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Role and Contribution of A2 Adenosine Receptors in the Regulation of Coronary Flow

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Role and Contribution of A$_2$ Adenosine Receptors in the Regulation of Coronary Flow

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Dissertation submitted to the School of Pharmacy at West Virginia University in partial fulfillment of the requirements for the degree of

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in

Pharmaceutical and Pharmacological Sciences

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ABSTRACT

Role of A_2 Adenosine Receptors in the Regulation of Coronary Flow

Maryam Sharifi Sanjani

One out of three individuals suffer from one form of cardiovascular disease, out of which, about 6,000,000 individuals suffer from coronary heart disease. Adenosine has long been known to play a role in coronary flow (CF) regulation; however, the individual role of A_{2A} and A_{2B} adenosine receptors (AR) and their contribution is yet to be fully elucidated. The purpose of this study was to characterize the pharmacology of both A_2 ARs in coronary arteries (CAs), in addition, to identifying their role in reactive hyperemia, and their signaling mechanisms. We hypothesized that A_2 ARs mediate an increase in CF, in addition, to being involved in metabolic control of CF. Therefore, in this study, we investigated CF changes due to exogenous activation of A_{2A} and A_{2B} ARs, in addition, to their endogenous activation during coronary reactive hyperemia, when metabolic factors, such as adenosine, play a pivotal role. We used the well established Langendorff isolated heart system as well as selective and non-selective AR agonists and antagonists, and A_{2A} and A_{2B} AR single and double knockout mice. We found that activation of both A_{2A} and A_{2B} ARs induces an increase in CF, albeit with different pharmacological profile. Further, in A_{2B}KO mice, A_{2A}ARs are up-regulated in order to compensate for deletion of A_{2B}ARs. These findings may suggest that both A_2ARs are capable of increasing the CF in conditions when a sufficient level of adenosine is available. However, we also found that only A_{2A}ARs contribute to CF changes in coronary reactive hyperemia, which may suggest that A_{2B}ARs may be activated in more severe conditions such as longer ischemic conditions, where adenosine levels are significantly augmented. We next investigated signaling pathways involved

in A_2AR-mediated effects. We found that non-mitochondrial K_{ATP} channels are a major end effectors in A_2AR-induced increase in CF. What is more interesting is that we illustrated that H_2O_2 mediates adenosine's effect on CF and that is coupled to adenosine-mediated effect on K_{ATP} channels. From these data, it can be concluded that A_{2A} and A_{2B} ARs may regulate CF in different conditions, albeit, maybe through the same signaling pathway.

To my grandmother, Sonia, Sister, Mana, Husband, Ali, and my father and mother,

Nasser and Shahnaz

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strength and driving force; I could not have reached here without their love, support and constant encouragement.

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LIST OF ABBREVIATIONS

μg	micro gram
μl	micro liter
A_1AR	A_1 adenosine receptor
$A_{2A}AR$	A_{2A} adenosine receptor
$A_{2B}AR$	A_{2B} adenosine receptor
A_3AR	A_3 adenosine receptor
AC	Adenylyl cyclase
ADA	Adenosine deaminase
AD	Adenosine
ADP	Adenosine diphosphate
ADR	Adrenoceptors
AK	Adenosine kinase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAY	BAY60-6583 compound
BKCa	Ca^{2+}-activated K channels
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CGS 21860	2-*p*-(2-carboxyethyl)phenethylamino-5' N-ethylcarboxy amidoadenosine hydrochloride

CF	Coronary flow
CA	Coronary artery
Cat	Catalase
CRC	Concentration-response curve
DAG	Diacylglycerol
DKO	Double knockout
DMSO	Dimethyl sulfoxide
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
EDHF	Endothelium-derived hyperpolarizing Factor
EETs	Epoxyeicosatrienoic acids
ERK1/2	Extracellular signal-regulated kinase 1/2
GB	Glibenclamide
GPCR	G protein coupled receptor
5-HD	5-Hydroxydecanoate
H_2O_2	Hydrogen peroxide
HR	Heart rate
IP_3	Inositol trisphosphate
K_{ATP}	ATP-dependent K^+ channels
kg	Kilogram
Kir	Inward rectifier K^+ channels
KO	Knock out
K_V	Voltage-dependent K^+ channels
LVDP	Left ventricle developed pressure
MAPK	mitogen-activated protein kinase

mg	milligram
ml	milliliter
mm	millimeter
mM	millimole
NADPH Ox	NADPH oxidase
NECA	N-ethylcarboxamide-adenosine
NO	Nitric oxide
PCR	Polymerase chain reaction
Pin	Pinacidil
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PO_2	myocardial oxygen tension
RH	Reactive hyperemia
ROS	Reactive oxygen species
SAH	S-Adenosyl-L-homocysteine
SAH-hydrolase	S-Adenosyl-L-homocysteine-hydrolase
SAM	S-Adenosyl-L methionine
SCH58261	2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine-5-amine
SEM	Standard error of the mean
Xanthine Ox	Xanthine oxidase

CHAPTER ONE

INTRODUCTION

Adenosine, produced by all cells, is an endogenous purine nucleoside with various physiological effects induced through its four subtypes of receptors A_1, A_{2A}, A_{2B}, and A_3. Figure 1.1 illustrates the chemical structure of adenosine. Adenosine induces its effects All adenosine receptors (AR) are G protein coupled receptors (GPCR) and have their own pharmacological profile and affinity for adenosine, tissue distribution, and effector coupling. Therefore, effects mediated through adenosine depend on the AR subtype activated.

Generation of adenosine and its metabolism

In physiological conditions, adenosine is produced from intracellular conversion of S-adenosyl-L methionine (SAM) to S-adenosyl-L-homocysteine (SAH) which then is converted to adenosine and homocysteine by SAH-hydrolase (30, 48) (Figure 1.2). Cytosolic adenosine can become phosphorylated via adenosine kinase to AMP in addition to getting converted to inosine by adenosine deaminse (ADA), especially at high levels of adenosine. Intracellular adenosine is transported down the concentration gradient into the extracellular space via equillibrative membrane transporters. Adenosine can also be produced extracellular through successive dephosphorylation of ATP to ADP and then ADP to AMP, which then goes under hydrolysis via membrane-bound ecto-5′-nucleotidases CD39 and CD73 in order to form adenosine. By the action of ADA, extracellularly produced adenosine can also be further converted to inosine which is finally broken down to uric acid that is excreted in urine. However, adenosine can also get re-converted to AMP via the enzyme adenosine kinase (AK). Nevertheless, extracellularly produced adenosine can also be transported into the cell via concentration- and/or sodium-dependent transporters (Figure 1.2). In addition to being metabolized,

adenosine acts on its specific receptors to induce various physiological effects (43, 48, 82).

Figure 1.1 Adenosine structure
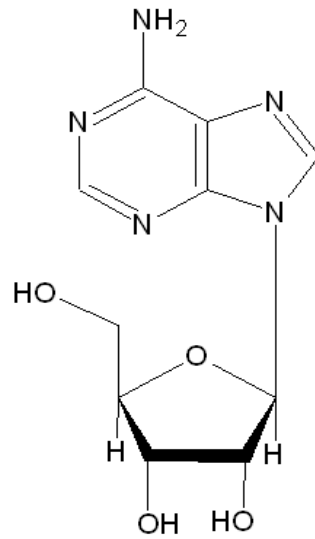
Figure 1.2 Production of adenosine under physiological conditions. Scheme adapted from Koos et al. (82)
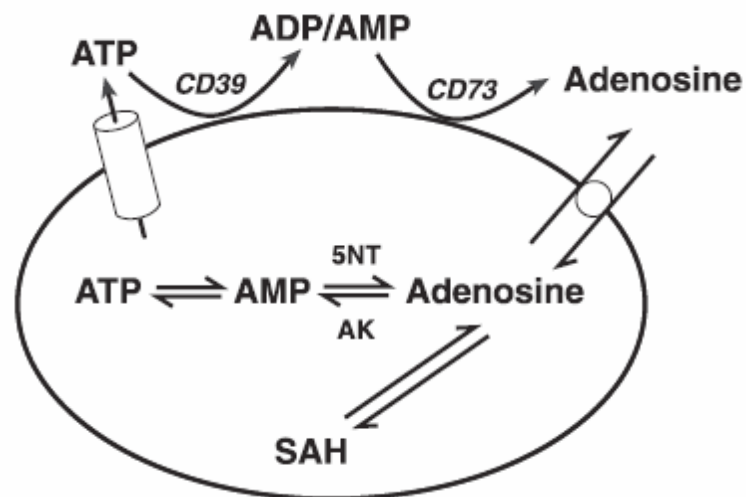
Coronary flow regulation

Coronary arteries (CAs) play a pivotal role in determining the oxygen and nutrient supply of the myocardium by regulating the coronary flow (CF). Myocardial perfusion is regulated by several factors released from endothelial cells, smooth muscle cells, and cardiomyocytes that affect the coronary vascular tone. It is well known that physical forces induce coronary responses known as myogenic and flow-induced (shear stress) vascular control that determine the vascular basal tone and CF (Figure 1.3). Also, metabolic factors such as adenosine have been shown to mediate CA responses during metabolic stimulation (active hyperemia), autoregulation, and/or reactive hyperemia (Figure 1.3). Moreover, sympathoneural factors such as the effect of β adrenoceptors (ADR) are reported to affect the coronary tone through endothelium-dependent and − independent manners (Figure 1.3).

Pressure-induced myogenic constriction importantly contributes to autoregulation of local blood flow, which also acts as a protective measure from sudden increases in arterial pressure (88). Given that this constriction allows resistance arteries, such as coronary arterioles, to constrict or dilate in response to vasoactive mediators and changes in intraluminal pressure, it counts as an essential component of basal microvascular tone where vascular resistance is tightly regulated (19). The myogenic response occurs independently of the endothelium and perivascular nerves and is, therefore, an inherent property of vascular smooth muscle cells (85, 98). Smooth muscle cells have stretch-activated cation channels that, when activated, induce an inward current leading to cell membrane depolarization and constriction (22-23, 160). Previous studies have shown that increased intraluminal pressure leads to depolarization of smooth muscle cells and the opening of Ca^{2+} channels, such as Ca^{2+}-activated K channels (BKCa), responsible for

increased intracellular Ca^{2+} leading to the contraction and the increase in myogenic tone (53-54, 108). Several endogenous mediators, such as cytochrome P450 metabolites, have been suggested to be involved in myogenic tone control via activation of BKCa channels (158). The CA myogenic tone is also proposed to be regulated by several molecular signaling pathways, such as, PKC pathway (29, 83, 99) and MAPK/ERK1/2 pathway (76-78). Moreover, the myogenic constriction in CA is suggested to antagonize EDHF-mediated dilation; it was reported that activation of BKCa channels reduces myogenic constriction and profoundly increases EDHF-mediated dilation in CA (54).

An increase in wall shear stress results in the release of various factors from the endothelium that subsequently act on smooth muscle cells in order to induce vasodilation (86, 122, 150, 165). This illustrates the release of endothelium-derived factors that may limit myogenic constriction. Endothelium-derived nitric oxide (NO), suggested as endothelium-derived relaxing factor (EDRF), was proposed to regulate the coronary reactive hyperemia responses (80, 150, 165). However, other non-NO and non-prostanoid endothelium-derived substances, referred to as endothelium-derived hyperpolarizing factors (EDHFs), were found to play an important role in the regulation of CA vascular tone. Epoxyeicosatrienoic acids (EETs) (46, 128), H_2O_2 (96, 101, 163), and K^+ (6) are a few suggested EDHFs in coronary arteries to highlight. Liu et al. showed that endothelial-derived H_2O_2 was critical in human CA dilation to shear stress (90, 101). The same group also suggested that mitochondria are the source of the H_2O_2 generation that plays a role in flow-induced human CA dilation (90). Miura et al. reported that shear stress induces endothelium-dependent vasodilation through CYP450 metabolites leading to hyperpolarization of smooth muscle cells through BKCa channels in human coronary arterioles (103). It should be highlighted that human CAs in these studies are dissected

from patients with cardiovascular disease, which may lead to different physiological responses than true controls, the tissues from healthy individuals. Nevertheless, previous studies have shown that endothelial production of NO can be triggered by mechanical shear forces or CF-induced pulsatile strain (154-155). It was demonstrated that NO contributes to the coronary responses in reactive hyperemia (167)and that adenosine is involved in CA flow-induced dilation and enhanced coronary response to pulsatile perfusion(87, 117, 169), which suggests the existence of a cooperative interaction between adenosine and the local flow regulation mechanisms.

It is well established that CF is regulated by the metabolic state of the heart (44) (Figure 1.3). Berne's adenosine hypothesis suggests that increases in myocardial oxygen consumption decreases myocardial oxygen tension (pO_2) which leads to the release of adenosine from cardiomyocytes (8, 43, 159). Furthermore, a role for H_2O_2 in metabolic CA vasodilation has been established (72, 116, 164). Chilian et al. suggested that H_2O_2 production increases in proportion to cardiac metabolism in order to couple the coronary blood flow to myocardial oxygen tension (131). On the other hand, increasing evidence has shown the involvement of K_{ATP} channels in metabolic CF adjustment through different approaches such as pacing-induced tachycardia (73), pharmacological metabolic stimulation (110), and exercise (36). Further, coronary arteriolar smooth muscle K_{ATP} channels (42, 124-125) and BKCa channels were reported to contribute to the basal CF regulation (53). There is evidence that during exercise, coronary NO production is increased in the heart (136), likely due to an increase in endothelial shear stress secondary to the increased CF rate (9, 149). Nevertheless, Chilian et al. demonstrated that CA vasoconstriction induced by inhibition of NO was counterbalanced by coronary vasodilation, indicating that many compensatory vasomotor adjustments occur in order to

maintain the CF (71). And finally, the knowledge that prostaglandins may also act as local chemical mediators to control coronary perfusion to adapt to the metabolic demands of the heart should be considered (135).

