs are important in modulating coronary vascular tone under resting conditions because baseline CF in A2A KO, A2B KO, and A2A/2B DKO mice was reduced (by 16%, 18%, and 24%, respectively) compared with WT mice (Figs. 3 and 4). Of interest, although double deletion of A2AARs and A2BARs further reduced baseline CF from either A2A KO or A2B KO mice, the extent of reduction in A2A/2B DKO mice (by 22%) was much less than the summed effect of A2AAR and A2BAR deletion (~34%), suggesting that other unknown compensatory mechanisms might be turned on to maintain resting CF when both ARs are deleted. Consistent with the results observed in AR KO mice, broad AR blockade by 8-SPT and selective A2AAR or A2BAR blockade by SCH-58261 (1 μM) or CVT-6883 (1 μM) also resulted in a significant reduction (by ~25%, ~35%, and ~18%, respectively) in resting CF (Figs. 1, 3, and 4). In agreement with our findings, other studies in humans (18), pigs (14), dogs (49), and isolated guinea pig hearts (21) have also reported that adenosine is involved in modulating CF under resting condition. Of note, a lower reduction in CF was observed in A2A KO mice (by ~18%) versus SCH-58261-treated animals (by ~35%; Fig. 3, A and C). The lower reduction in baseline CF might be due to upregulated A2BARs in A2A KO mice compared with WT mice, as previously reported (45). Regarding the downstream

Fig. 5. Effect of catalase on pacing-induced coronary hyperemia. A–C: relationship between HR and CF (A), RPP and CF (B), and HR and LVDP (C) before and after the addition of catalase (the enzyme that decomposes H2O2 to water, 2.500 U/ml, n = 6). *P < 0.05, significant difference in the slope of the linear curves (A and B) and significant difference versus control (C, paired t-test).

Fig. 6. Effect of ATP-sensitive K+ channel blockade on pacing-induced coronary hyperemia. A–C: relationship between HR and CF (A), RPP and CF (B), and HR and LVDP (C) before and after the addition of glibenclamide (ATP-sensitive K+ channel blocker, 5 μM, n = 6). *P < 0.05, significant difference in the slope of the linear curves (A and B) and significant difference versus control; #P < 0.05 vs. LVDP at a HR of 400 beats/min.
mechanisms of adenosine in modulating resting CF, our results (45) and those of others (56) suggest that A2AR-mediated NO production may be responsible for resting tone regulation because 8-SPT (nonselective adenosine antagonist; Fig. 2) or SCH-58261 (selective A2AR antagonist) (56) failed to further decrease baseline CF in the presence of a NO synthase inhibitor. Concomitant with the decreased baseline flow, a significant reduction in LVDP (Table 1 and Figs. 1C, 3C, and 4D) and a relatively but not statistically lower \( +dP/dt \) (Table 1) were observed upon blockade of adenosine either pharmacologically or genetically. The correlated decrease in cardiac contraction with the decreased CF suggest that adenosine is an important mediator involved in modulating resting CF and that the decreased cardiac function is associated with the decreased CF.

Despite the decrease in resting CF, pacing-induced hyperemia was not affected after AR blockade or deletion, in as much as the linear curve of the relationship between HR and/or RPP and CF was downward shifted in a parallel manner and the slope of the curve was not altered before or after AR blockade or deletion (Figs. 1, 3, and 4). These results, obtained from targeted AR KO mice, add further evidence to the previous findings that an AR blocker failed to inhibit exercise-induced coronary hyperemia in chronically instrumented dogs, although it lowered the balance between \( O_2 \) supply and delivery under resting conditions (47, 49). The approach of using targeted AR KO mice allowed us to rule out the possibility that increased endogenous adenosine overcomes the competitive receptor blockade, thus masking the direct AR blockade effect. Additionally, in the presence of a NO synthase inhibitor, 8-SPT did not further decrease resting flow as well as pacing-induced coronary hyperemia, indicating both NO and adenosine act as tonic vasodilator but are not required for coronary metabolic hyperemia.

Increasing evidence suggests that \( H_2O_2 \), an endogenous metabolite, acts as a pivotal mediator responsible for metabolic coronary vasodilation (37, 42, 53, 54). In support of this idea, our results showed that catalase significantly decreased resting CF (by \( \sim 16\% \)) as well as the pacing-induced increase in CF (by \( \sim 37\% \); Fig. 5, A and B). It should be pointed out that although A2AR-mediated \( H_2O_2 \) production in the coronary endothelium and smooth muscle cells has been previously reported to play an important role in coronary ischemic hyperemia (42, 57), the failure of AR blockade or deletion to reduce metabolic hyperemia (discussed above) suggest that other mechanisms than adenosine-mediated signaling are responsible for \( H_2O_2 \) production, thus contributing to coronary metabolic vasodilation. Regarding the source of \( H_2O_2 \), we speculate that mitochondrial electron transport in cardiomyocytes might be an important source for pacing-induced \( H_2O_2 \) production (37).

