Published in final edited form as:

J Mol Cell Cardiol. 2016 January ; 90: 30–37. doi:10.1016/j.yjmcc.2015.11.033.

Enhanced A_{2A} adenosine receptor-mediated increase in coronary flow in type I diabetic mice

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Abstract

Adenosine A_{2A} receptor (A_{2A}AR) activation plays a major role in the regulation of coronary flow (CF). Recent studies from our laboratory and others have suggested that A_{2A}AR expression and/or signaling is altered in disease conditions. However, the coronary response to AR activation, in particular A_{2A}AR, in diabetes is not fully understood. In this study, we use an STZ mouse model of type 1 diabetes (T1D) to look at CF responses to the nonspecific AR agonist NECA and the A_{2A}AR specific agonist CGS 21680 *in-vivo* and *ex-vivo*. Using immunofluorescence, we also explored the effect of diabetes on A_{2A}AR expression in coronary arteries. NECA mediated increase in CF was significantly increased in hearts isolated from STZ-induced diabetic mice. In addition, both in *in-vivo* and *ex-vivo* responses to A_{2A}AR activation using CGS 21680 were significantly higher in diabetic mice when compared to their controls. Immunohistochemistry showed an upregulation of A_{2A}AR in both coronary smooth muscle and endothelial cells (~160% and ~140%, respectively). Our data suggest that diabetes resulted in an increased A_{2A}AR expression in coronary arteries which resulted in enhanced A_{2A}AR-mediated increase in CF observed in diabetic hearts. This is the first report implying that A_{2A}AR has a role in the regulation of CF in diabetes, supporting recent studies suggesting that the use of adenosine and its A_{2A} selective agonist (regadenoson, Lexiscan®) may not be appropriate for the detection of coronary artery diseases in T1D and the estimation of coronary reserve.

Keywords

Diabetes; coronary flow; A_{2A} adenosine receptor; NECA; CGS 21680

Introduction

Type 1 diabetes (T1D) is a metabolic disease characterized by an increase in blood glucose levels due to a deficit in insulin secretion. In this country, recent published study has shown that the prevalence of T1D in youth has increased by 21.1% [1]. Additionally, long-term

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diabetes causes damage to several organ systems. In the vasculature, diabetic complications can be divided into microvascular (retinopathy and nephropathy) and macrovascular (cardiovascular diseases and erectile dysfunction), with cardiovascular disease being the leading cause of morbidity and mortality in diabetic patients [2]. In the heart, vascular and endothelial dysfunction is a well-established complication of diabetes, resulting in coronary artery disease (CAD) and may be in part responsible for the increased incidence of ischemic heart disease in the diabetic population. One of the most important factors regulating coronary artery (CA) vascular tone is adenosine, which is a metabolite released locally under physiologic and pathophysiologic conditions. In diabetes, studies have shown a crucial role of adenosine signaling in the regulation of glucose homeostasis and pathophysiology of the disease. In a model of T1D, non-selective adenosine receptor ligand NECA was able to promote pancreatic β cell regeneration *in-vivo*, which was countered by gene ablation of A_{2A}AR, suggesting an important role for this receptor in promoting the restoration of normoglycemia [3–5]. Adenosine is an autocoid that exerts its effects through activation of four G-protein coupled receptors: A₁, A_{2A}, A_{2B}, and A₃. While activation of A_{2A}AR and A_{2B}AR results in vasodilation, A₁AR and A₃AR receptor activation results in vasoconstriction. Within the heart, adenosine plays an important role in cardiac contractility, coronary flow (CF), inflammation, cell growth, tissue remodeling, and substrate utilization [6]. In the coronary vasculature, vasodilation was shown to be predominantly mediated through A_{2A}AR activation, while A₁AR activation was shown to negatively regulate vasodilation in mice [6–12]. A_{2B}AR and A₃AR play a lesser role in the regulation of CF [6, 11, 13]. In the clinic, adenosine has long been used for super ventricular tachycardia as well as detection of CAD [14–16]. Because of adenosine's frequent undesirable side-effects, regadenoson (Lexiscan[®], approved by FDA in 2008), an A_{2A}AR selective agonist, is preferred for myocardial perfusion imaging due to its minimal side effects [17]. Our laboratory has recently shown an increase of A_{2A}AR-mediated CF in a mouse model of hyperlipidemia and/or atherosclerosis, suggesting that possible A_{2A}AR upregulation should be taken into consideration while using A_{2A}AR agonists for cardiac imaging to assess coronary reserve in disease states [18]. The effect of diabetes on AR subtype expression is not well studied, with only a few studies reporting cell and tissue specific changes in AR expression in the heart, kidney, and liver [19–21]. We and others have demonstrated that the A_{2A}AR plays a major role in the regulation of coronary microvascular dilation; however, no studies have investigated the effect of T1D on the AR regulation of CF. We sought to determine the effect of T1D on A_{2A}AR expression and on CF response to A_{2A} activation *in-vivo* and *ex-vivo* to examine the hypothesis that the A_{2A}AR-mediated increase in CF is impaired.