Figure 1.3 Several reported pathways for metabolic vasodilation of coronary arteries. Scheme adapted from Koos et al. (82).
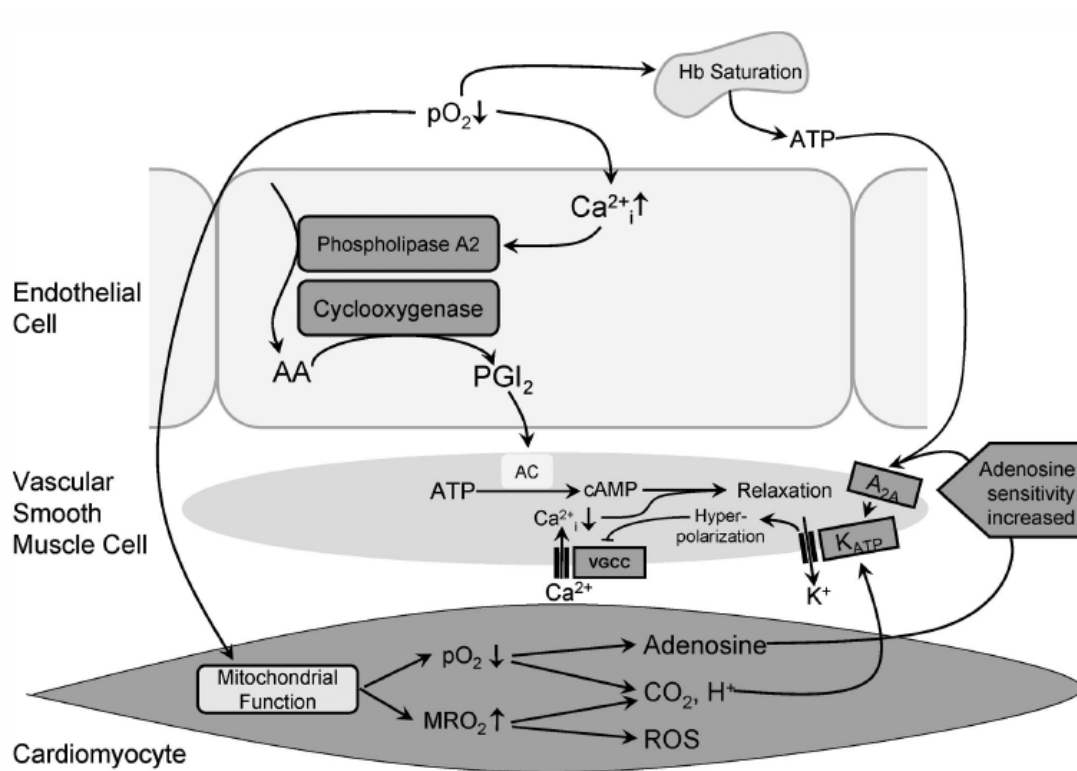
It has been known that neurotransmitters released from nerve endings are known to affect the coronary vascular tone. Sympathetic stimulation-mediated coronary vasoconstriction is capable of competing with local metabolic control to lower coronary venous oxygen tension (44). The autonomic effects on CF regulation have been studied at rest and during exercise through different approaches such as after surgical or chemical denervation of the heart or in the presence of selective autonomic receptor antagonists. It was reported that cardiac neural ablation did not impair the ability to maintain the exercise level, although the initial hemodynamic adjustment to exercise was delayed (52). It is established that blockade of α_2-adrenoceptors (ADR) can affect the CF through three mechanisms: a) blockade of prejunctional αADR which interrupts the negative-feedback control of norepinephrine release leading to an increase in norepinephrine levels and induction of cardiac ßADR stimulation and an increase in CF (60, 62); b) αADR blockade can increase CF by interrupting vasoconstriction mediated by postjunctional α_1 and α_2ADRs located on CA smooth muscle cells; and c) α_2ADR on CA endothelial cells can stimulate release of nitric oxide (NO), which can oppose the αADR-mediated vasoconstriction (20, 65). On the other hand, it was reported that norepinephrine brings about human coronary vasodilation through $ß_2$ADR (139) that involves NO and K_{ATP} channels (100). Previous studies suggested that $ß_2$ADR-mediated CF regulation is minimal under resting conditions in dogs (5, 61) while, βADRactivation was shown to contribute to coronary vasodilation during exercise in a feed-forward manner (104-105, 153). $β_2$ADR activation during exercise causes a significant degree of CA dilatation independent of myocardial effects of $β_1$ADR stimulation (31, 94). Overall, the consensus is that βADR blockade causes a greater reduction in CF compared to myocardial oxygen

consumption, resulting in increased oxygen extraction by the heart which demonstrates a direct feed-forward for βADR CA vasodilatory effect.

In this thesis, based on previous studies on adenosine and the existence of broad factors involved in the regulation of CF, we elaborate on the role of adenosine, K_{ATP} channels, and H_2O_2 in addition to a brief look at β adrenoceptors, as a sympathoneural factor, in light of adenosine as the main focus.

Figure 1.4 Previously reported major signal transduction pathways in the regulation of coronary flow. Adapted from Komaru et al. (81)
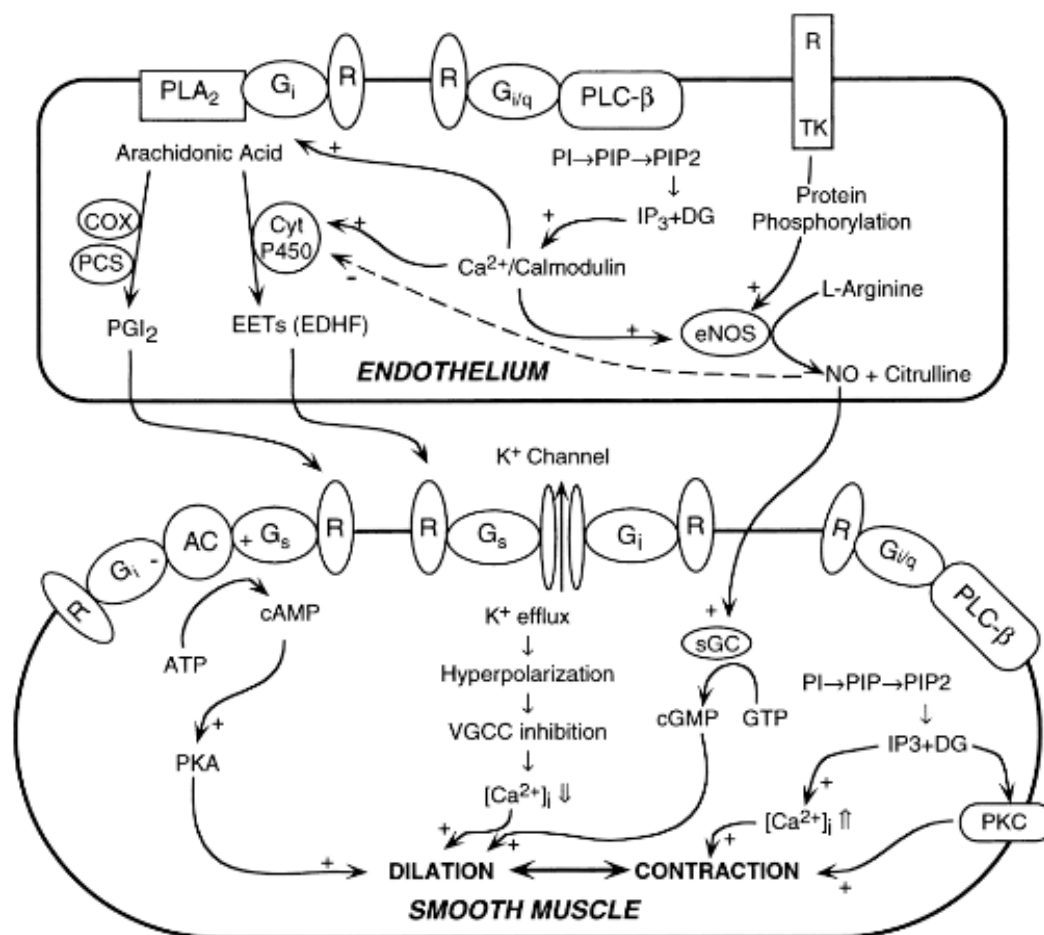
Role of adenosine in coronary flow regulation

The heart has a small anaerobic metabolic capacity and, therefore, is essentially dependent on CF in order to provide the heart with oxygen on a beat to beat basis. Some also say that heart function is comprised of continuous sequential episodes of transient increase in CF, called reactive hyperemia (RH), with the decreased flow occurring during the systole and the RH response occurring during the diastole. Additionally, during conditions such as coronary vasospasm or even exercise the myocardium is also responsive to acute periods of decreased CF by means of RH. Nevertheless, as mentioned, the increase in CF during more aggressive conditions such as exercise or angina plays a crucial role in providing the heart with necessary oxygen. However, it is interesting to note that the myocardium is reported to only extract about 75% of the delivered oxygen at rest and that the seemingly high level of repayment during episodes of RH only provides oxygen repayment ratios of between 26-82% (129), which suffice to say that myocardium has a small oxygen extraction reserve and that the contribution of any vasodilatory factor plays a crucial role for the regulation of CF and the efficient oxygen supply and, hence, cardiac function. Needless to say that although the myocardial oxygen supply (in the form of blood flow or oxygen extraction) is small, the myocardial oxygen consumption can increase up to five fold when needed (43).

In 1963, Berne et al. proposed the adenosine hypothesis (7). The idea was that the myocardial oxygen supply over oxygen consumption ratio is reflected in the intracellular myocardial oxygen tension (pO_2). For example, if myocardial oxygen supply over consumption ratio decreases (as during exercise), the myocardium encounters a fall in pO_2 which leads to the breakdown of adenine nucleotides (ATP, ADP, AMP) and, hence, generation of adenosine. The produced adenosine then diffuses out of the cell to act on its

receptors on coronary arteriolar smooth muscle cells (through an endothelium-dependent and –independent manner) in order to cause vasodilation. This increase in CF delivers more oxygen to the myocardium and, thus, returns myocardial pO_2 back toward the normal range (Figure 1.5). The adenosine hypothesis is also a transmitter hypothesis which suggests that adenosine transmits information regarding the status of one cell type such as cardiomyocyte to another cell type such as arteriolar smooth muscle by diffusing across the interstitial space (43, 48). Therefore, Berne's adenosine hypothesis suggests that adenosine coordinates the myocardial oxygen consumption to CF (Figure 1.5). The role of each AR subtype in the regulation of CF has been extensively studied.

Adenosine receptors classification and characteristics

Adenosine receptors were initially classified into A_1 and A_2 subtypes based on their potency for ligands and antagonism by methylxanthines (91). Subsequently subclassification of A_2 into A_{2A} and A_{2B} subtypes was pharmacologically based upon the high affinity A_{2A} and low affinity A_{2B} binding sites in rat brain (24). All ARs are seven transmembrane GPCR with approximately 21–28 amino acids in each transmembrane region. The $A_{2A}AR$ is 409 and 410 amino acids long in human and mouse, respectively. $A_{2A}AR$ is the largest of all ARs due to its extended C-terminus of no known function (121). Overall, there is relatively low level of amino acid homology between different ARs in a single species in addition to the same AR between species which may contribute to the different ARs-mediated effects observed in different species.

The N-terminus of ARs are extracellular and it was shown that all ARs other than $A_{2A}AR$ have a palmitoylation site near the C-terminus (1). Furthermore, all ARs are glycosylated on their 2nd extracellular loop (121) and although the 3rd intracellular loop is involved in both A_1 and $A_{2A}AR$ G protein coupling, the C-terminus is only involved in

A_1AR G protein coupling (151). It was also reported that the $A_{2A}AR$ phosphorylation of intracellular loop is involved in this AR desensitization (118). Currently, all four ARs are cloned and their expressions in various tissues have been extensively studied.

The $A_{2A}AR$ gene has been localized to chromosome 22q11.23 (27, 92) and chromosome 10 in human and mouse, respectively (162). However, the $A_{2B}AR$ gene has been localized to chromosome 17p12-11.2 (69) chromosome 11 in human and mouse, respectively (162). $A_{2A}AR$ was the first AR genetically modified in a murine model (89). The $A_{2A}AR$ knockout (KO) mice were developed in three different genetic backgrounds of CD1, congenic C57B L/6, and pure 129-Steel (17-18, 89) while $A_{2B}AR$ has been recently developed only in C57BL/6 background. $A_{2A}KO$ animals demonstrated several central nervous system disturbances such as decreased exploratory activity, increased aggressiveness and hypoalgesia (89). Furthermore, $A_{2A}KO$ mice with CD1 genetic background were demonstrated to have increased blood pressure, heart rate, and platelet aggregation (89) while an $A_{2A}KO$ mice on either a mixed 129Steel-C57BL/6 or congenic C57BL/6 genetic background did not show any change in blood pressure(17, 26). While there are relatively a large number of studies on $A_{2A}KO$, there are not many studies using $A_{2B}KO$ mice and, therefore, a better understanding of its functional effects or compensatory mechanisms due to its genetic modification still remains to be fully elucidated.

A_2ARs: The presence of both A_{2A} and $A_{2B}ARs$ on coronary endothelial and smooth muscle cells and the pivotal vasoregulatory role of adenosine in human CA is well established (37-38, 113, 130, 133). The involvement of $A_{2A}AR$ in the regulation of CF in various other species has been also broadly reported (11, 37-38, 74, 106, 130, 133, 137, 142, 145, 167). Previous studies further suggest that metabolic vasodilatory factors

such as adenosine are the primary mediators responsible for the CF regulation during RH (21, 63, 114-115). *In vivo* studies in dogs showed a role for $A_{2A}AR$ in CF regulation during RH (32), which further confirms the physiological role of adenosine. Zatta et al. also confirmed this finding by using pharmacological approaches (11, 167). Furthermore, it is proposed that $A_{2A}AR$ contributes to the coronary circulation basal tone since deletion of $A_{2A}AR$ and application of SCH58261 (A_{2A} selective antagonist) diminished the baseline CF compared to WT hearts (137, 145).

Due to its lower affinity for adenosine, $A_{2B}AR$ is activated in conditions where a significant increase in adenosine levels is observed, such as in ischemia (48). Also, a previous study showed that $A_{2B}AR$ may not play a role in basal tone regulation of CF since targeted deletion of $A_{2B}AR$ had no effect on the baseline CF (137). Additionally, Tune et al. suggested no role for $A_{2B}AR$ in RH by means of indirect measures such as using non-selective antagonist (11). Nevertheless, $A_{2B}AR$ is shown to be involved in the regulation of CF, albeit may be with a lesser contribution compared to $A_{2A}AR$ (13, 106, 137, 142). Furthermore, recent studies suggested an interrelationship between A_{2A} and $A_{2B}ARs$ subtypes since one compensates for the down-regulation/deletion of the other (137, 145) through their up-regulation in coronary arterioles. In fact the involvement of A_{2A} and $A_{2B}ARs$ and their down- or up-regulation is reported in many pathophysiological conditions (2, 4, 41, 75). Regardless of this relationship, the differential expression of ARs is shown to contribute to functional heterogeneity of human endothelial cells (45) and the impairment of adenosine-related signaling contributes to the pathophysiology of congestive heart failure (2). By benefiting from gene-modified models and pharmacological approaches and by using selective adenosine analogs, we were allowed

to better assess the role of A_2ARs in the regulation of CF and, therefore, to better understand the heterogeneity of CF in relation to A_2ARs responses.

A_1AR: There are previous reports from our laboratory suggesting that A_1AR negatively modulates the role of $A_{2A}ARs$ and probably $A_{2B}ARs$ in CF regulation (141, 144). The presence of only $A_{2A}ARs$ and $A_{2B}ARs$ on coronary endothelial cells may also indirectly support this finding (113). Additionally, it was recently reported that in Ossabaw swine A_1AR antagonism by DPCPX positively regulated basal CF. However, although A_1AR may not play a major role in CF regulation, it has been shown that this receptor mediates negative chronotropic effects which may indirectly affect the CF in conditions where adenosine concentration is elevated enough to induce the chronotropic effect of this AR subtype. Nevertheless, A_1ARs are known to protect the heart from ischemia-reperfusion (107, 161, 168) and coronary atherosclerosis (preliminary data from our laboratory (146)).

A_3AR: Zhao et al. showed that high concentrations of A_3AR agonist, Cl-IBMECA, induced coronary vasodilation which was further blocked by $A_{2A}AR$ antagonism (171). However, A_3AR targeted deletion increased the $A_{2A}AR$-mediated response. This finding may suggest that A_3AR also participate in CF regulation of isolated murine hearts via negative modulation of $A_{2A}AR$-mediated coronary vasodilation (141). Additionally, single-nucleotide polymorphism (SNP) of I248L in A_3AR gene is shown to be associated with coronary heart disease (120). Further studies are required to better understand the role of A_3AR in CF regulation and responses.