The \( K_{ATP} \) channel has been reported to be involved in coronary ischemic vasodilation (24, 41, 42, 57), although its role in metabolic vasodilation remains controversial (13, 15, 23, 35, 47, 55). In isolated mouse hearts, the present study showed that glibenclamide (5 \( \mu M \)) significantly reduced CF (by \( \sim 46\% \); Fig. 6A) and LVDP (by \( \sim 36\% \); Fig. 6C) at rest. In agreement with our results, studies in dogs have reported that intracoronary infusion of glibenclamide (50–80 \( \mu g \cdot kg^{-1} \cdot min^{-1} \)) significantly decreased baseline flow (by \( \sim 20–50\% \)) (38) and was associated with cardiac contractile dysfunction (systolic wall thickening decreased by \( \sim 43\% \)) (15). Importantly, recovery of the CF rate to preglibenclamide levels after the addition of sodium nitroprusside reversed the cardiac dysfunction, suggesting that \( K_{ATP} \) channels exert essential roles in maintaining resting CF and that the decreased CF upon \( K_{ATP} \) channel blockade in the coronary vasculature causes ischemia-induced cardiac contractile dysfunction (15). It is important to note, however, that \( K_{ATP} \) channels at concentrations of \( >10 \mu M \) have been reported to inhibit other \( K^+ \) and \( Ca^{2+} \) channels (5, 36). The concentration we used in the present study (5 \( \mu M \)) is within its selectivity range (5, 36), thus strengthening our conclusion that the \( K_{ATP} \) channel is important in modulating resting CF. Interestingly, in disagreement with the majority of studies showing that blockade of \( K_{ATP} \) channels did not attenuate the increase in CF during treadmill exercise or paired cardiac pacing in dogs and pigs (13, 15, 34, 35, 47), the present study on isolated mouse hearts demonstrated that blockade of \( K_{ATP} \) channels not only reduced resting flow but also markedly inhibited pacing-induced functional hyperemia by \( \sim 94\% \) (Fig. 6, A and B). The discrepancy among the studies could be due to 1) species differences (mice vs. pigs and dogs) or 2) the difference in animal models (isolated hearts vs. whole animals). In isolated hearts from mice deficient in \( K_6.1 \) (one of the \( K_{ATP} \) subunits), isoprenaline-induced coronary vasodilation was significantly attenuated, indicating an important role of \( K_{ATP} \) channels in coronary metabolic hyperemia (55). Additionally, the redundant mechanisms responsible for CF regulation in whole animals, including local and neurohormonal control mechanisms, may complicate the explanation of the data, e.g., the reduction of coronary metabolic hyperemia by the \( K_{ATP} \) channel blocker might be compensated by an enhanced neurohormonal effect or other unknown mechanisms to maintain the balance between \( O_2 \) supply and demand, thus resulting in unchanged metabolic hyperemia in vivo even after \( K_{ATP} \) channel blockade. Concomitant with the reduced metabolic hyperemia in isolated mouse hearts, we also observed dramatic cardiac contractile dysfunction. In contrast to significant increases in LVDP upon pacing in control condition, \( K_{ATP} \) channel blockade resulted in a significant reduction in LVDP upon pacing (Fig. 6C), suggesting potential ischemic cell damage related to a decrease in CF.

It is well established the \( K_{ATP} \) channels play important roles in adenosine-mediated coronary reactive hyperemia (1, 8, 22, 41, 42, 57). Our recent studies (42, 58) have demonstrated that A2AR-mediated \( H_2O_2 \) production opens \( K_{ATP} \) channels, partially contributing to coronary reactive hyperemia. In these studies, although adenosine-induced \( K_{ATP} \) current was reduced in A2AR KO and A2A/2B DKO animals, pinacidil (\( K_{ATP} \) opener)-induced current was not affected after deletion of both A2ARs and A2BARs, suggesting that A2ARs and A2BARs are not required for \( K_{ATP} \) activation and that the absence of ARs does not affect channel expression, as we previously reported (33, 42). Additionally, we demonstrated that \( K_{ATP} \) channel blockade inhibited coronary reactive hyperemia (57) to a higher extent than A2AR blockade, indicating that other factors, including adenosine, can also activate \( K_{ATP} \) channels, thus contributing to coronary vasodilation. In the present study, \( K_{ATP} \) channel blockade dramatically decreased metabolic hyperemia, whereas only a parallel downward shift of the relationship between CF and RPP was observed upon A2AR and/or A2BAR blockade or ablation, suggesting that other unknown mediators than adenosine released during increased cardiac metabolic demand can activate \( K_{ATP} \) channels, contrib-
uturing to the increased flow during metabolic hyperemia. One of the mediators appears to be H$_2$O$_2$, because scavenging of H$_2$O$_2$ by catalase significantly decreased metabolic hyperemia by $\sim$37% (Fig. 5, A and B) and to a lesser extent than K$_{ATP}$ channel blockade (by $\sim$94%; Fig. 6, A and B). Taken together, our results suggest that while adenosine-mediated H$_2$O$_2$ production and K$_{ATP}$ channel opening are important for coronary reactive hyperemia, adenosine appears to be not required for metabolic hyperemia, and other unknown mediators act together with H$_2$O$_2$ to open K$_{ATP}$ channels and are thus responsible for metabolic hyperemia in isolated mouse hearts.