Methods

All experimental protocols were performed according to West Virginia University guidelines and with approval of the Animal Care and Use Committee.

STZ mice and induction of diabetes

T1D was induced in 7 to 9-week-old male mice following the protocol of the Animal Models of Diabetic Complications Consortium using multiple low-dose streptozotocin

(STZ; Sigma, St. Louis, MO) injections as previously described [22]. Briefly, injections of 50 mg/kg body weight STZ dissolved in sodium citrate buffer (pH 4.5) were performed daily for 5 consecutive days after 4 to 5 h of fasting. Mice that served as vehicle controls were given the same volume per body weight of sodium citrate buffer. Blood glucose levels >300 mg/dL were considered diabetic. Eight weeks post-STZ injections, animals were killed for further experimentation [23].

Materials

5'-*N*-ethylcarboxamidoadenosine (NECA) and 4-[2-[[6-Amino-9-(*N*-ethyl- β -D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS 21680) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NECA and CGS-21680 were prepared as 10 mM stock solutions using DMSO (Sigma, St. Louis, MO) followed by serial dilutions with 50% DMSO and distilled water and a further dilution to the desired concentration was achieved with distilled water (final DMSO concentration of <1%) [9]. Anti-A_{2A}AR antibody was purchased from EMD Millipore. Alexa 533-conjugated goat anti-mouse secondary antibody was purchased from Invitrogen, and DRAQ5 was purchased from Abcam.

Langendorff-perfused mouse heart preparation

Isolated heart experiments were performed in accordance with our previously published methods [9, 11–13]. In brief, mice were anesthetized with pentobarbital sodium (50 mg/kg ip). A thoracotomy was performed, and hearts were removed into heparinized (5 U/mL), ice-cold Krebs-Hensleit (KH) buffer. Hearts were rapidly perfused retrogradely through the aorta cannulated with a 20-gauge, blunt-ended needle at a constant pressure of 80 mmHg and continuously gassed with 95% O₂-5% CO₂ KH buffer containing 119 mM NaCl, 11 mM glucose, 22 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 2 mM pyruvate, and 0.5 mM EDTA at 37°C in a standard Langendorff fashion and allowed to beat spontaneously. The left atrium was removed, and the left ventricle (LV) was vented with a small polyethylene apical drain. A water-filled balloon made of plastic wrap was inserted into the LV across the mitral valve, which was connected to a fluid-filled pressure transducer by polyethylene tubing for the continuous measurement of LV developed pressure (LVDP). CF was measured via a Transonic flow probe (Transonic Systems, Ithaca, NY) in the aortic perfusion line. Baseline coronary flow, LVDP, and heart rate (HR; derived from the ventricular pressure trace) were monitored for 30–40 min during the equilibration period and recorded on a Power Lab data-acquisition system (AD Instruments, Colorado Springs, CO) [9–13, 18, 24].

Langendorff experimental protocols

After equilibrium, concentration response curves (CRC) (10^{-10} – 10^{-6} M) to non-selective AR agonist NECA and to the A_{2A}AR agonist CGS 21680 were acquired in perfused hearts from wild type mice. These agonists were infused into the coronary perfusate through an injection port directly proximal to the aortic cannula. The infusion rate was controlled to a maximum of 1% of the total CF by a microinjection infusion pump. Each concentration of

either agonist was infused for 5 min or until the flow is plateaued followed by a minimum of 5 min of perfusion for drug washout [12, 13, 18, 24].