Figure 1.5 Berne's adenosine hypothesis. Scheme adapted from Feigl et al. (43)
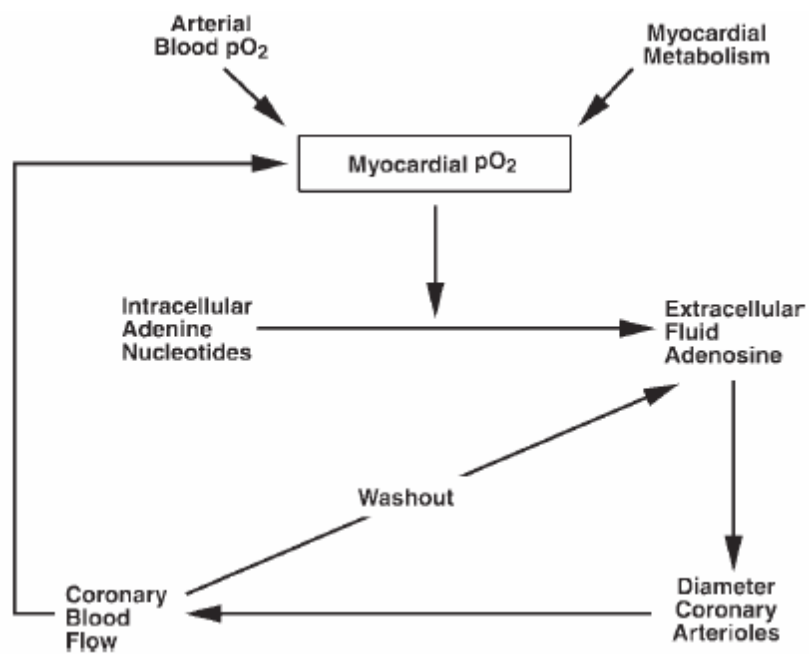
Classically known signaling of adenosine receptors

As mentioned, each AR has its own pharmacological profile. Adenosine at its basal concentration is sufficient to activate A_1, A_{2A}, and A_3 ARs. However, due to its low affinity for adenosine, A_{2B}ARs are activated in conditions where significant high levels of adenosine are produced, such as in pathophysiological conditions. It is well established that A_1 and A_3ARs are associated with G_i proteins while A_{2A} and A_{2B} ARs are coupled to G_s proteins. The A_1 and A_3ARs signal through $G_{i/o}$ proteins and its activation leads to an inhibition of adenylyl cyclase (AC) which causes a lowering of cyclic AMP (cAMP) levels. The A_{2A} and A_{2B}ARs signal through the Gs pathway and its activation leads to stimulation of AC resulting in increased production of cAMP. Classically, ARs were thought to induce their effects through inhibition (G_i) or stimulation (G_s) of adenylyl cyclase. However, it is becoming more and more evident that these receptors also activate other pathways such as phospholipase C (PLC), mitogen-activated protein kinase (MAPK), etc. In fact, signaling through the A_1AR can also lead to activation of IP_3/DAG through the PLC pathway (68, 143). Additionally, the A_{2B} receptor signals through $G_{s/q}$ and its activation can either result in increased cAMP or IP_3/DAG and Ca^{2+} levels. Furthermore, ARs are now shown to be associated with the K^+ channels, production of reactive oxygen species (ROS) and activation of the arachidonic pathway (34, 50, 59, 111, 137).

Role of K^+ channels in A_{2A}AR-mediated coronary flow regulation

K^+ channels play an essential role in determining the cell membrane potential and regulating the contractile tone of arterial smooth muscle cells through controlling the Ca^{2+} entry via voltage-dependent Ca^{2+} channels and, thereby, the contractility (67). There are four types of K^+ channels on CA smooth muscle and endothelial cells: voltage-

dependent K^+ (K_V), Ca^{2+}dependent K^+ (BKCa), ATP-dependent K^+ (K_{ATP}), and inward rectifier K^+ (Kir) channels. K_{ATP} channels are shown to be present on isolated coronary artery cells (51, 140), in addition to K_V channels which are known to be expressed on coronary arteries (33, 66, 140, 156, 166). Additionally, BKCa channels are known to be abundantly expressed on coronary smooth muscle cells and contribute to CA vasodilation in different species (15, 64, 102), in addition, to Kir channels which are reported to be present on both coronary smooth muscle and endothelial cells (119, 126, 140, 157, 166).

It is worth mentioning that K^+ was suggested to be the EDHF in various vascular beds (39-40). Additionally, in general, K^+ channels have been suggested to be involved in A_{2A} and A_{2B}ARs-mediated hyperpolarization of CA (55-56, 74, 109, 112, 137, 170) and in adenosine's role in potentiating flow-induced CA vasodilation (84). Coronary arteriolar smooth muscle K_{ATP} channels are tonically active under physiological conditions. Indeed, in humans as well as in different animal species, application of glibenclamide, a K_{ATP} channel blocker, resulted in a decrease in resting blood flow (42, 124-125, 137). Additionally, a primary role for K_{ATP} channels in mediating the sustained dilation of CA during RH was suggested (167).

Adenosine is reported to activate K_{ATP} channels in rabbit isolated CA smooth muscle cells (140) and in the A_{2A}-induced effect in RH (11, 167). Additionally, a recent study proposed that the decrease in CF produced by intracoronary glibenclamide may cause metabolic changes of ischemia including the release of adenosine (35, 132). This is an important observation, since it indicates that blockade of the endogenous vasodilator system associated with K_{ATP} channel activity can cause coronary vasoconstriction sufficient to result in myocardial ischemia (132). Adenosine-mediated activation of K_{ATP} channels may be indirectly through release of some other mediators such as H_2O_2 (please

see the role of H_2O_2 in A_{2A}AR-mediated CF regulation section)(50). A study reported that the effect of adenosine on endothelial K_{ATP} channels may be mediated via pertussis toxic-sensitive G proteins and not through the release of NO and cGMP production, whereas activation of smooth muscle cell K_{ATP} channels may not involve G proteins (59). Moreover, the enhanced responsiveness to adenosine at lower CA intraluminal pressure was suggested to be due to increased smooth muscle cell K_{ATP} channels activity (169). In addition to the A_2AR-mediated pathway, one study suggested that adenosine also activates K_{ATP} currents in single CA smooth muscle cells via A_1ARs (25). Nevertheless, the signaling pathway due to the activation of endothelial K_{ATP} channels is yet to be fully explored.

To date no patch clamp study has demonstrated the effect of adenosine on K_V currents in CA smooth muscle cells. However, *in vivo* studies have shown that 4-aminopyridine, K_V channel blocker, inhibited CA vasodilation induced by adenosine (11, 32, 56-58). Previous studies on CA smooth muscle cells also indicate the involvement of PKC and PKA in the K_V channel-mediated pathway (138, 140),which renders the question whether adenosine induces the activation of K_V channels through the PKC pathway.

Role of H_2O_2 in A_{2A}AR-mediated coronary flow regulation

H_2O_2 is suggested to act as EDHF in humans and mice (95, 97, 163). Additionally, it was reported that H_2O_2 induces CA vasodilation through both endothelium-dependent and –independent manners (147). Recent reports have established the contribution of H_2O_2 in flow-induced dilation of isolated human CA (101) and in reactive dilatation of rat isolated coronary arterioles (79). Yada et al. proposed that H_2O_2 (as an EDHF *in vivo*) plays an important role in CA autoregulation in cooperation with

adenosine and NO (163). However, so far there are no reports showing the relationship between H_2O_2 and adenosine-mediated coronary vasodilation, which lead to the basis of our study.

It is worth mentioning that Berne's adenosine hypothesis suggests that increases in myocardial oxygen consumption decrease myocardial oxygen tension which leads to the stimulation of the release of adenosine from cardiomyocytes (152), where $A_{2A}ARs$ have also been found to be expressed in both human and mouse (16, 93). Adenosine induces CA dilatation through activation of sites on cardiomyocytes in addition to the arteriole vascular bed itself (148). Furthermore, as mentioned, a role for H_2O_2 in metabolic vasodilation has already been proposed (164) and Chilian et al. suggested that H_2O_2 production increases in proportion to cardiac metabolism in order to couple the coronary blood flow to myocardial oxygen tension (131). What is interesting to appreciate is that both H_2O_2 and adenosine are released from cardiomyocytes and that they may modulate the vascular tone through the cardiac myocyte (72). Therefore, myocardium may be partly the source of both adenosine and H_2O_2 released for the regulation of CF. Our study is based on such similarities between adenosine and H_2O_2 (please also refer to chapter 4).

Relationship between adrenoceptors (ADR) and A₂ARs in coronary flow regulation

Endogenous adenosine was reported to affect the norepinephrine release from sympathetic nerve endings (127). The $A_{2A}AR$-mediated facilitation of norepinephrine release was also suggested to require activation of the PKC and PKA pathways (49) which itself is proposed as an adenosine signaling pathway. It was reported that αADR-mediated coronary vasoconstriction during exercise limits coronary perfusion in impaired endothelial function situations (70). DeFily et al. further added to this finding by showing

that, even in normal conditions, stimulation of αADR-induced vasoconstriction is masked by autoregulatory control, by means of NO release and endogenous adenosine (28). Furthermore, it was demonstrated that coronary RH responses due to adenosine are blocked by αADRs when NO synthesis is interrupted (14). On the other hand, the cardioprotective effect of carvedilol, a βADR blocker, was shown to be through an adenosine-dependent mechanism (3). Additionally, it was demonstrated that βADR-mediated inotropic responses were attenuated by adenosine (134). Therefore, overall there is some evidence on similarities between ADR and AR cardiovascular effects and signaling. However, there are no studies on the interaction or cross talk of ARs and ADRs in the regulation of CF which was the basis of our new study (Please see our appendix A).

Clinical applications

Adenosine and its agonist are currently being used in clinical settings; The vasoregulatory properties of A_{2A}ARs are used clinically in humans for the diagnosis of coronary artery disease through the use of adenosine (Adenoscan) as a substitute for exercise stress testing for people who can't exercise in myocardial perfusion imaging (12). The use of Adenoscan has its own limitations due to its blocking effect on AV conduction [due to activation of A_1ARs (10, 12)] and inducing bronchospasm [probably due to activation of A_1, A_3 or A_{2B}ARs (47, 123)]. However, these side effects may be alleviated by the development of more A_2ARs subtype selective agonists. A new A_{2A}AR selective agonist called Regadenoson (Lexiscan®) has been developed and approved by FDA which is being used clinically in myocardial perfusion imaging. Additionally, in many cardiovascular diseases such as coronary atherosclerosis, angina, and hypertension, an attenuated vascular response to adenosine has been demonstrated where functional

disturbance of smooth muscle or endothelial cells may be the underlying cause. Therefore, it is important to better understand adenosine's role in the regulation of CF, its mechanism of action, and its interplay with other AR receptors, and/or its interaction with the ADRs.

Project Summary

Adenosine plays an important role in coronary flow regulation in physiological and pathological conditions where AR expression modification may occur. Therefore, the purpose of this investigation was to better understand the individual role and contribution of A_2ARs in the regulation of coronary flow and their mechanism of action during their exogenous and endogenous activation. In addition to better understand the heterogeneity of CF responses by ARs, elucidation of ARs' role in CF regulation would enable us to develop therapies with less unwanted side effects or new enhanced medications for the diagnosis and/or treatment of cardiovascular disorders. With this purpose in mind, the following aims were developed:

Specific Aim 1: Elucidation of the role of A_2 ARs in the regulation of coronary flow

Specific Aim 2: Identification of A_2 ARs signaling pathways in coronary flow regulation

Specific Aim 3: Characterize the role of A_2 ARs in coronary reactive hyperemia

Specific Aim 4: Identify the role of H2O2 in A_2 ARs' signaling pathway during reactive hyperemia

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CHAPTER TWO

RATIONALE AND LIMITATIONS

The isolated perfused heart (Langendorff heart system), especially for small animals, is one of the optimal approaches, having in mind, the quantity and quality of data that can be obtained in a laboratory setting. Nevertheless, this approach has its own advantages and disadvantages.

The isolated heart provides us with types of data, such as heart rate, left ventricle pressure, and coronary flow, in the absence of the confounding effects of other organs,systemic circulation, neurohormonal factors, etc. This characteristic is considered as an advantage since it allows the distinction of aforementioned factors from cardiac responses. The absence of neurohormonal and peripheral factors can often be compensated for, if required; for example catecholamines or other neurotransmitters may be included in perfusate and many other peripheral mediators can be added exogenously. However, this approach can also be considered a disadvantage in the sense that it makes the preparation one step further away from the physiological *in vivo* state. Nonetheless, without a doubt, the isolated heart system provides us with the ability of performing metabolic or pharmacological dose-response studies. Although one must take into account the fact that, as an *ex vivo* preparation, the isolated heartundergoes deterioration in time, but it is capable of providing data up to 3-4 hours.

Using the isolated heart system, we are also able to induce whole heart ischemia through flow occlusion; this is an approach that we used for our reactive hyperemia experiments. Anoxia or hypoxia at various degrees of oxygen deprivation (in the presence of normal flow) can also be easily imposed through manipulation of oxygenation rate or amount. Therefore, this approach provides us with a powerful tool for assessing many aspects of ischemia/hypoxia or even, if interested, ischemia-reperfusion-induced injury, in which case single coronary occlusion can be performed.

It is important to know that we have two modes of operation of the isolated perfused heart systems: constant flow and constant pressure. Whereas constant flow perfusion eliminates a variable from the experiment, it also has the disadvantage that, unlike constant pressure perfusion, autoregulatory coronary flow mechanisms are overridden and, therefore, it does not provide us with data regarding coronary flow regulation. Switching between constant flow and constant pressure modes of perfusion is not very straightforward so that it can be feasible within a single experimental protocol. However, currently, this system is now commercially available through ADInstruments Ltd. which allows us to switch instantly between constant pressure and constant flow modes of perfusion, enabling perfusion pressures and coronary flow to be controlled over a wide range. This feature is of considerable advantage in many studies of vascular function.

Using isolated perfused heart, several types of data can be gathered; for the purpose of morphology and vascular anatomy studies, heart or vascular fixation can be easily performed through coronary perfusion of required agents and a sequential analysis using light or electron microscopy. Additionally, the heart can be sliced, starting at the apex of the heart and working towards the base, for histology studies of coronary arteries. The whole heart can also be used for gross morphology such as is required during infarct size or cardiac hypertrophy studies. From a biochemistry/physiology point of interest, arterio-venous substrate concentration differences for metabolites, oxygen, and a host of other factors involved during physiological and extreme metabolism can be measured. Additionally, measurement of cellular constituents such as enzymes and proteins can be performed for the assessment of tissue injury due to the influence of, for example, various conditions such as ischemia/reperfusion or hypoxia. Microelectrodes can further be used

for continuous measurement of interstitial K^+, Ca^{2+}, pH or monophasic action potentials. With all that in mind, using isolated heart perfusion the coronary perfusion, can be used for delivery of vectors in gene transfer studies where adenovirus or other vectors can be selectively delivered to the coronary artery vasculature and hence providing us with a so called "organ specific gene modification", which may replace whole animal knockout models as an approach which may exclude the effect of compensatory mechanisms due to the general whole animal gene modification.

From a more functional standpoint, using the isolated heart system, electrocardiographic recordings allow the detection, identification, and quantification of cardiac arrhythmias. Furthermore, the Langendorff preparation provides valuable information on left ventricular systolic and diastolic pressures and derivatives such as dp/dt. Various ultrasound techniques, such as echocardiography and Doppler, can also be used for measurements of cardiac wall thickening, stroke volume, cardiac output, coronary flow velocity, etc. While most studies with the isolated perfused heart are focused on the function and malfunction of the cardiomyocyte, using contractile and metabolic endpoints, it should be highlighted that Langendorff heart system can be used to study vascular reactivity, endothelial and smooth muscle function, and the effect of a variety of agents and drugs on coronary flow. Indeed, the isolated heart has been the foundation of much of the work on the no reflow phenomenon where A_2ARs agonists are currently being clinically investigated as an approach for reduction of reperfusion injury. Nevertheless, even from basic therapeutic approaches, the isolated heart preparation is reliable for assessing the direct cardiovascular effects of various therapeutic agents in terms of contractile function, electrical activity, or metabolic function. This preparation allows us to perform dose-response studies with reproducibility and precise control of

agent concentration. An additional advantage is that we are able to washout drugs from the circulation by replacing perfusion fluids which itself can be an excellent approach to study drug tachyphylaxis or desensitization.

There are also further important issues to consider when using the isolated heart system. Like so many other aspects of heart perfusion, the decision of whether to pace the heart or not, is based on a compromise between protocol requirements and quality of data. Heart rate in isolated hearts, when left to contract spontaneously, undergoes a progressive time-dependent decline. Spontaneous heart rate, in a perfused heart system, is usually significantly below the physiological normal rate. Mouse heart rate, in an *in vivo* preparation, is about 500 beats/min, whereas heart rate of about 380 beats/min, can be expected in the *ex vivo* preparation. This decline in baseline heart rate, of course, adds an additional variable, which itself may also vary among the animals. Moreover, this decline in heart rate also adds variability to the measurements obtained for left ventricular pressure. During an experiment, the worsening of HR depends on a large number of factors including the skills of the worker, avoidance of injury, the species being used, the composition of the perfusion fluid, the drugs used, age, HR and work load, and the temperature at which the studies are carried out. During a Langendorff heart preparation experiment, a deterioration rate of contractile function of about 5-10% per hour is expected. Therefore, the rate of deterioration should be taken into account for the interpretation of studies and the experiments should be more or less time-matched with corrections for baseline deterioration when comparing groups.