It should be pointed out that there are some limitations regarding the translation of our findings based on our present experimental model into the clinical applications regarding the pathophysiology of CF regulation. First, although the effects of neurohormonal and blood components were excluded to scrutinize the metabolic control mechanism in CF regulation, buffer-perfused isolated hearts did not allow us to clearly separate the contribution of shear- and/or pressure-induced CF changes from local metabolic effects. Second, the continuously oxygenated (95% O$_2$) Krebs buffer used in isolated hearts has much less O$_2$ carrying capacity than blood, which might cause hypoxia and relatively lower cardiac contractile function compared with blood-perfused hearts (40). However, in buffer-perfused hearts paced from 400 to 600 beats/min, we observed a correlated increase in mvo$_2$ from 160 to 230 $\mu$mol min$^{-1}$ g$^{-1}$ as well as CF (increased by $\sim$40%). More importantly, CF increased more than twofold over baseline after 15-s flow occlusion (42, 57), indicating that buffer-perfused hearts have a minimum hypoxia and coronary vessels have the capacity to further dilate. However, due to the relatively lower myoglobin O$_2$ saturation in buffer-perfused hearts (39), the cardiac interstitial adenosine level might be higher compared with blood-perfused hearts, which may overestimate the role of adenosine in modulating resting coronary blood flow in our model. Future studies are needed to compare adenosine levels in buffer-versus blood-perfused hearts. Finally, to extend our study to an atherosclerotic model, where blunted metabolic hyperemia has been reported, it should be noted that other factors, such as phenotypic changes of coronary vessels (more stiff and fibrotic) and hemodynamic alterations due to collateral circulation and/or coronary steal (less flow to the ischemic region where the vascular network is already maximally dilated), need to be considered for studies.

In conclusion, blockade of ARs, in particular, A$_{2A}$ARs and A$_{2B}$ ARs using combined pharmacological compounds with targeted gene deletion, decreased CF at rest but failed to reduce the pacing-induced increase in CF. Inhibition of NO synthase decreased resting CF without an effect on pacing-induced hyperemia, which was not altered by combined blockade of ARs, suggesting both NO and adenosine are not required for coronary metabolic hyperemia. The mechanisms responsible for pacing-induced coronary hyperemia involve mostly K$_{ATP}$ channels and, to a lesser extent, H$_2$O$_2$, since blockade of K$_{ATP}$ channels by glibenclamide almost abolished pacing-induced coronary vasodilation, whereas scavenging of H$_2$O$_2$ by catalase only partially inhibited the response. Thus, although adenosine-mediated H$_2$O$_2$ production and the subsequent opening of K$_{ATP}$ channels in the coronary vasculature play an important role in coronary ischemic vasodilation, other mechanisms, but not adenosine, are responsible for H$_2$O$_2$ production and opening of K$_{ATP}$ channels, leading to coronary metabolic vasodilation in isolated mouse hearts.

A compromised coronary vasodilation may not necessarily cause ischemia under resting condition. However, during increased myocardial demand, e.g., exercise, the coronary vascular dysfunction will lead to a mismatch between O$_2$ demand and supply, manifesting the clinical symptom known as angina. Although mice differ significantly from humans in many aspects, which may limit the extrapolation of present study to clinical situations, our model, using paced mouse hearts to gradually increase rates that are within the range of HR change during exercise in humans, will provide an important basis for further mechanistic studies in humans. The different mechanisms regarding the roles of adenosine, H$_2$O$_2$, and K$_{ATP}$ channels in CF regulation during ischemic versus metabolic hyperemia may add new knowledge to the understanding of pathophysiology of coronary artery diseases.

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