In-vivo assessment of echocardiography and coronary blood flow Doppler measurement

Each mouse was anesthetized in an induction chamber with inhalant isoflurane at 3% in 100% oxygen. When fully anesthetized, the mouse was transferred to dorsal recumbency, placed on a heated imaging platform, and maintained at 1–1.25% isoflurane for the duration of the experiment. The hair of the mouse chest wall was carefully removed, and warm electrode gel was applied to the limb leads, allowing for an electrocardiogram and the respiration rate to be recorded during ultrasound imaging. A rectal probe was used to monitor the temperature of the mouse. Ultrasound images were acquired using MS550D transducer (22–55 MHz) on the Vevo2100 Imaging System (Visual Sonics, Toronto, Canada). Placing the transducer to the left of the sternum allowed us to obtain images of the aortic outflow tract, the apex of the heart, and LV along its longest axis (i.e., long-axis B-mode images). Once all long-axis B-mode images were attained, the transducer was rotated 90 degrees to acquire short-axis B-mode images at the mid-papillary muscle level then moved up till left coronary artery (LCA) was visible for the measurement of the size of the vessel. Then transducer was rotated back to long axis parasternal view with the probe lateralized and the ultrasound beam anteriorly tilted. In this image window, the entire LCA, from the aortic sinus to the distal branch site, could be visualized using color Doppler echocardiography. The course of the LCA was typically parallel to the Doppler beam, which also facilitates Doppler measurements without any angle correction. Then the system was switched to pulse-wave Doppler mode with a gate size of 0.65 mm. CF signals were identified on the Doppler spectral display by flow toward the probe peaking in early diastole and then decaying and being minimal during systole as illustrated in figure 3. The flow velocity measurements were made at the same vessel site at baseline and during CGS 21680-induced hyperemia (fig. 4). Measurements were averaged from three cardiac cycles.

Coronary Blood Flow (CBF) was calculated using following formula: $\text{Flow}_{\text{CBF}} (\text{mL/min}) = ((\pi/4) \times D^2 \times \text{VTI} \times \text{HR})/1,000$ where D is the internal coronary diameter (in mm) measured in B-mode ultrasound images, VTI is the velocity–time–integral (in mm), or area under the curve of the doppler blood flow velocity tracing, and HR is heart rate. Coronary Flow Reserve (CFR) = $\text{CBF}_{\text{CGS 21680}}/\text{CBF}_{\text{baseline}}$ where $\text{CBF}_{\text{CGS 21680}}$ is the peak coronary flow measured after 0.5 mg/kg CGS 21680 femoral vein IV injection. The peak CBF was usually reached within 1 ½ mins after injection. After peak CBF was reached, cardiac function and coronary artery size were measured again for the measurement of the CGS 21680 effect [25].

Fluorescence immunostaining for the A_{2A}AR in isolated mouse coronary arteries

Left coronary arteries (70–150 µm) from vehicle and STZ mice were isolated and fixed with 2% ice-cold paraformaldehyde for 30 min and permeabilized for 10 min with 0.1% Triton X-100. The vessels were then blocked with 5% goat serum for 1 h before overnight incubation at 4°C with anti-A_{2A}AR (mouse monoclonal, 1:300 dilution; Upstate) antibodies. The vessels were washed for 1 h with PBS and then incubated for 2 h with PBS buffer containing Alexa 533-conjugated goat anti-mouse secondary antibodies, as well as DRAQ5

(a nuclear stain, 1 μ M; Invitrogen). The vessels were washed again for 1 h with PBS and then mounted on slides for imaging with a confocal microscope (LSM 510, Zeiss). Each stack of images was acquired by optical sectioning at successive *x-y* focal planes with a vertical depth of 1 μ m using a Zeiss objective (EC Plan-Neofluar $\times 40/1.30$, oil differential interference contrast) and a 1,024 $\times$ 1,024 scanning format. The mean fluorescence intensity (FI) of each stack of regions of interest (ROIs) that cover the area of individual endothelial cells (ECs) and smooth muscle cells (SMCs) was quantified using ImageJ. A mean of the FIs averaged from four ROIs of each vessel segment was calculated and is presented in arbitrary units (AU) [11].

Statistical analysis

For CRC, data were analyzed using a nonlinear interactive fitting program (GraphPad Prism, Graph Pad Software Inc., San Diego CA). Data are expressed as mean $\pm$ SEM (n), where n is the number of mice. Statistical analysis of agonist CRC was performed using the *F*-test for $E_{\max}$ and EC_{50} values obtained from best-fit analysis. In addition, data were further analyzed using analysis of variance (ANOVA) followed by the Bonferroni correction to compare between groups at the same concentration, and Dunnett's test to compare within the same group to baseline. Other parameters were analyzed using Student's *t*-test for significance. Values of $p < 0.05$ were considered a statistically significant difference.