Use of the isolated heart perfusion approach is reported in many large animals such as pigs, monkeys, sheep, dogs and even humans. Nevertheless, such studies are less frequently performed due to many limiting factors such as high cost, presence of greater

variability in results, need forf large volumes of perfusion fluid and specialized equipment required. The availability of transgenic technology for mice undoubtedly resulted in increasing numbers of studies using murine preparations. Indeed, one of the reasons to use mouse, in our laboratory, was the availability of AR KO mice. Currently, the literature consists of an increasing number of studies which employ mouse hearts although the high HR ofmice may set some limitations for translation of data obtained from this species to human studies. Another limitation in the use of mice, in some studies, is the very small size of all organs or tissues. For example, the size of many arteries, such as large conduit coronary arteries, is in ~80 µm range, which shows a very smaller size range for the coronary resistance vessels. The size of these vessels somehow limits the study of coronary arteries in that the isolation of the actual resistance vessels is extremely challenging. Also, the use of a larger number of animals is required in order to obtain sufficient amount of tissue for the purpose of even basic experiments such as RT-PCR or western blot. Another limitation with the use of mice is the miniaturization of equipment; handling of rat hearts, for example, has a great advantage over smaller hearts from mice where intraventricular pressure recordings are more difficult. The rat does, however, suffer from very short action potential duration which can limit its value in terms of translation of data to human studies of arrhythmia and antiarrhythmic drugs. Other species, such as the rabbit, suffer problems with anesthesia and the guinea-pig heart differs from other species in that the coronary vasculature is totally collateralized, effectively preventing the study of regional ischemia in this species. Thus, as no ideal species or setting exists, in selecting a species or preparation for a specific study, it is crucial to recognize and appreciate the disadvantages and advantages.

CHAPTER THREE

Contributions of A_{2A} and A_{2B} adenosine receptors in coronary flow responses in relation to K_{ATP} channel using A_{2B} and $A_{2A/2B}$ double knockout mice

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Abstract

Adenosine plays a role in physiological and pathological conditions and A_2 adenosine receptors (AR) expression is modified in many cardiovascular disorders. In this study, we elucidated the role of $A_{2B}AR$ and its relationship to $A_{2A}AR$ in coronary flow (CF) changes using A_{2B} single (KO) and $A_{2A/2B}$ double (DKO) knockout mice in Langendorff setup. We used two approaches: a) selective and non-selective AR agonists and antagonists, and b) A_{2A} and $A_{2B}KO$ and $A_{2A/2B}DKO$. BAY60-6583 (selective A_{2B} agonist) had no effect on CF (ml/min/g) in $A_{2B}KO$ while it significantly increased the WT CF (maximum of 23.3 ± 9). NECA (non-selective AR agonist) increased the CF in $A_{2B}KO$ (maximum of 34.6 ± 4.7) to a significantly higher degree compared to WT (maximum of 23.1 ± 2.1). Also, CGS21680 (selective A_{2A} agonist) increased the CF in $A_{2B}KO$ (maximum of 29 ± 1.9) to a significantly higher degree compared to WT (maximum of 25.1 ± 2.3). SCH58261 (A_{2A} selective antagonist) inhibited the NECA-induced increase in CF to a significantly higher degree in $A_{2B}KO$ (19.3 ± 1.6 *vs.* 0.5 ± 0.4) compared to WT (19 ± 3.5 *vs.* 3.6 ± 0.5). NECA did not induce any increase in CF in $A_{2A/2B}DKO$ while a significant increase was observed in WT (maximum of 23.1 ± 2.1). Furthermore, mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate had no effect on NECA-induced increase in CF in WT while NECA-induced increase in CF in WT (17.6 ± 2), $A_{2A}KO$ (12.5 ± 2.3), and $A_{2B}KO$ (16.2 ± 0.8) was significantly blunted by K_{ATP} channel blocker, glibenclamide, to 0.7 ± 0.7, 2.3 ± 1.1, and 0.9 ± 0.4, respectively. Also, CGS21680 (22 ± 2.3), and BAY60-6583 (16.4 ± 1.60)-induced increase in CF in WT was significantly blunted by glibenclamide to 1.2 ± 0.4 and 1.8 ± 1.2, respectively. In conclusion, this is the first evidence supporting the compensatory up-regulation of $A_{2A}AR$ in $A_{2B}KO$ mice and demonstrating that both A_{2A} and $A_{2B}ARs$ induce CF changes

through K_{ATP} channels. These results identify AR-mediated CF responses which may lead to better therapeutic approaches for the treatment of cardiovascular disorders.

Introduction

Adenosine is an endogenous nucleoside which is released through the breakdown of adenine nucleotides. Adenosine's cardiovascular effects are mediated through activation of its four subtypes of receptors (AR), namely, A_1, A_{2A}, A_{2B} and A_3. Activation of A_1AR results in negative chronotropic and ionotropic effect, and a decrease in coronary flow (CF) (68) while other studies suggest that the activation of both A_1 or A_3ARs prior to ischemia is cardioprotective (6, 30). However, adenosine is shown to play a vasoregulatory role in human coronary arteries (16-17, 62-63). It is well established that the activation of $A_{2A}AR$ induces positive inotropic effect and that $A_{2A}AR$ plays a major role in the regulation of CF in *ex vivo* models (67, 69). Additionally, *in vivo* studies in dogs showed the involvement of $A_{2A}AR$ in CF (reactive hyperemia) regulation (5, 77) and thus, supporting a physiological role for adenosine. Adenosine-induced effects are species-dependent (20, 23). For example it is reported that guinea pig coronary vasodilation is more sensitive to adenosine compared to rats (72). Furthermore, few studies show that $A_{2B}AR$ acts as a vasoconstricting as well as a vasodilating factor in different vascular beds (13, 57), in addition, to playing a cardioprotective role during ischemia/reperfusion (14-15, 30). Nevertheless, there are no reports fully elucidating the individual and relative role of $A_{2B}AR$ in relation to $A_{2A}AR$ in coronary vasodilation. Our group and others have previously suggested a role for $A_{2B}AR$ in coronary artery vasodilation by means of indirect measures such as using non-selective agonist and antagonists and $A_{2A}KO$ mice (48, 67) which are in accordance with previous reports

showing A_2AR-mediacted vasodilation in isolated human coronary arteries (39, 63). Furthermore, this laboratory has shown the presence of A_{2B} and $A_{2A}ARs$ in human and porcine coronary artery endothelial and vascular smooth muscle cells that may further support the involvement of A_{2A} and $A_{2B}ARs$ in the regulation of CF and a further possible interaction between these two receptor subtypes (53, 71). Coexpression of more than one AR subtype is also reported from other laboratories on human endothelial cells (36, 53) however, there are no reports studying whether the coexpressed receptors interact. We have recently shown an up-regulation of $A_{2B}AR$ in $A_{2A}KO$ mouse coronary artery suggesting an interrelationship between A_{2A} and $A_{2B}ARs$ subtypes since $A_{2B}AR$ compensated for the down-regulation/deletion of $A_{2A}AR$ (69). Additionally, since the involvement of A_{2A} and $A_{2B}ARs$ and their down- or up-regulation is reported in many pathophysiological conditions (2, 40) and differential expression of ARs contributes to functional heterogeneity of human endothelial cells (21), elucidating the presence of such compensatory mechanisms and signaling pathways for ARs is essential for better understanding of the heterogeneity of vascular responses and the regulation of CF.

While $A_{2B}AR$ is reported to induce its cardiovascular effects through activation of arachidonic acid pathway in vasculature (13), $A_{2A}AR$-induced vascular effects are demonstrated to partly involve CYP expoxygenases (50). We and others have previously demonstrated that $A_{2A}AR$-induced vasodilation is partly due to release of nitric oxide in mouse aorta and coronary arteries (69). However, $A_{2B}AR$-induced vasodilation is both nitric oxide-dependent and –independent in mouse aortic conduit artery and coronary arteries, respectively (1, 69). Although K_{ATP} channels are shown to be present in coronary artery cells (28, 65) and K^+ channels in general are known to be involved in A_{2A} and $A_{2B}ARs$-mediated hyperpolarization of coronary arteries (31, 39, 52), the involvement of

K_{ATP} channels in adenosine-induced coronary artery responses remains poorly understood.

The purpose of the present study was to directly and more fully elucidate the individual contribution of $A_{2B}AR$ and its relationship to $A_{2A}AR$ in A_2ARs-mediated induction of coronary artery vasodilation in light of the K_{ATP} channel involvement. The hypotheses to be tested were; a) $A_{2B}AR$ contributes to coronary vasodilation, b) A_{2A} and $A_{2B}ARs$ interact to induce coronary artery vasodilation and they each compensate for the deletion/down-regulation of the other, and c) A_{2A} and $A_{2B}ARs$-induced increase in CF is the result of activation of K_{ATP} channels. Since there are inconsistencies between the results obtained from transgenic models compared to those obtained using pharmacological approaches (55), in this study, we have used both pharmacological and molecular (targeted gene deleted mouse model) approaches. Furthermore, we used $A_{2A/2B}DKO$ mice to remove any possible compensatory up-regulation of either A_{2A} or $A_{2B}AR$ in CF responses.

Methods

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

Generation of DKO mice. A_{2A} -/- mice (backcrossed 12 generations to the C57BL/6 background) were bred with A_{2B} -/- mice (backcrossed 12 generations to the C57BL/6 background) to generate A_{2A} +/- A_{2B} +/- double heterozygotes. These double heterozygotes were then intercrossed, and 1/16 of the resultant offspring were A_{2A} -/-A_{2B} -/- DKOs. Male and female DKO breeding pairs were established from these animals to

produce DKO mice. Since both A_{2A} -/- and A_{2B} -/- mice were inbred on C57BL/6, DKO are also inbred.

WT, $A_{2A,}$ and A_{2B}KO and $A_{2A/2B}$DKO mice. Hearts were isolated from age-matched 10 to 14 weeks old mice. WT mice of a mixed C57BL/6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, Maine). A_{2B}KO and $A_{2A/2B}$DKO mice of the same background were generously provided by Dr. Stephen Tilley, Department of Medicine, University of North Carolina, Chapel Hill, NC. A_{2A}KO mice of the same background were generously provided by Dr. Catherine Ledent, Universite Libre de Bruxelles, Brussels, Belgium. All four strains were bred at the West Virginia University animal facility as a sub-colony of the original strain. The mice were kept in cages with 12:12-hr light-dark cycles and maintained on a standard laboratory diet with access to water *ad libitum*. All animal care and experimentation were in accordance with the West Virginia University Institutional Animal Care and Use Committee and the principles of the National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

Chemicals. All chemicals were prepared as 10 mM stock using Dimethyl Sulfoxide (DMSO, Sigma) followed by serial dilution with 50% DMSO and distilled water and further dilution to desired concentration was achieved with distilled water (final DMSO concentration of < 1%). NECA (5'-N-ethylcarboxamido adenosine), CGS21680, 5-hydroxydecanoate, and glibenclamide were bought from Sigma (St. Louis, MO, USA) and SCH58261 was a gift from Schering-Plough, Italy. A_{2B} agonist, BAY60-6583, was a gift from Bayer (Germany). All stated concentrations are the actual drug concentration delivered as 1% of coronary flow.

Langendorff-perfused mouse heart preparation. Isolated heart experiments were performed in accordance with the methods published earlier from our lab (48). In brief, mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). A thoracotomy was performed, and the hearts were removed into heparinized (5U/ml) ice-cold Kreb-Hensleit (KH) buffer. The hearts were retrogradely perfused rapidly through the aorta cannulated with a 20-gauge, blunt-ended needle at a constant pressure of 80 mmHg with continuously gassed with 95% O_2 and 5% CO_2 KH buffer containing (mM): 119 NaCl, 11 Glucose, 22 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 2 Pyruvate and 0.5 EDTA at 37°C in a standard Langendorff fashion and allowed to beat spontaneously. The left atrium was removed and the left ventricle was vented with a small polyethylene apical drain. A water-filled balloon made of plastic wrap was inserted into the left ventricle across the mitral valve, which was connected to a fluid-filled pressure transducer by polyethylene tubing for continuous measurement of left ventricular developed pressure (LVDP). Left ventricle diastolic pressure was adjusted to a pressure of 2-5 mmHg by filling up the left ventricle balloon. Coronary flow was measured via a Transonic flow probe (Transonic Systems; Ithaca, NY) in the aortic perfusion line. Baseline coronary flow, LVDP, and heart rate (derived from the ventricular pressure trace) were monitored for 30 min during the equilibration period, which were recorded on a Power Lab data acquisition system (AD Instruments; ColoradoSpring, CO). Hearts with persistent arrhythmias or poor LVDP (<80 mmHg) during the equilibration period were excluded from the study. To rule out the tachyphylaxis or desensitization, at the end of each antagonist experiment, after another washout period, a repeat infusion of the agonist was performed.

Experimental Protocols. After 30 minutes of equilibrium, baseline coronary flow, heart rate, and developed pressure were measured in WT, $A_{2B}KO$, and $A_{2A/2B}DKO$ isolated hearts. Concentration-response curve (CRC) (10^{-10}-$10^{-6}M$) were performed for BAY60-6583 (A_{2B} selective agonist), NECA (non-selective AR agonist) and CGS21680 (selective A_{2A} agonist) by infusing the drugs into the coronary perfusate through an injection port directly proximal to the aortic cannula using a microinjection infusion pump (Harvard Apparatus; Holliston, MA). After equilibration period, each heart was perfused with increasing concentration of selected drug to develop a concentration-response relationship curve. Each drug concentration was given for 5 min with 10 min intervals to allow complete drug washout (or using recovery to baseline parameters). All concentrations are the actual concentration in the perfusate. Data were collected at the end of each infusion period and washout time, which was used as a reference for normalizing responses to the subsequent drug concentration. In the result section, actual values from CRCs (figures 1-2,4, and 6) are presented as the maximum response and values from the use of a single drug concentration in the presence and absence of an antagonist (Figures 5 and 7-9) are presented as the actual drug-induced effect (maximal response minus the baseline).

Antagonist experiment protocol. After 30 min equilibration period and measurement of baseline parameters, agonists, NECA ($10^{-8}M$, in A_{2B} KO mice, or $10^{-8}M$, in A_{2A} KO mice, closest concentration to the EC_{50} obtained from previously performed experiments) (Figures 7 and 8), BAY60-6583 ($10^{-7}M$) (Figure 9), and CGS21680 ($5\times10^{-9}M$) (Figure 9), were infused for 5 min at 1% rate of coronary flow. The plateau effect of coronary flow, heart rate, and developed pressure were recorded and a 10 min washout interval was allowed to reach the baseline. This was followed by infusion of antagonists, SCH58261

(10^{-6}M, A_{2A} selective antagonist) (71) (Figure 5) or glibenclamide (10^{-5}M) (Figures 7-9), at 1% rate of coronary flow for at least 10 min after which, the agonist was also added to the infusion line for an additional 5 min (for a total of 15 min). The baseline at the end of antagonist infusion was treated as the new baseline for the subsequent agonist responses. The data at the end of the 15 min (the end of infusion of both agonists and antagonists) were used to compare with data obtained from the first infusion of the agonist alone. At the end of the experiment, after at least 10 min of washout and reaching the baseline, the agonist was again infused in order to check for tachyphylaxis or desensitization.

Western Blot. Mouse mesenteric arterioles (up to third branch arterioles) homogenates were obtained from isolated and cleaned tissue using ice-cold lysis buffer consisting of 0.05 M Tris-buffered saline, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM phenylmethylsulfonyl and Halt Protease Inhibitor Cocktail (Thermo Scientific) by way of glass homogenizer. Samples were then centrifuged for 15 min at 13,000 rpm and the supernatant was stored at -80°C. Protein extracts (30 μg protein per well) were separated on NuPAGE 4-12% Bis-Tris Gels (Invitrogen) along with Novex Sharp Protein Standard, 3.5–260-kDa, (Invitrogen) run in parallel. Proteins were then transferred to a PVDF membrane (Millipore), blocked in 5% milk for 1 hour and then probed with anti-A_{2A}AR Rabbit Polyclonal IgG antibody (45) developed in our laboratory for detection of A_{2A}AR protein with a dilution of 1:1000 in TBST + 0.5% milk overnight at 4°C or with anti-β-actin (Santa Cruz Biotechnology) at a dilution of 1:5000 at room temperature for 1 hour. This was followed by incubation with a secondary horseradish peroxidase-conjugated antibody (anti-mouse and anti-rabbit immunoglobulins, respectively; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. For detection of bands, the membranes

were treated with an enhanced chemiluminescence reagent (GE Healthcare) for 1 min and subsequently exposed to ECL Hyperfilm (GE Healthcare). Relative band intensities were quantified by densitometry, and each sample was normalized to the β-actin values and calibrated to $A_{2A}AR$ protein expression in WT.

Data and Statistical analysis. Western blot data and baseline functional data of $A_{2A}KO$, $A_{2B}KO$, and $A_{2A/2B}DKO$ groups were compared to WT, analyzed by using *t*-test. Differences in dose response curves and responses to each drug at the same concentration between WT, $A_{2B}KO$, $A_{2A}KO$, and $A_{2A/2B}DKO$ hearts were analyzed using two-way ANOVA for repeated measures. Antagonist-induced responses (HR, LVDP, and CF) for WT, $A_{2A}KO$, and $A_{2B}KO$ hearts were compared with *t*-test. Statistical comparisons were done on percent changes. Results were considered significant when $P < 0.05$. Values are means ±SEM. The EC_{50}s were calculated using Prism (Graphpad Software, La Jolla, CA).

Results

Baseline function in WT, $A_{2A}KO$, $A_{2B}KO$, and $A_{2A/2B}DKO$ isolated hearts. Table 1 demonstrates the baseline parameters for coronary flow (CF), heart rate (HR), and left ventricle developed pressure (LVDP) for $A_{2A}KO$, $A_{2B}KO$, $A_{2A/2B}DKO$, and WT mice after 30 min of equilibration of the isolated hearts as mentioned in the "materials and methods". The average body weight of $A_{2B}KO$ mice was significantly lower compared to the WT while heart weight to body weight ratio were not significantly different in any KOs compared to WT. Furthermore, no significant baseline differences were observed in CF and HR in any of the KOs compared to WT while LVDP was significantly higher in $A_{2A/2B}DKO$ compared to WT hearts (Table 1).

Effect of A$_{2B}$AR activation on coronary flow. BAY60-6583 (A$_{2B}$ selective agonist) caused a concentration-dependent increase in CF in WT whereas no effect was observed in A$_{2B}$KO mice (Figure 1A). BAY60-6583 increased the CF in WT mice (with a maximum of 23.3±9ml/min/g, n=7) to a significantly higher degree compared to A$_{2B}$KO (no response) (Figure 1A). BAY60-6583 had no effect on HR of either WT or A$_{2B}$KO (Figure 1B) while it induced a significant increase in LVDP from baseline in the WT animals to a maximum of 95.6±14.9 mmHg (Figure 1C, n=7). BAY60-6583 had no effect on LVDP in A$_{2B}$KO hearts (Figure 1C).

Contribution of A$_{2A}$AR in ARs-mediated coronary vasodilation of A$_{2B}$KO mice. Since we wanted to test if A$_{2A}$AR compensates for A$_{2B}$AR in producing coronary vasodilation in A$_{2B}$KO mice, we used NECA (non-selective AR agonist) in A$_{2B}$KO and WT mice. NECA increased the CF in A$_{2B}$KO mice (with a maximum of 34.6±4.7ml/min/g, n=8, and EC$_{50}$: 6.8×10^{-9}±0.8M) to a significantly higher degree compared to WT mice (with a maximum of 23.1±2.1ml/min/g, n=9, and EC$_{50}$: 3.5×10^{-9}±0.6M) (Figure 2A). Furthermore, NECA induced a significant increase in LVDP of A$_{2B}$KO compared to WT with a maximum increase of 115.5±8.5mmHg and 77.5±11.6mmHg, respectively (Figure 2C), while a decrease in HR was noted in both WT and A$_{2B}$KO mice with no significant difference between the two groups (Figures 2B).

Up-regulation of A$_{2A}$AR expression in A$_{2B}$KO isolated mesenteric arterioles. We used mesenteric arterioles as resistance vessels to analyze A$_{2A}$AR expression level in A$_{2B}$KO mice. The A$_{2A}$AR protein was significantly increased in A$_{2B}$KO isolated mesenteric arterioles compared to WT (25.7% higher expression, Figure 3, n=4, $p < 0.05$)

Up-regulation of $A_{2A}AR$ in $A_{2B}KO$ mice. CGS21680 increased the CF (ml/min/g) in A_{2B}KO mice (27.2 ± 2.6 at 5×10^{-8}M and 29 ± 1.9 at 10^{-7}M with EC_{50}: $3.9\times10^{-9}\pm0.7$M, n=7) to a significantly higher degree compared to WT (22.4 ± 1.5 at 5×10^{-8}M and 25.1 ± 2.3 at 10^{-7}M with EC_{50}: $2.6\times10^{-9}\pm0.7$M, n=7) (Figure 4A). Additionally, CGS21680 caused a significant increase in LVDP (mmHg) in A_{2B}KO (111.5 ± 6.7 at 5×10^{-8}M and 113 ± 8.1 at 5×10^{-7}M) compared to WT (97.9 ± 4.9 at 5×10^{-8}M and 93 ± 6.1 at 5×10^{-7}M) while there were no difference between the HR induced by CGS21680 when comparing WT and A_{2B}KO (Figures 4B-C).

Up-regulation of $A_{2A}AR$ in $A_{2B}KO$ mice. To further confirm the up-regulation of A_{2A}AR in A_{2B}KO mice as a compensatory response for the regulation of CF, we used SCH58261, A_{2A} selective antagonist, and tested its effect on NECA (10 nM)-induced increase in CF in WT and A_{2B}KO. SCH58261 (1 μM) significantly decreased the baseline CF (ml/min/g) of WT (from 9.9 ± 0.7 to 6.8 ± 0.7) and A_{2B}KO (from 12.3 ± 0.6 to 7.7 ± 1.1, (Figure 5A). SCH58261 also caused a significantly higher inhibition of NECA-induced increase in CF (ml/min/g) in A_{2B}KO (19.3 ± 1.6 *vs.* 0.5 ± 0.4 n=4) compared to WT (19 ± 3.5 *vs.* 3.6 ± 0.5, n=6) (Figure 5A). NECA at 10 nM concentration and SCH58261 had no significant effect on HR in both WT and A_{2B}KO animals (Figure 5B). However, NECA significantly increased the LVDP (mmHg) in both A_{2B}KO (from 99.9 ± 11.5 to 141 ± 20.8, n=4) and WT (from 89.5 ± 4.4 to 123.1 ± 4.5, n=6) hearts (Figure 5C). SCH58261 significantly reduced the baseline LVDP (mmHg) in both A_{2B}KO (from 99.9 ± 11.6 to 84.2 ± 12.2) and WT (from 89.5 ± 4.4 to 80.6 ± 5.2) hearts. Additionally, SCH58261 significantly reduced the NECA-induced increase in LVDP in both A_{2B}KO and WT groups (34.9 ± 5.9 *vs.* 2.8 ± 1.5, n=4, and 33.6 ± 5.7*vs.* 6.7 ± 4, n=4, respectively) compared to their respective controls (Figure 5C).

Involvement of A_{2A} and A_{2B}ARs in producing coronary vasodilation. In order to rule out the compensatory response between A_{2A} and A_{2B}ARs, NECA was used in $A_{2A/2B}$DKO mice (Figure 6). NECA did not induce any increase in CF in $A_{2A/2B}$DKO (n=5) while a concentration-dependent response was observed in WT with a maximum increase up to 23.1 ± 2.1ml/min/g (n=9) (Figure 6A). Additionally, NECA-induced effect on LVDP (mmHg) was significantly lower in $A_{2A/2B}$ DKO (53 ± 2 at 10^{-7}M and EC_{50}:$1.3\times10^{-8}\pm0.4$) compared to WT (82.5 ± 11.6 at 10^{-7}M and EC_{50}:$1.8\times10^{-8}\pm0.2$) (Figure 6C). However, the decrease in HR in WT was not different compared to $A_{2A/2B}$DKO hearts (Figure 6B).

Involvement of K_{ATP} channels in A_{2A} and A_{2B} ARs-induced increase in coronary flow. We used 5-hydroxydecanote (mitochondrial K_{ATP} channel blocker, 10^{-4}M) to test its effect on NECA (10^{-8}M)-induced increase in CF in WT mice. The NECA concentrations were chosen based on the EC_{50} obtained from previously performed experiments (10^{-8}M in WT and A_{2B}KO and 5×10^{-8}M in A_{2A}KO). 5-hydroxydecanote had no effect on NECA-induced increase in CF (ml/min/g) (15.3 ± 2.8 *vs.* 18.5 ± 3.1, n=5, Figure 7A). Additionally, 5-hydroxydecanote had no effect on NECA-induced changes in HR and LVDP (Figures 7B-C). Furthermore, we used glibenclamide (K_{ATP} channel blocker) to test its effect on NECA-induced increase in CF in WT, A_{2A} and A_{2B}KO mice (Figures 7A and 8A). Glibenclamide significantly reduced NECA-induced increase in CF (ml/min/g) in WT (17.6 ± 2 *vs.* 0.7 ± 0.7, n=4, Figure 7A), A_{2A}KO (12.5 ± 2.3 *vs.* 2.3 ± 1.1, n=5, Figure 8A), and A_{2B}KO (16.2 ± 0.8 *vs.* 0.9 ± 0.4, n=5, Figure 8A). However, glibenclamide did not change NECA-induced effect on HR (bpm) in A_{2A} and A_{2B}KO while it significantly reduced the NECA-induced effect on HR in WT compared to its control (28.5 ± 9.7 *vs.* -8.1 ± 7.6) where it also significantly reduced the basal HR from 315.5 ± 11.1 to 236.6 ± 15.2 (Figures

7B and 8B). Furthermore, glibenclamide did not change NECA-induced effect on LVDP in A_{2A}KO while significantly reduced the LVDP (mmHg) in WT (26.4 ± 9.9 *vs.* 1.2 ± 3.6) and A_{2B}KO (16.7 ± 3.3 *vs.* 2.4 ± 2.3, n=5) (Figures 7C and 8C). To confirm the effect of glibenclamide we tested its effect on pinacidil (K_{ATP} channel opener as positive control). Glibenclamide significantly reduced pinacidil-induced increase in CF (ml/min/g) from baseline in WT (19 ± 3 *vs.* 10.2 ± 1.8, n=6). However, glibenclamide did not change pinacidil-induced effect on HR and LVDP in WT mice (Figure 7).

Additionally, we tested the effect of glibenclamide on CGS21680 (A_{2A} selective agonist) and BAY60-6583(A_{2B} selective agonist)-induced increase in CF in WT (Figure 9A). Glibenclamide significantly reduced the effect of CGS21680 (5×10^{-9}M) (22 ± 2.3, n=5) and BAY60-6583 (10^{-7}M) (16.4 ± 1.6, n=4) on CF (ml/min/g) to 1.2 ± 0.4 (n=5) and 1.8 ± 1.2 (n=4), respectively (Figure 9A). In the presence of glibenclamide both the baseline and drug-induced effects on HR were significantly decreased compared to their control (Figure 9B). Additionally, glibenclamide significantly reduced CGS21680 and BAY-induced increase in LVDP (mmHg), 31.6 ± 13.1 vs. 1.9 ± 4.7 (n=5) and 20.1 ± 1.7 vs. 7.7 ± 5.5 (n=4), respectively (Figure 9C).

Figure 3.1 Effect of BAY 60-6583 (BAY) in A$_{2B}$KO and WT mice on coronary flow (A), heart rate (B) and LVDP (left ventricle developed pressure) (C). *Significant difference compared to WT, $p<0.05$.