Results

Effect of diabetes on metabolic and physiological parameters

Compared to age-matched control mice, diabetic mice exhibited a significant decrease in body weight (26.79 ± 0.37 g in vehicle vs. 21.71 ± 0.46 g in STZ, $p < 0.05$) and heart weight (0.117 ± 0.003 g in vehicle vs. 0.095 ± 0.002 g in STZ, $p < 0.05$), while the ratio of heart to body weight was not significant between the two groups. As expected, STZ injection resulted in a significant increase in blood glucose levels compared to vehicle injection in mice (149 ± 7 mg/dL in vehicle vs. 507 ± 14 mg/dL in STZ, $p < 0.05$). Basal CF was not different between the two groups (1.90 ± 0.06 mL min $^{-1}$ in vehicle vs. 1.92 ± 0.08 mL min $^{-1}$ in STZ, $p > 0.05$). However, when corrected to heart weight, diabetic mice exhibited a significant increase in basal CF when compared to vehicle-injected mice (16.29 mL min $^{-1}$ g $^{-1} \pm 0.46$ vs. 20.46 mL min $^{-1}$ g $^{-1} \pm 0.95$, $p < 0.05$). No significant difference was observed in both groups in contractility: max dp/dt (5140 ± 149 vs. 4874 ± 328 , $p > 0.05$) and min dp/dt (-4107 ± 209 vs. -4401 ± 286 , $p > 0.05$) (Table 1).

Ex-vivo effect of the non-selective AR agonist NECA on CF, heart rate and contractile function

NECA infusion resulted in a concentration-dependent increase in CF in both vehicle and STZ mice. Although EC_{50} values were not significantly different between groups (EC_{50} -8.62 ± 0.18 in vehicle vs. -8.49 ± 0.29 in STZ), the perfused hearts from STZ mice exhibited a significant increase in maximal CF in response to NECA when compared to those from vehicle-treated animals ($E_{\max}$ 39.83 ± 1.83 mL min $^{-1}$ g $^{-1}$ vs. 28.21 ± 0.72 mL min $^{-1}$ g $^{-1}$ respectively, $p < 0.05$, figure 1A). NECA infusion caused a concentration-dependent decrease in HR, which was not significantly different between the two groups

(HR -216 ± 112 bpm in vehicle vs. -198 ± 18 bpm in STZ at $10 \mu\text{M}$ NECA, $p > 0.05$, figure 1B). NECA infusion also caused a concentration-dependent increase in LVDP, which was not significantly different between the two groups (LVDP 143 ± 8 mmHg in vehicle vs. 135 ± 14 mmHg in STZ at $10 \mu\text{M}$ NECA, $p > 0.05$, figure 1C).

Ex-vivo effect of the selective A_{2A}AR agonist CGS 21680 on CF, heart rate and contractile function

CGS 21680 infusion resulted in a concentration-dependent increase in CF in both vehicle and STZ mice. The perfused hearts isolated from STZ mice exhibited a significant increase in maximal CF in response to A_{2A}AR receptor agonists CGS 21680 when compared to those from vehicle-treated animals (E_{max} $41.32 \pm 0.82 \text{ mL min}^{-1}\text{g}^{-1}$ vs. $30.97 \pm 1.26 \text{ mL min}^{-1}\text{g}^{-1}$ respectively, $p < 0.05$, figure 2A), however, EC_{50} values were not significantly different between groups (EC_{50} -8.49 ± 0.26 in vehicle vs. -8.93 ± 0.13 in STZ). Although no significant difference between the two groups was noted (HR 19 ± 9 bpm in vehicle vs. 30 ± 13 bpm in STZ at $10 \mu\text{M}$ CGS 21680, $p > 0.05$, figure 2B), CGS 21680 infusion caused a slight increase in HR, which was significant at the high concentration. LVDP was also slightly increased; however, it was not significantly different between the two groups nor from baseline (LVDP 109 ± 6 mmHg in vehicle vs. 121 ± 11 mmHg in STZ at $10 \mu\text{M}$ CGS 21680, $p > 0.05$, figure 2C).