Figure 3.2 Effect of NECA in A$_{2B}$KO and WT mice on coronary flow (A), heart rate (B), and LVDP (C). *Significant difference compared to WT, *p*<0.05.
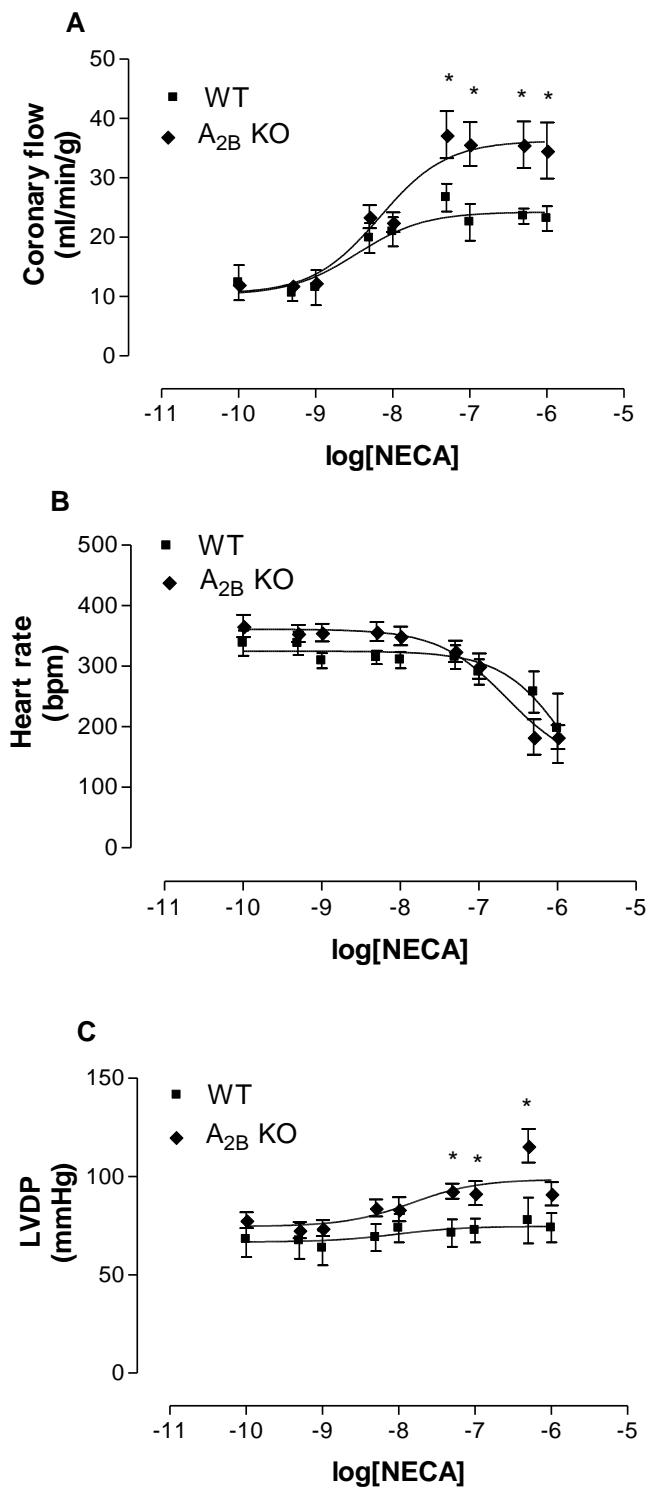
Figure 3.3 A_{2A}AR expression levels in WT and A_{2B}KO isolated mesenrteric arterioles.*Significant difference compared to WT, *p*<0.05.
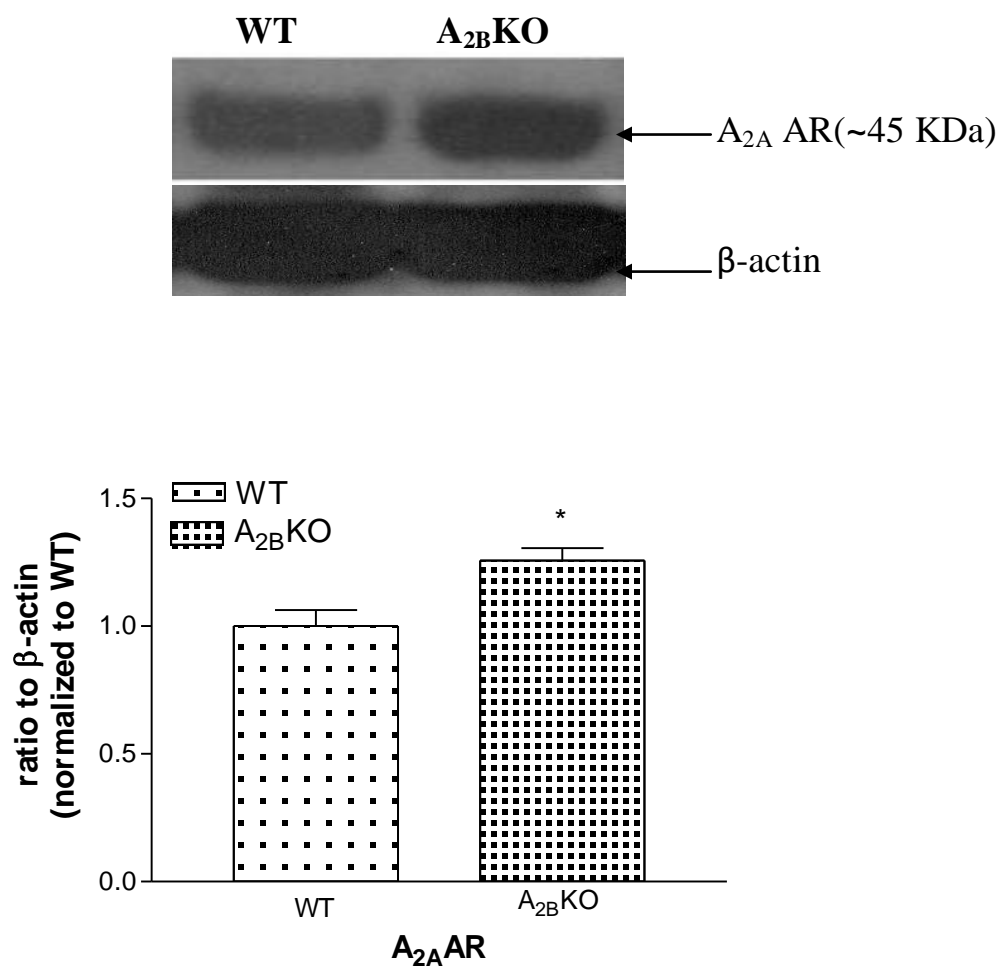
Figure 3.4 Effect of CGS 21680 in A$_{2B}$KO and WT mice on coronary flow (A), heart rate (B), and LVDP (C).*Significant difference compared to WT, *p*<0.05.
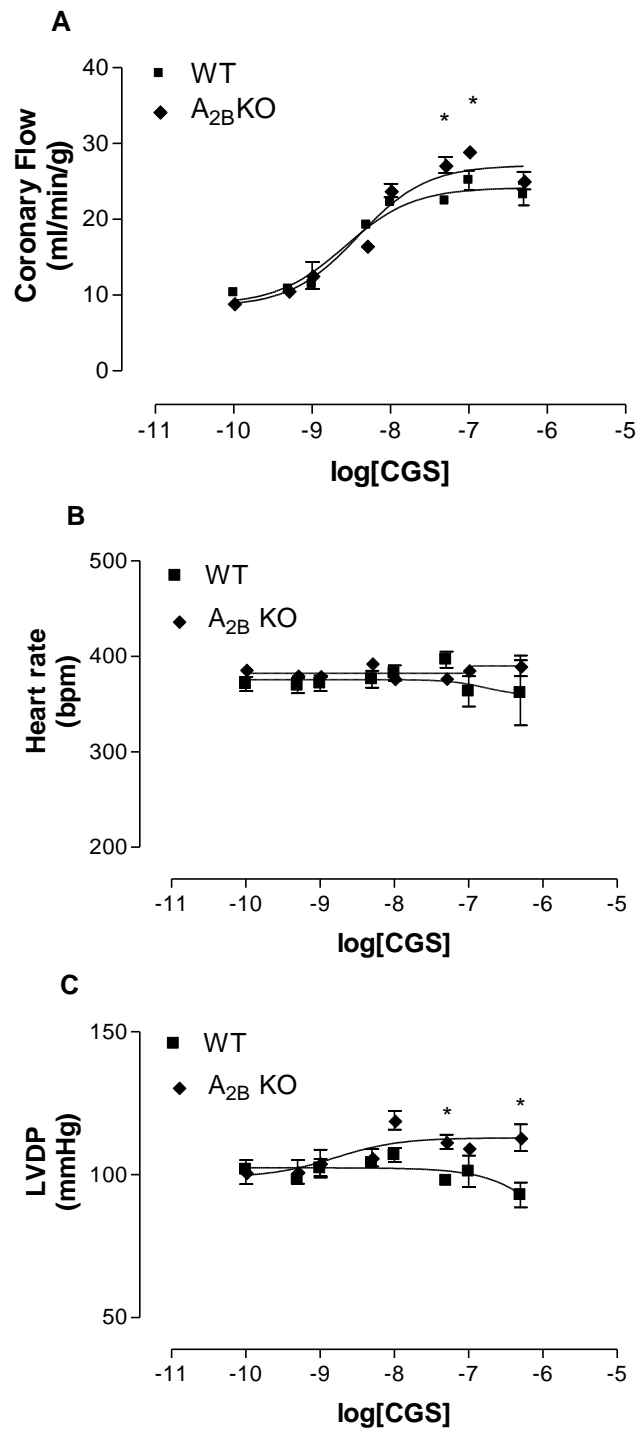
Figure 3.5 Effect of SCH 58261(1µM) on NECA-induced increase in coronary flow (A), heart rate (B), and LVDP (C) in WT and A$_{2B}$KO. *Significant difference between drug-induced effects in the presence of antagonist compared to their corresponding control. #Significant difference in baselines in the presence of antagonist compared to their corresponding control. $Significant difference between WT and A$_{2B}$KO antagonist treated groups, *p*<0.05.
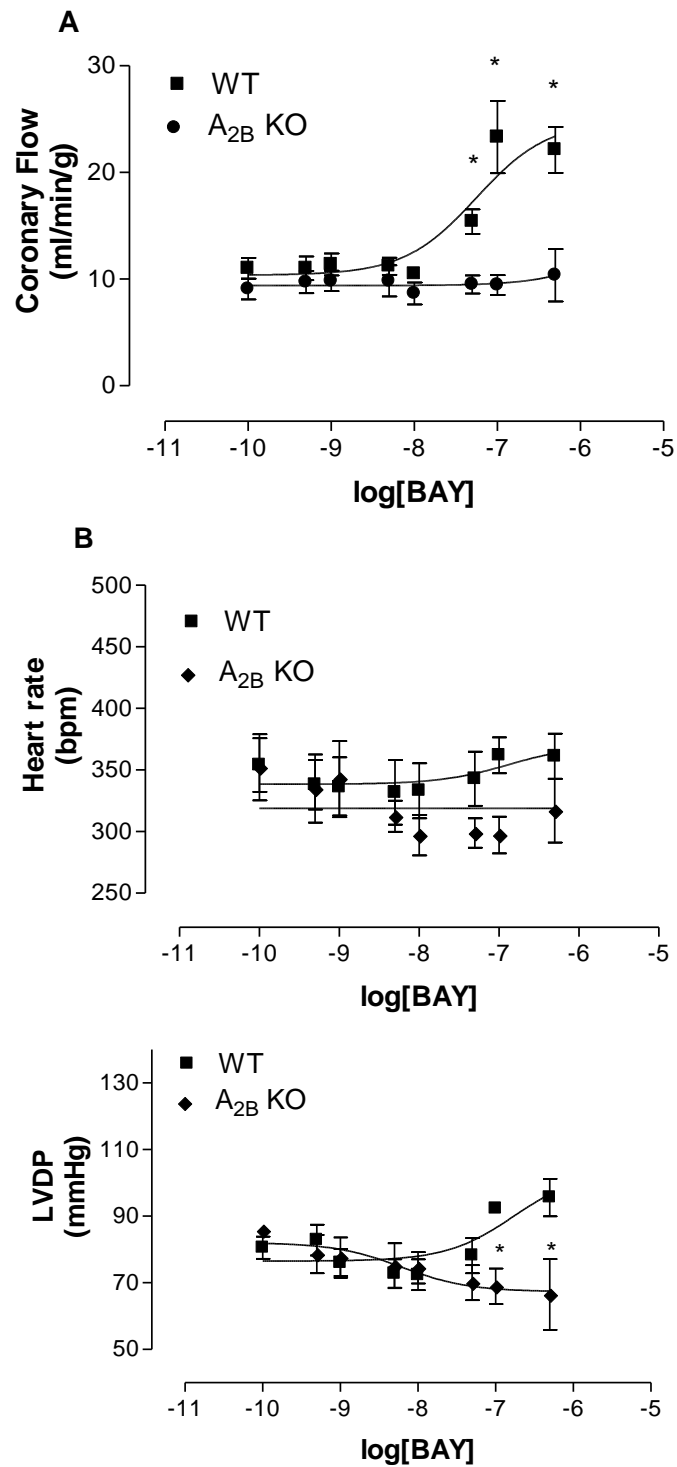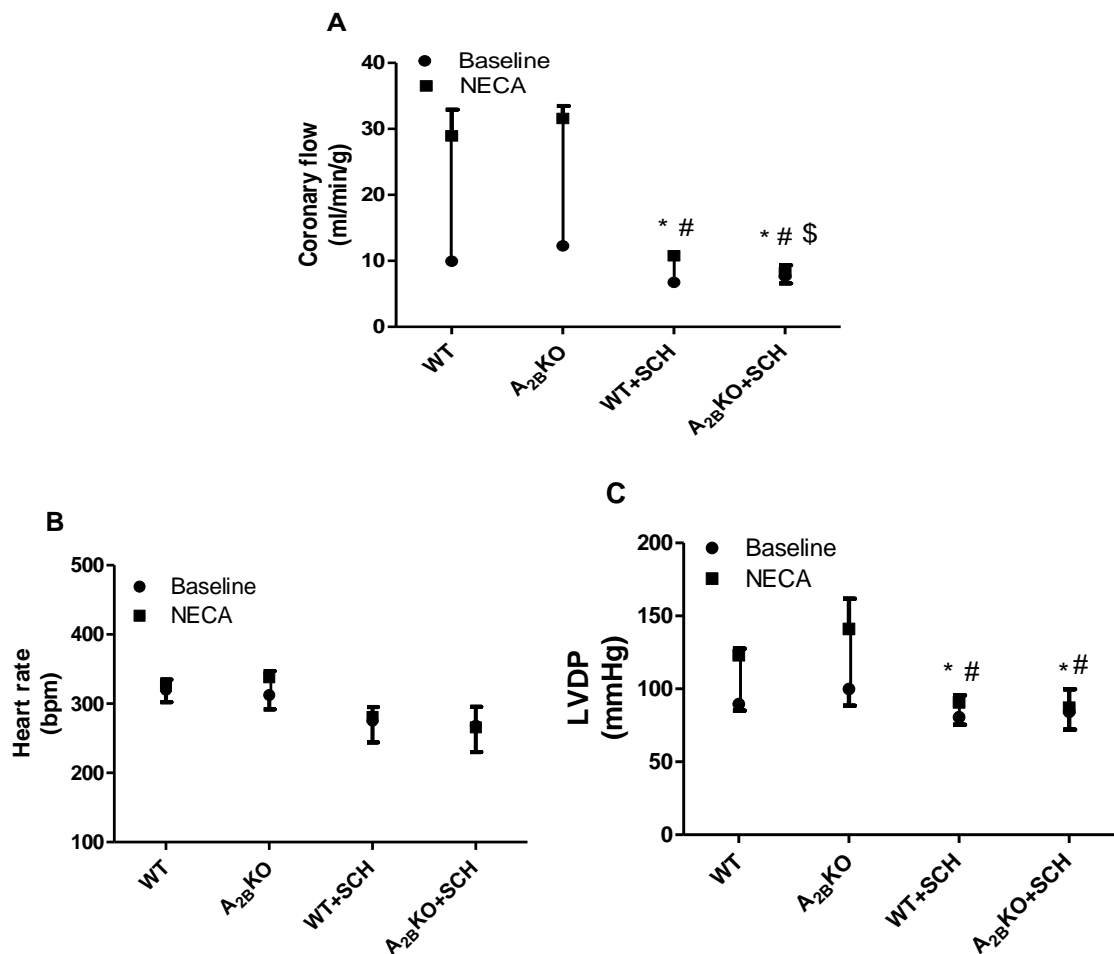
Figure 3.6 Effect of NECA in A$_{2A/2B}$DKO and WT mice on coronary flow (A), heart rate (B), and LVDP (C). *Significant difference compared to WT, $p<0.05$.
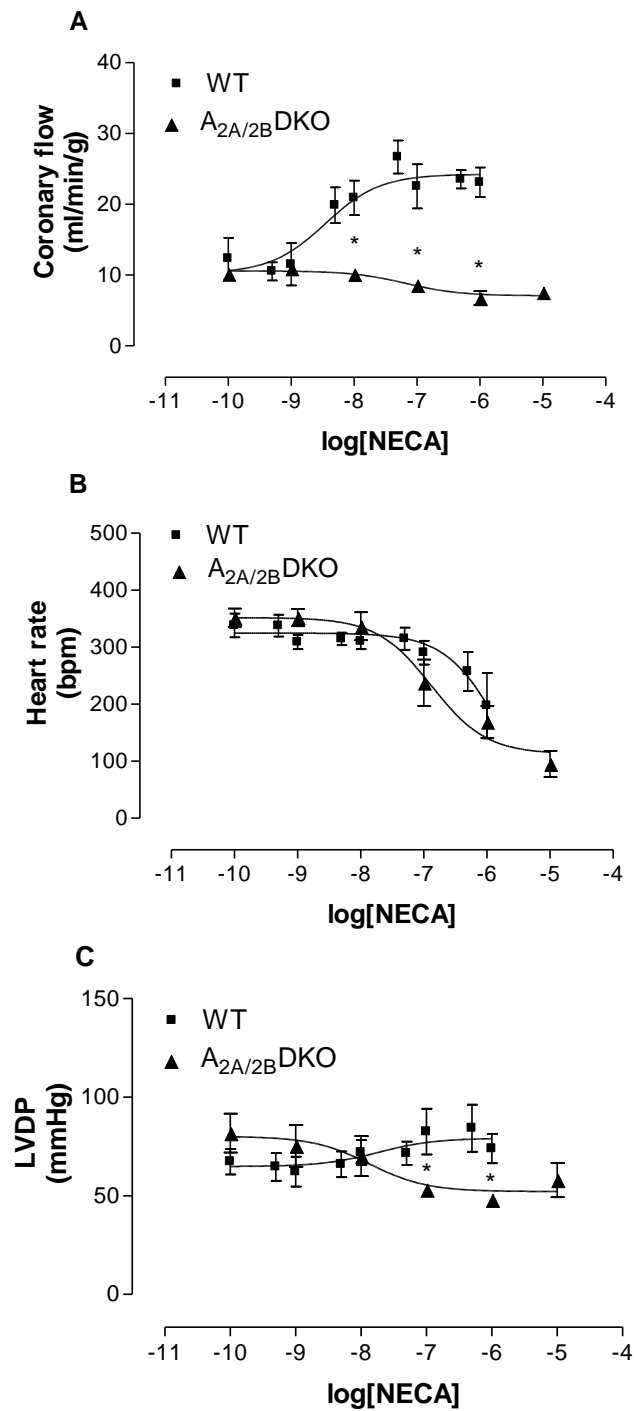
Figure 3.7 Effect of 5-hydroxydecanote (5-HD) on NECA-induced increase in coronary flow (A), heart rate (B), and LVDP (C) in WT and the effect of glibenclamide (GB) on NECA and pinacidil (PIN)-induced increase in coronary flow (A), heart rate (B), and LVDP (C) in WT.*Significant difference in drug-induced effects in the presence of antagonist compared to their corresponding control. #Significant difference in baselines in the presence of antagonist compared to their corresponding control, $p<0.05$.
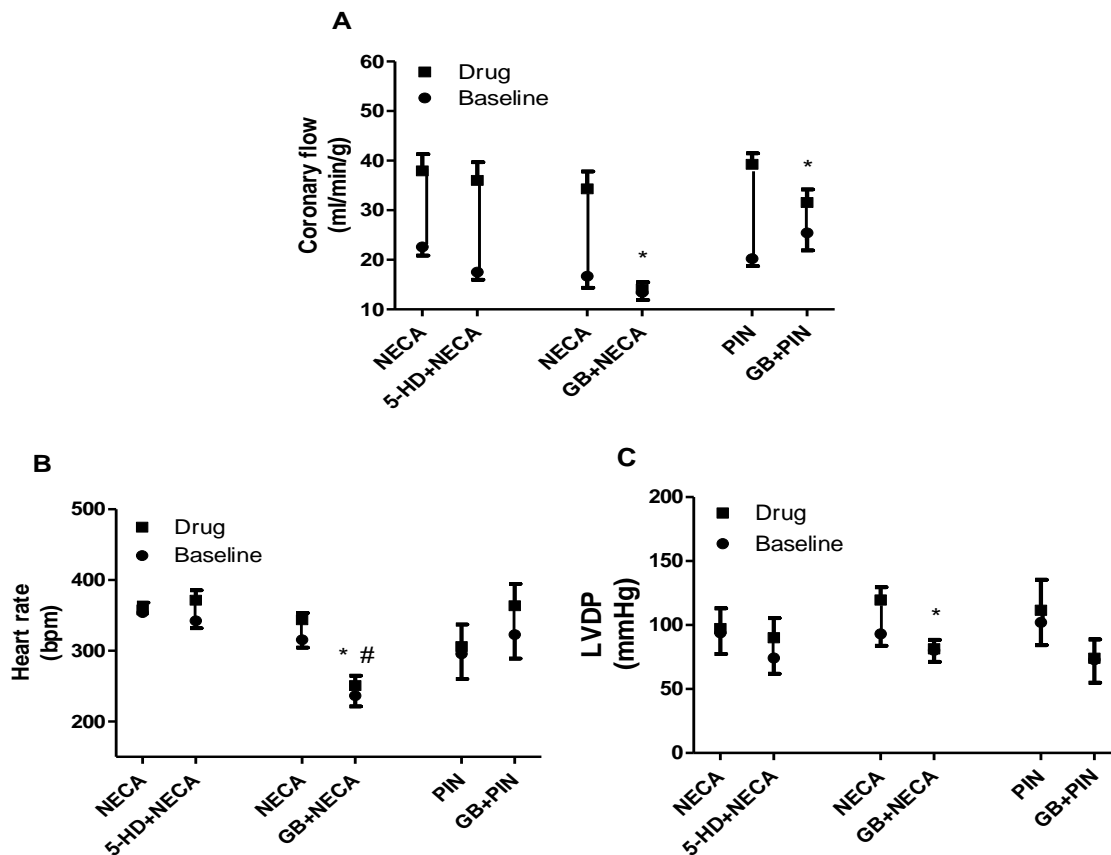
Figure 3.8 Effect of glibenclamide (GB) on NECA-induced increase in coronary flow (A), heart rate (B), and LVDP (C) in A$_{2A}$ KO and A$_{2B}$ KO. *Significant difference in drug-induced effects in the presence of antagonist compared to their corresponding control, $p<0.05$.
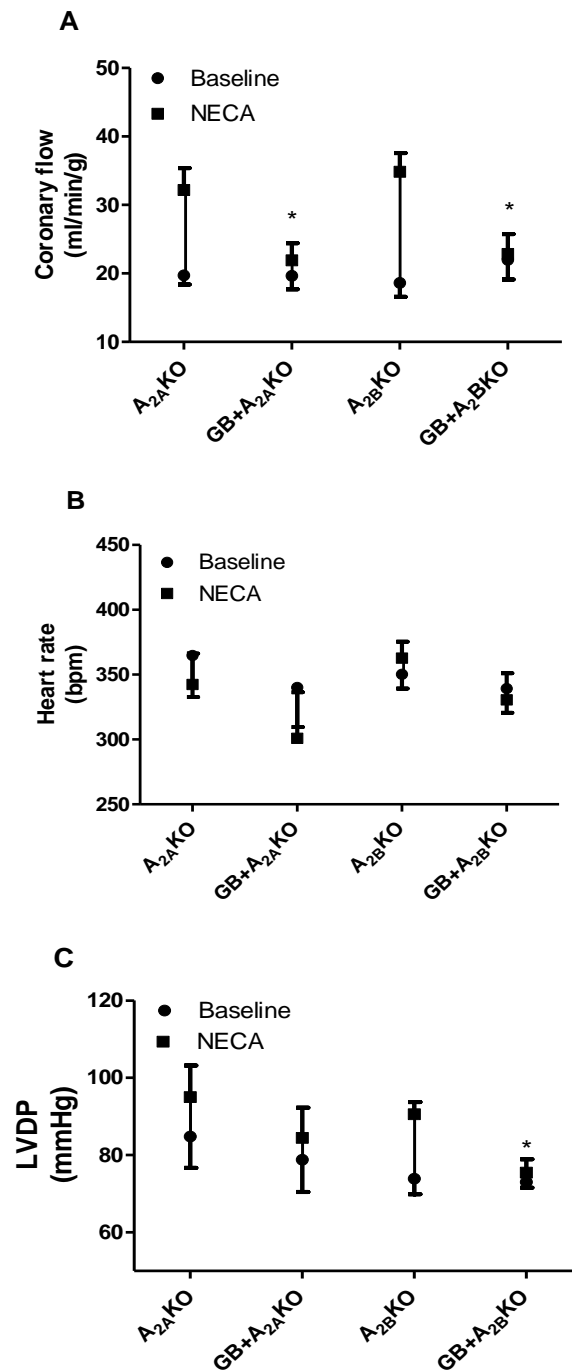
Figure 3.9 Effect of glibenclamide (GB) on CGS 21680 (CGS) and BAY 60-6583 (BAY)-induced increase in coronary flow (A), heart rate (B), and LVDP (C) in WT. *Significant difference in drug-induced effects in the presence of antagonist compared to their corresponding control. #Significant difference in baselines in the presence of antagonist compared to their corresponding control, $p<0.05$.
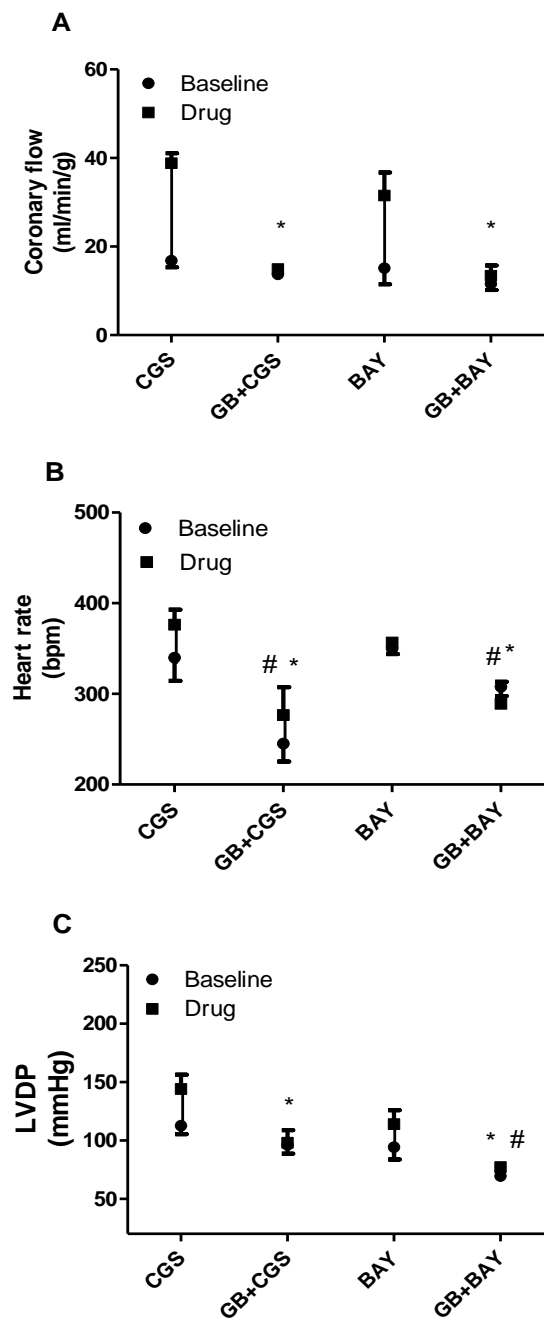
Table 3.1