In-vivo effect of the selective A_{2A}AR receptor agonist CGS 21680 on CF and cardiac function

Coronary flow (expressed in mL min^{-1})—Baseline CF was not significantly different between the two groups ($0.30 \pm 0.08 \text{ mL min}^{-1}$ vs. $0.28 \pm 0.10 \text{ mL min}^{-1}$, $p > 0.05$, figure 3A). Following an injection of CGS 21680 (0.5 mg/kg), CF was increased in both groups; however, the increase in CF was significantly higher in the STZ group when compared with the vehicle group (3 times increase in STZ vs. 2 times increase in vehicle, figure 3B).

Cardiac function—Baseline HR was not significantly different between the two groups (423 ± 22 bpm in vehicle vs. 397 ± 43 bpm in STZ, $p > 0.05$, figure 4A). However, A_{2A}AR receptor agonists, CGS 21680, resulted in a significant increase in HR in vehicle-treated mice (516 ± 72 bpm with CGS 21680 vs. 423 ± 22 bpm at baseline, $p < 0.05$, figure 4A) and STZ (595 ± 31 bpm with CGS 21680 vs. 397 ± 43 bpm at baseline, $p < 0.05$, figure 4A). There was no significant difference in HR between the two groups following CGS 21680 injection (516 ± 72 bpm in vehicle vs. 595 ± 31 bpm in STZ, $P > 0.05$, figure 4A). Stroke volume (SV) was slightly but significantly higher in STZ-treated mice when compared to vehicle ($27.75 \pm 1.24 \mu\text{L}$ in vehicle vs. $33.09 \pm 3.66 \mu\text{L}$ in STZ, $p < 0.05$, figure 4B) but CGS 21680 injection did not affect SV in vehicle-treated mice ($27.75 \pm 1.24 \mu\text{L}$ at baseline vs. $24.09 \pm 7.56 \mu\text{L}$ with CGS 21680, $P > 0.05$, figure 4B). However, CGS 21680 significantly reduced SV in STZ mice to level comparable to that of vehicle ($33.09 \pm 3.66 \mu\text{L}$ at baseline vs. $25.81 \pm 4.28 \mu\text{L}$ with CGS 21680, $p < 0.05$, figure 4B).

Ejection fraction (EF) was significantly higher in STZ-treated mice when compared to vehicle ($50.81 \pm 1.55\%$ in vehicle vs. $59.43 \pm 2.45\%$ in STZ, $p < 0.05$, figure 4C), but CGS 21680 injection did not affect EF in vehicle-treated mice ($50.81 \pm 1.55\%$ at baseline vs.

56.49 ± 10.68% with CGS 21680, $p > 0.05$, figure 4C). However, CGS 21680 significantly increased EF in STZ mice (59.43 ± 2.45% at baseline vs. 73.23 ± 4.60% with CGS 21680, $p > 0.05$, figure 4C). Together, the cardiac function and CF data suggest that hearts from STZ mice are more sensitive to A_{2A}AR activation compared to non-diabetic control mice.

Cardiac output (CO) was not different between the two groups (11.73 ± 0.30 mL/min vs. 13.22 ± 2.45 mL/min, $p > 0.05$, figure 4D). CGS 21680 injection did not affect CO in neither vehicle-treated mice (11.73 ± 0.30 mL/min at baseline vs. 12.53 ± 4.75 mL/min with CGS 21680, $p > 0.05$, figure 4D) nor in STZ mice (13.22 ± 2.45 mL/min at baseline vs. 15.32 ± 2.27 mL/min with CGS 21680, $p > 0.05$, figure 4D).

Immunohistochemistry for A_{2A}AR expression in WT and STZ coronary arteries

To determine whether diabetes induced by STZ resulted in an alteration of A_{2A}AR expression, we performed immunofluorescence staining in the CA. As shown in figure 5, CA isolated from STZ-treated mice exhibited a significant increase of A_{2A}AR expression in both vascular smooth muscle cells (SMC) (~160% vs vehicle) and endothelial cells (~138 % vs vehicle), suggesting a role for A_{2A}AR upregulation in the response observed in response of AR to agonists *in-vivo* and *ex-vivo*.

Discussion

The major finding of this study is that CF response to AR activation, namely A_{2A}AR, is increased in T1D due to increased A_{2A}AR expression in coronary vessels. This effect was observed both *in-vivo* and *ex-vivo* without a major change in cardiac function by STZ-induced diabetes in mice. Given that diabetes is a major risk factor for CAD, and given that adenosine or regadenoson (a selective A_{2A}AR agonist, Lexiscan®) are routinely used for diagnosis of CAD, it is imperative to study the effect of disease states such as diabetes on the AR signaling, especially A_{2A}AR.

As expected, STZ treatment resulted in an increase in blood glucose level accompanied by a significant decrease in body weight, which has been well documented by other investigators [19–22, 26]. As demonstrated by our *ex-vivo* data, diabetes did not affect cardiac function; no significant differences in contractility (+dp/dt and –dp/dt) were observed between vehicle and STZ-treated mice (Table 1). While our *in-vivo* data showed no significant difference in HR or cardiac output between vehicle and STZ-treated mice, there was a significant increase in SV and EF in diabetic mice (Figure 5). Although previous studies using the STZ diabetic model have shown either a decrease or no change in cardiac function in diabetic animals, this discrepancy might be explained by different experimental settings (mice vs. rats, different mouse strains and duration of diabetes) [27–30]. In fact, a recent study measuring cardiac function in diabetic mice every 4 weeks post-STZ injection (up to 32 weeks), indicated that the decrease in left ventricular ejection fraction was observed only at 16 weeks post-STZ injection, while the alteration of contractile function was observed starting 20 weeks post-STZ injection in male diabetic mice [31]. Further studies are needed to clarify these results.

Diabetes is a major risk factor for cardiovascular diseases (CVDs) such as CAD, increasing both morbidity and mortality incidence. T1D is characterized by insulin deficiency and hyperglycemia, which results in decreased production of nitric oxide (NO), increased production of reactive oxygen species (ROS) leading to increased advanced glycosylation end products (AGEs) and inflammation, culminating in endothelial and vascular dysfunction as well as abnormal platelets function [34–36]. STZ-induced diabetes has also been shown to affects AR expression in the heart [19], kidney [21], liver [20], and hippocampus [37]. In our present study, we reveal an increase in A_{2A}AR expression in coronary vascular and endothelial cells in an STZ model of T1D. Although the mechanism of the A_{2A}AR upregulation is unclear, the pattern in this study is similar to that in the heart, kidney, liver, and hippocampus of STZ diabetic animals where A_{2A}AR expression increased [19–21, 36, 37]. This could mean that increased A_{2A}AR expression and signaling during diabetes may act as protective mechanisms to counter the effect of diabetes in different organs [36, 38–40]. In our study, hearts from diabetic mice exhibited a significant increase in flow response to the non-specific AR agonist, NECA (*ex-vivo*) as well as the A_{2A}AR specific agonist CGS 21680 (*ex-vivo and in-vivo*). This increase was accompanied with an increase of both vascular and endothelial A_{2A}AR expression in coronary arteries isolated from diabetic mice when compared to those isolated from non-diabetic mice. Our data in this study also support our previous results where A_{2A}AR expression and signaling increased in a hyperlipidemic/ atherosclerotic mouse model [18]. Therefore, we speculate that increased A_{2A}AR expression and signaling in coronary arteries may compensate for the insulin deficiency. In fact, in addition to its metabolic effect, insulin also causes vasodilation by increasing NO production and increasing L-arginine transport into endothelial cells, which requires A_{2A}AR activation [41]. In T1D, where the production of insulin is decreased, A_{2A}AR upregulation and signaling would compensate by increasing NO-dependent vasodilation, as A_{2A}AR was shown by our lab and others to cause vasodilation partially in an NO-dependent manner [10, 18, 42]. Also, diabetes and hyperglycemia were shown to cause endothelial dysfunction as a result of a decrease in endothelial-dependent vasodilation. In this study, mesenteric arteries isolated from STZ-treated mice exhibited a decrease in acetylcholine-mediated relaxation ($70.19 \pm 2.68\%$ in vehicle *vs.* $51.94 \pm 4.86\%$ in STZ), so the increase in A_{2A}AR expression can be a compensatory mechanism to counter the endothelial dysfunction at least at the early onset of diabetes. Furthermore, an earlier study has shown that aortic SMC from STZ-diabetic mice exhibited enhanced growth, which was susceptible to the growth inhibitory effect of adenosine in an A₂R-guanylyl cyclase-dependent pathway, suggesting that A₂R can regulate abnormal proliferation of SMC within the arterial wall in diabetes [26]. Given that diabetes is associated with increased inflammation; a third explanation is that increased A_{2A}AR expression in diabetes may be protective – at least at an early stage of diabetes-since A_{2A}AR activation was shown to be immunosuppressive inhibiting activation of different immune cells and pro-inflammatory cytokine production (such as TNF- α) while increasing anti-inflammatory cytokine production [43, 44].