Baseline data for WT, $A_{2A}KO$, $A_{2B}KO$, and $A_{2A/2B}DKO$ mice hearts.

	Wild-Type n=30	A_{2A}KO n=12	A_{2B}KO n=29	$A_{2A/2B}$DKO n=15
Body weight, g	25±0.6	25±1.2	23.8±0.6*	24.7±1.1
Heart weight, g	0.1	0.1	0.1	0.1
Heart-to-body weight ratio, %	0.5±0.4	0.4	0.4±0.1	0.4
Coronary flow, ml.min^{-1}.g^{-1}	15.2±0.8	17.2±0.9	14.5±0.7	15.6±1.8
Heart rate, beats/min	341.6±10.3	356.8±14.5	345.1±7.8	330.4±17.8
Developed pressure, mmHg	80.3±4.2	81.2±4.8	83.3±3.3	97.1±6.2*

All parameters were collected after 30 min of equilibration in langendorff preparation.

All values are means± S.E.M.* significantly different compared to WT.

Discussion

In this study we have further elucidated the contribution of $A_{2B}AR$ and its relationship to $A_{2A}AR$ in A_2ARs-mediated coronary artery vasodilation. With the use of $A_{2A/2B}DKO$ mice, we also showed for the first time that A_{2A} and $A_{2B}ARs$, out of four ARs subtypes, contribute to coronary artery vasodilation and down-regulation/deletion of either of A_{2A} and $A_{2B}ARs$ leads to a compensatory up-regulation of the other AR and thus, suggesting an interrelationship between these two receptor subtypes. Furthermore, we showed for the first time the involvement of non-mitochondrial K_{ATP} channels in $A_{2B}AR$-mediated coronary artery vasodilation, and finally showed the direct involvement of K_{ATP} channels in $A_{2A}AR$-mediated increase in coronary flow using AR KO mice.

Adenosine's role in tissue protection may involve mechanisms such as increasing the tissue blood flow and protecting against ischemic damage (37). Adenosine is well known to play a vasoregulatory role in human coronary arteries (16-17, 62-63). We and others have previously also demonstrated the pivotal role for $A_{2A}AR$ in CF regulation (5, 63, 67, 69). We also have preliminary data showing the involvement of A_2AR in coronary reactive hyperemia that indirectly supports a physiological role for adenosine in CF regulation (data not shown) which is in support of previous *in vivo* and *ex vivo* reactive hyperemia studies (5, 12, 77). Additionally, it is suggested that $A_{2A}AR$ activation contributes to basal tone in coronary circulation through the release of nitric oxide (69). Our present data (Figure 5A) also support such a role for $A_{2A}AR$ since SCH58261 ($A_{2A}AR$ selective antagonist) significantly reduced the baseline CF.

Due to its low affinity for adenosine, $A_{2B}AR$ is known to be activated in conditions where a significant increase in adenosine levels is observed, such as in ischemic conditions (25). Therefore, $A_{2B}AR$ may be more involved in pathophysiological

conditions rather than physiological situations. However, cardiovascular effects of $A_{2B}AR$ and its role in coronary vasodilation still remain to be fully elucidated. It was reported that $A_{2B}AR$ plays a role in ischemia/reperfusion and preconditioning (15). Additionally, previous studies have used indirect measures such as non-selective agonist/antagonist (alloxazine), which is only about 10 fold more selective towards $A_{2B}AR$ relative to $A_{2A}AR$ (8), or $A_{2A}KO$ mice (without the use of A_{2B} knockout) to indirectly study the role of $A_{2B}AR$ in CF regulation (48, 67). In this study, we demonstrated the contribution of both A_{2A} and $A_{2B}ARs$ in coronary artery vasodilation where we further clarified the individual role of $A_{2B}AR$ with the use of selective $A_{2B}AR$ agonist BAY 60-6583 and $A_{2B}KO$ mice (Figure 1). We also demonstrated that A_{2A} and $A_{2B}ARs$, out of all ARs, are involved in inducing coronary artery vasodilation with the use of $A_{2A/2B}DKO$ mice (Figure 6). Reports suggesting that A_1 and A_3ARs negatively modulate the role of A_{2A} and $A_{2B}ARs$ in CF regulation (66, 68) and the presence of only A_{2A} and $A_{2B}ARs$ on human and porcine coronary endothelial cells (53) may also indirectly support their involvement in coronary artery vasodilation.

Azakura et. al. suggested that the impairment of adenosine-related signaling contributes to the pathophysiology of congestive heart failure (2). In addition, we have previously demonstrated that $A_{2A}AR$ is up-regulated in high-salt compared to normal-salt fed mice (51) and failure to up-regulate $A_{2A}AR$-induced pathway contributes to the development of hypertension (44). Furthermore, few studies have shown that $A_{2B}AR$ is selectively up-regulated in ischemic mouse hearts and by hypoxic conditions (3, 18) and differential expression of ARs contributes to functional heterogeneity of human endothelial cells (21). We have recently shown the up-regulation of $A_{2B}AR$ in $A_{2A}KO$ mice (69) and in this current study we demonstrated the up-regulation of $A_{2A}AR$ in

A_{2B}KO mice (Figures 2-5), suggesting the presence of a relationship between these two ARs. Therefore, further understanding of the relationship between A_{2A} and A_{2B}ARs may help us towards development of newer therapeutic approaches. Also, the observation that the down-regulation/deletion of A_{2A} or A_{2B}AR leads to compensatory up-regulation of A_{2B} and A_{2A}AR, respectively, itself supports an important regulatory function for each of these AR subtypes. It is interesting to mention that this trend is observed in hypoxia of human umbilical vein endothelial and bronchial smooth muscle cells where hypoxia modulates the expression of ARs by decreasing A_{2A}AR mRNA but increasing A_{2B}AR mRNA levels (22). It is also noteworthy to mention that adenosine acts as a feed-back mediator in heart during hypertrophy and hypoxia (34) during which conditions other studies have shown the modification of ARs' expression (2-3, 18, 22). These kinds of interactions have also been observed between other receptor subtypes such as adrenoceptors (ADR), where α_1ADR compensates for the down-regulation of βADR in pathological conditions such as cardiac remodeling (75).