Our results showed that basal CF was not different between the two groups (Table 1). In addition, the response to non-specific AR agonist NECA increased in hearts isolated from STZ treated mice (Figure 1). Also, response to A_{2A}AR specific agonist CGS 21680 significantly increased in diabetic mice compared to control both *ex-vivo and in-vivo*

(Figures 2 and 3). NECA- and CGS 21680-mediated increases in CF seem to be mediated by an increase in $A_{2A}AR$ expression in the coronary artery. In contrast to our results, a recent study using db/db mice as model of type II diabetes (T2D) showed a decrease in basal CF and an adenosine-mediated increase in CF in hearts isolated from db/db mice when compared to non-diabetic mice [45]. This difference could be explained by the distinct etiology of the two diseases. In fact, T2D is characterized by a pre-diabetes phase with an early metabolic imbalance (increased insulin resistance and slight increase in fasting glucose levels), which is associated with systemic innate inflammation in the vasculature, particularly the coronary arteries [46]. In contrast, T1D-associated microvascular and cardiac complications occur a while after the onset of diabetes with a strong correlation between the CVD and the duration of T1D [46]. Further studies using mice from an early to a late stage of T1D (i.e. 4 to 32 weeks post STZ injection) in relation to $A_{2A}AR$ expression and signaling in the CF are necessary to understand the role of adenosine mediated CF in T1D. Also, while $A_{2A}AR$ activation was shown to be beneficial, studies suggested that $A_{2A}AR$ activation can also be detrimental in chronic disease states promoting fibrosis [43]. Again, further studies looking at adenosine signaling in early vs. late stages of T1D are important.

In addition to changes in CF, NECA induced a concentration dependent decrease in HR; however, no difference was observed in the NECA response between the two groups (vehicle vs. STZ) (Figure 1). Our results are in accordance with previous studies that showed NECA exerting a negative chronotropic response in isolated hearts [12, 18, 47]. The negative chronotropic effect of NECA is mainly attributed to the A_1R activation resulting in the blockade of the atrioventricular nodal conduction [18, 48]. A study using hearts isolated from A_1R knockout mice showed no effect of NECA on HR, further suggesting the role of A_1R in the chronotropic effect of NECA [12]. High dose CGS 21680 increased HR in both groups (Figure 2), with no difference between the groups, suggesting a minimal role of $A_{2A}AR$ in the regulation of HR (compared to A_1). *In vivo*, similar to our findings, CGS 21680 was shown by others to result in tachycardia [49]. The increase in heart rate following CGS 21680 administration is probably the result of the reflex response to hypotension. While these data suggest augmented chronotropic and EF responses to CGS21680 in STZ animals, we cannot ascertain here whether this reflects a direct cardiac change and/or an altered vascular response to $A_{2A}AR$ agonism in STZ animals. Although there was no difference in the contractility response to AR agonists between the two groups (STZ vs. vehicle, Figures 1 and 2), NECA caused an increase in contractility in perfused hearts, corroborating the effect of NECA in accordance with previous studies. On the other hand, CGS 21680 tended to increase the contractility in both groups; however, it was not significant. Our data suggests that NECA-mediated increases in contractility may be mediated through A_1 activation and may reflect a negative staircase phenomenon in rodent hearts due to A_1 mediated bradycardia. The role of A_1AR 's agonist-mediated increase in contractility effects has also been previously reported [50]. Given that our lab and others have shown a cross talk between $A_{2A}AR$ and A_1AR signaling in the heart, we looked at the effect of A_1AR -mediated changes in CF, HR, and LVDP using 2-Chloro-N-cyclopentyladenosine (CCPA; A_1R specific agonist). No significant difference between the two groups was observed in CF, HR or LVDP (data not shown). Taken together, our data

suggest a role for A_{2A}AR expression and signaling in increased CF in an STZ model of T1D.

An important outcome of our study is its clinical implication; adenosine (nonspecific AR agonist) and regadenoson (A_{2A}AR selective agonist Lexiscan®) are routinely used in clinics for the detection of CAD, with the latter being more favorable due to its minimal systemic and side effects [17, 52]. However, our study in addition to others suggest that adenosine [53] and regadenoson [18] may not be appropriate for the detection of coronary vascular diseases and estimation of coronary reserve in an early stage of the disease in which the change in AR expression and/or signaling may mask an underlying disease state [18, 53]. Future studies to understand the functional changes involved in the early stages are necessary for an early detection and treatment of the CAD.

Limitations

It should be pointed out that there are some concerns regarding the translational value of our findings due to the limitation of our current experimental model. First, as noted in discussion, our model showed changes in EF, observed *in vivo*, which was opposite to what was conventionally observed in prior studies of T1D and may complicate the explanation of the current findings. In addition, while the STZ model is a valuable model for T1D, we also acknowledge that the nature of T1D induction, duration of the study, and the lack of insulin replacement treatment may complicate the translational value of the study regarding human T1D.

Acknowledgments

This study was supported by NIH grants of HL027339, HL094447 and U54GM104942. Small animal imaging and image analysis were performed in the West Virginia University Animal Models & Imaging Facility, which has been supported by the Mary Babb Randolph Cancer Center and NIH grants P20 RR016440, P30 GM103488 and S10 RR026378.

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Highlights

- Type 1 diabetic animals showed enhanced coronary flow response to A_{2A}AR agonist.
- Type 1 diabetes resulted in an increased A_{2A}AR expression in coronary arteries.
- Adenosine and A_{2A}AR agonist may not be appropriate for estimation of CAD.

Figure 1.

Concentration–response curve for coronary flow (A), change in heart rate (B), and LVDP (C) for the non-specific AR agonist NECA in isolated perfused hearts from vehicle and STZ-injected mice. Results are expressed as mean $\pm$ standard error of the mean (SEM) ($n = 7$). * $p < 0.05$ vs. vehicle group, ^a $p < 0.05$ vs. vehicle's baseline and ^b $p < 0.05$ vs. STZ's baseline.

Figure 2.

Concentration–response curve for coronary flow (A), change in heart rate (B), and LVDP (C) for A_{2A}R specific agonist CGS 21680 in isolated perfused hearts from vehicle and STZ-injected mice. Results are expressed as mean ± standard error of the mean (SEM) (n = 6–7). **p* < 0.05 vs. vehicle group, ^a *p* < 0.05 vs. vehicle's baseline and ^b *p* < 0.05 vs. STZ's baseline.

Figure 3.

In vivo assessment of basal coronary flow and coronary reserve in vehicle and STZ-injected mice with representative tracing for the data represented in bar figures (A) basal CF and (B) fold increase in CF to a single dose of CGS 21680. Results are expressed as mean $\pm$ standard error of the mean (SEM) (n =4). * $p < 0.05$ vs. vehicle group.

Figure 4.

In vivo assessment of cardiac function in vehicle and STZ-injected mice (A) HR pre and post CGS 21680 injection, (B) SV pre and post-CGS 21680 injection, (C) EF pre and post-CGS 21680 injection and (D) CO pre and post-CGS 21680 injection. Results are expressed as mean ± standard error of the mean (SEM) (n = 4). * $p < 0.05$ vs. baseline and # $p < 0.05$ vs. vehicle.

Figure 5.

A_{2A} R expression is increased in coronary arteries isolated from STZ diabetic mice. *Top panel* is a representative confocal immunofluorescence image showing expression of A_{2A}R (red) in isolated coronary arteries of vehicle and STZ injected mice. *Lower panel*: fluorescence intensity of A_{2A}R in coronary smooth muscle (A) and endothelial cells (B) ($n = 5$) of isolated coronary arteries. * $p < 0.05$ vs. vehicle group.

Table 1

Changes in physiological and metabolic parameters: Diabetic mice exhibit an increased blood glucose levels accompanied by a significant decrease in heart and body weight. Diabetes did not affect basal coronary flow or the contractility of the heart. Results are expressed as mean $\pm$ standard error of the mean (SEM) (n = 16–18).

	Vehicle	STZ
Age (Weeks)	15.51 $\pm$ 0.32	15.77 $\pm$ 0.26
Body weight (g)	26.79 $\pm$ 0.37	21.71 $\pm$ 0.46 *
Heart weight (g)	0.117 $\pm$ 0.003	0.095 $\pm$ 0.002 *
Blood Glucose (mg/dL)	149 $\pm$ 7	507 $\pm$ 14 *
Basal coronary Flow (ml min ⁻¹)	1.90 $\pm$ 0.06	1.92 $\pm$ 0.08
Positive dP/dt (mmHg/s)	5140 $\pm$ 149	4874 $\pm$ 328
Negative dP/dt (mmHg/s)	4107 $\pm$ 209	4401 $\pm$ 286

*
p < 0.05 vs. vehicle group.