Adenosine and its agonist are currently being used in clinical settings; The vasoregulatory properties of ARs are used clinically in humans for the diagnosis of coronary artery disease through the use of Adenosine (Adenoscan) as a substitute for exercise stress testing in myocardial perfusion imaging (7). The use of Adenoscan has its own limitations due to it blocking the AV conduction (due to activation of A_1AR (4, 7)) and inducing bronchospasm (probably due to activation of A_1, A_3 or A_{2B}ARs (24, 59)). However, these side effects may be alleviated by the development of more A_2AR subtype selective agonists. A new A_{2A}AR selective agonist called Regadenoson (Lexiscan®) has been developed and approved by FDA which is being used clinically in myocardial perfusion imaging. During this use of adenosine and its agonist in determining coronary

reserve, the assessment of the true maximal dilatation of coronary arteries is an important factor (34). However, it is reported that maximal dilatation of coronary arteries with vasodilators such as adenosine does not eliminate all coronary vasomotor tone and that the role of hemodynamic changes should also be considered (34, 73). Nevertheless, the knowledge of the presence of a compensatory interaction between A_{2A} and A_{2B}ARs may be helpful since most cardiac imagings are done in patients with vascular disease where the AR expression may have been already modified due to the disease condition. Furthermore, adenosine is being investigated as a treatment during percutaneous coronary intervention in order to reduce the myocardial reperfusion injury in patients with myocardial infarction (which is usually associated with worsening of cardiac injury and function). In these patients, intracoronary injection of adenosine is followed by a better myocardial salvage and thus prevention of left ventricular remodeling, ejection fraction, decreased infarct size, and ST-segment elevation resolution (10, 26, 46). However, Desmet et. al. demonstrated that adenosine significantly ameliorates ST-segment resolution while it does not improve myocardial salvage or coronary blood flow (11). Additionally, in another study, Ross et. al. showed a relationship between infarct size and primary clinical end point (death or heart failure) where adenosine significantly reduced the infarct size while, it did not improve the clinical outcome (61).

It is well known that arterioles, compared to coronary conduit arteries, are the major players in flow regulation and as previously reported, A_{2A}AR may be the major coronary artery vasodilator compared to A_{2B}AR. Furthermore, large coronary arterioles were shown to be less responsive to adenosine than small ones and Hein et. al. have reported that mainly A_{2A}ARs are expressed in coronary arterioles (32, 42). The same disparity of receptor subtype distribution has also been seen with adrenoceptors, with

adrenergic constriction of large arterioles being mediated by α_1 while in terminal arterioles α_2 exert this function (56). Additionally, in coronary arterioles, a greater α_2 response has been shown compared to larger coronary arteries (70). Thus, showing the up-regulation of $A_{2A}AR$ in $A_{2B}KO$ mice would most likely not be reflected on larger coronary conduit arteries such as LAD. Isolation of mouse LAD and other large coronary conduit arteries is technically difficult but possible, however isolation of coronary resistance arterioles from mouse heart is almost impossible and to our knowledge no one has been able to isolate coronary arterioles so far which is a limitation in our study. However, $A_{2A}AR$ protein expression in mouse mesenteric artery resistance vessels (up to third branch of arterioles) shows the up-regulation of $A_{2A}AR$ in $A_{2B}KO$ mice which confirms the compensatory up-regulation of $A_{2A}AR$ in $A_{2B}KO$ mice resistance vessels (Figure 3).

The occurrence of the up-regulatory mechanism phenomenon in our studies may be TNF-α-induced and post-transcriptionally regulated (41) or due to DNA methylation since this may regulate AR cell surface expression levels (9). Furthermore, HIF-1 has also been suggested to up-regulate the expression of $A_{2B}AR$ in hypoxic conditions (76). Although, the underlying mechanism may not be understood at this time, it seems that this relationship only exists between A_{2A} and $A_{2B}ARs$ and not between A_1 or A_3ARs since there was no significant difference between the concentration response curve for CCPA (A_1AR selective agonist) and Cl-IBMECA (A_3AR selective agonist) in $A_{2A/2B}DKO$ mice compared to WT (data not shown).

Potassium channels have been suggested to be involved in regulating blood flow and blood pressure and in AR signaling pathways (29, 49, 78). For example, inward rectifier K^+ (K_{IR}) channels may play a role since their presence has been reported on

coronary artery smooth muscle cells (CASMCs) (54, 60, 65), in addition, to their role as the dominant K^+ conductance in resting membrane potential (38). Moreover, adenosine is reported to activate ATP-sensitive potassium (K_{ATP}) channels in rabbit mesenteric arteries and isolated CASMCs (58, 65). Others have demonstrated the presence of K_{ATP} channels on coronary arteries and CASMCs (28, 65) and their role in A_{2A}-induced effect in reactive hyperemia and retinal microvessels dilation (5, 33, 77). However there are no reports to our knowledge suggesting the involvement of K_{ATP} channels in A_{2A} and A_{2B}ARs-induced changes in mouse CF.

In this study, we used mitochondrial K_{ATP} channel blocker, 5-hydroxydeconoate (5-HD) and glibenclamide to test K_{ATP} channels involvement in A_{2A} and A_{2B}ARs-induced CF changes. Glibenclamide blocked the NECA-induced increase in CF in A_{2A} and A_{2B}KO and BAY60-6583-, and CGS21680-induced increase in CF in WT mice. However, 5-HD had no effect on NECA-induced increase in CF, which may suggest a role for non-mitochondrial K_{ATP} channels in both A_{2A} and A_{2B}ARs signaling pathways (Figures 7-9A). However, this study does not show if the K_{ATP} channels are located on endothelial or SMCs. This is important since endothelial dysfunction is an early risk factor for cardiovascular diseases where a reduced adenosine response has been reported (19). Further, Wang et. al. suggested that activation of endothelial K_{ATP} channels might result in the protection against endothelial dysfunction (74). We think that adenosine-induced activation of K_{ATP} channels may be indirectly through release of some other mediators such as hydrogen peroxide (27), which was previously suggested to be the endothelium-derived hyperpolarizing factor (47) and that may be involved in cardiovascular dysfunction (64). Elucidations of these signaling pathways will help us

better understand the underlying cause of reduced adenosine response in cardiovascular diseases.

The availability of gene deleted knockout mice has been an important tool to dissect the physiological and pharmacological pathways for elucidating the role of a single receptor. However, in some instances, mice lacking a specific receptor gene exhibit phenotypic differences such as higher blood pressure in $A_{2A}KO$ mice (43). Therefore, in this study we used both pharmacological and molecular (single and double gene knockout) approaches to confirm our results. We also evaluated the baseline parameters of WT and KO hearts (Table 1). The baseline LVDP of $A_{2A/2B}DKO$ was significantly higher compared to WT, which may be attributed to alterations at second messenger or translational levels. The relationship between A_{2A} and $A_{2B}ARs$ and activation of other signaling pathways such as an increase in the levels of cAMP and Ca^{2+} may compensate for the deletion of $A_{2A/2B}ARs$ (35, 37). Even adrenoceptors may compensate for the absence of A_{2A} and $A_{2B}ARs$ since there are reports showing direct and indirect anti β-adrenergic effect of adenosine, suggesting the presence of an interaction between these two different GPCRs (49). Further experiments are required to better understand the relationship between these two receptor classes. There are no significant differences in baseline HR, LVDP, and CF of $A_{2A}KO$ and $A_{2B}KO$ mice compared to WT. We also tested the $A_{2B}KO$ mice for endothelium functionality using bradykinin where we found no significant difference between the $A_{2B}KO$ and WT groups, thus, showing the presence of a normal functional endothelium in $A_{2B}KO$ (data not shown).

CGS21680 and NECA induced an increase in LVDP of WT and $A_{2B}KO$ but not $A_{2A/2B}DKO$. BAY60-6583 also induced an increase in LVDP of WT mice. These data support our previous report that A_{2A} and $A_{2B}ARs$ may be involved in cardiac contractility

and positive inotropic effect (Figures 1, 2, and 4C) (69). Changes in CF can affect the contractility (Gregg effect) which is an inherent problem with the isolated perfused heart preparation. However, as shown in this study (Figures 2 and 6), the increase in CF comes earlier than the changes in LVDP which may suggest that the observed effects of A_2ARs activation on LVDP may not be due to Gregg's effect. The opening of K_{ATP} channels may also affect the contractility independent of CF changes which may be independent of non-mitochondrial K_{ATP} channels in this study since there is no difference observed in NECA-induced changes in LVDP in the presence of 5-HD (mitochondrial K_{ATP} channel blocker) while a significant decrease in NECA-induced changes in LVDP was shown in the presence of glibenclamide (non-selective K_{ATP} channel blocker) (Figure 7). Additional studies are needed to define the role of A_2ARs on LVDP. However, our data from $A_{2A/2B}DKO$ are very important since they show that only A_{2A} and $A_{2B}ARs$ are involved in LVDP and coronary vasodilation which could be further exploited for therapeutic approaches such as heart failure.

Our current study also confirms our previous finding that A_1AR plays a role in HR (68) while A_{2A} and $A_{2B}ARs$ do not since BAY60-6583 (Figure 1B) and CGS21680 (Figure 4B) did not affect HR while NECA (non-selective agonist) decreased the HR (Figure 2B). Additionally, our data showed a decrease in baseline HR in the presence of glibenclamide (Figures 7 and 9B) which could be due to the presence of K_{ATP} channels on atria and ventricle (79) and the K_{ATP} channel-dependent potassium efflux-induced shortening of cardiac action potentials and hence a decrease in HR (79).

In conclusion, we showed for the first time, the individual role of $A_{2B}AR$ and the contribution of $A_{2A}AR$ in A_2ARs-mediated coronary vasodilation using $A_{2B}KO$ and $A_{2A/2B}DKO$ mice along with the use of $A_{2B}AR$ and $A_{2A}AR$ selective agonists. We found

that A_{2A} and A_{2B}ARs contribute to coronary artery vasodilation which involves K_{ATP} channels and that A_{2A}AR is up-regulated in A_{2B}KO mice. These findings are another step toward a better understanding of ARs' pharmacology in coronary artery and the heterogeneity of CF responses by ARs which may lead to better therapeutic approaches for the treatment of cardiovascular disorders.

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CHAPTER FOUR

Interactions between A_{2A} adenosine receptor, hydrogen peroxide, and K^+-ATP channel in coronary reactive hyperemia

Abstract

Myocardial metabolites such as adenosine mediate reactive hyperemia (RH), partially, by activating ATP-dependent K^+ (K_{ATP}) channels in coronary smooth muscle. In this study, we investigated the role of A_2 adenosine receptors (ARs) and their downstream signaling mechanisms in RH. We tested the hypothesis that coronary RH involves A_{2A}ARs, hydrogen peroxide (H_2O_2), and K_{ATP} channels using A_{2A} and A_{2B} single (KO) and $A_{2A/2B}$ double knockout (DKO) mice hearts in a Langendorff heart. Flow debt-to-repayment ratio (RPA//DA) following a 15s occlusion was 1.3±0.1 in WT which was reduced in hearts from A_{2A}KO (0.9±0.1), but not A_{2B}KO (1.2±0.1) mice. Catalase (1250 U/ml), an enzyme that breaks down H_2O_2, significantly reduced the baseline flow (ml/min/g) in A_{2A}KO hearts (16.8±0.9) compared with control (19.4±1.6) and WT in the presence and absence of catalase (21.4±2.1 and 19.8±0.9, respectively). However, catalase significantly reduced the RPA/DA in WT (0.8±0.1) but, not in A_{2A}KO (0.9±0.1) mice compared with its control. Patch clamp experiments demonstrated that adenosine (10μM) activated a glibenclamide (10μM; a K_{ATP} channel blocker)-sensitive conductance (nS/pF) in smooth muscle cells from WT (0.048±0.01) but not A_{2A}/A_{2B}DKO (0.016±0.01) mice. Importantly, however, conductance activated by pinacidil (10 μM; a K_{ATP} channel opener) was similar in cells from WT and A_{2A}/A_{2B}DKO mice (0.013±0.034 vs. 0.012±0.04 nS/pF). Additionally, in WT smooth muscle cells, H_2O_2 (1 mmol/L) activated a glibenclamide-sensitive conductance (0.074±0.02 nS/pF). Our data indicate that A_{2A}ARs are coupled to K_{ATP} channels in RH, in part, via the production of H_2O_2 as a signaling intermediate.

Keywords: coronary circulation, ischemic vasodilation, adenosine receptors, knockout mice, reactive oxygen species

Introduction

The heart responds to acute ischemia by transiently increasing blood flow in a phenomenon called reactive hyperemia (RH) (7). The temporary reduction in coronary vascular resistance is mediated by chemical signals released into blood (47), including adenosine (44). Adenosine induces its effects through activation of its receptors (AR) namely A_1, A_{2A}, A_{2B}, and A_3. Adenosine's cardiovascular effects depend on the activation of the subtype of receptor involved, where A_2ARs are known to play a role in coronary vasodilation through their action on both endothelial and smooth muscle cells. Our laboratory and others have shown that $A_{2A}ARs$ play a pivotal role in the regulation of coronary flow (CF) (48, 50), while, $A_{2B}ARs$ are also involved, albeit with a lesser contribution compared to $A_{2A}ARs$ (32, 48, 50). Additionally, pharmacological studies indicate that A_{2A} and $A_{2B}ARs$ are the subtypes most likely involved in coronary RH (2, 11, 62). However, undesirable overlap in the pharmacological profiles of adenosine receptor antagonists obfuscates the relative contribution of A_{2A} and $A_{2B}ARs$ in RH. Thus, in order to more specifically determine the roles of A_{2A} and $A_{2B}ARs$ in coronary RH, we used Langendorff-perfused hearts from wild type (WT) and A_{2A}, A_{2B} knockout (KO) and A_{2A} and A_{2B} double knockout (DKO) mice.

Numerous mediators and end effectors of coronary RH have been proposed. Importantly, however, information remains scarce regarding the role of H_2O_2, a coronary metabolic and endothelium-dependent vasodilator (29, 45, 58), and its interactions with ARs and K_{ATP} channels. Some support for H_2O_2 in RH has been provided by studies of skeletal muscle and isolated coronary arterioles (3, 20). The idea that K_{ATP} channels participate in coronary RH is well established (1, 12), but it is unknown whether H_2O_2 couples adenosine receptor activation to K_{ATP} channel activity. K_{ATP} channels are known to mediate, at least in part, H_2O_2-induced dilatation of arterioles from skeletal muscle and brain (25, 55) and cardiac myocytes (18). However, to our knowledge, direct evidence of this mechanism in smooth muscle is still to be elucidated. Thus, we

ascertained whether H_2O_2 is involved in coronary RH and whether H_2O_2 mediates the coupling of adenosine receptors to activation of K_{ATP} in coronary RH.

Materials and Methods

Animals. An Institutional Animal Care and Use Committee at 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. A_{2A} and A_{2B} knockout mice, both backcrossed 12 generations to the WT C57BL/6 background (Jackson Laboratory; Bar Harbor, ME), were bred to generate A_{2A}/A_{2B} double heterozygotes. Double heterozygotes were intercrossed, 1/16 of the offspring were A_{2A}/A_{2B} double knockouts, and A_{2A}/A_{2B} knockout breeding pairs were established. Mice were caged in a 12:12-hr light-dark cycle with free access to standard chow and water.

WT, $A_{2A,}$ and $A_{2B}KO$ and $A_{2A/2B}DKO$ mice. WT mice of a mixed C57/BL6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, Maine). All four WT, $A_{2A}KO$, $A_{2B}KO$, and $A_{2A/2B}DKO$ strains were bred at the West Virginia University animal facility as a sub-colony of the original strain. All animal care and experimentation were in accordance with the West Virginia University Institutional Animal Care and Use Committee and the principles of the National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

Langendorff-perfusion. Mice (10-14 wks of age) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and hearts were excised into heparinized (5 U/ml) ice-cold Krebs-Henseleit (KH) buffer. KH contained (mmol/L) 119 NaCl, 11 glucose, 22 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 2 pyruvate and 0.5 EDTA. Hearts beat spontaneously when retrogradely perfused (80 mmHg) with 37°C KH bubbled with 95% O_2 and 5% CO_2. The left atrium was removed and the left ventricle drained. A fluid-filled balloon was inserted into the left ventricle and connected to a transducer for pressure measurements. Left ventricular diastolic

pressure was adjusted to 2-5 mmHg. Coronary flow was measured with a probe (Transonic Systems; Ithaca, NY) in the aortic perfusion line. Hearts were paced to 400 beats/min and function allowed to stabilize. Hearts with persistent arrhythmias or developed pressure less than 80 mmHg were excluded from the study. Baseline coronary flow and cardiac function were measured using a Power Lab data acquisition system (AD Instruments; Colorado Springs, CO). Hearts were then subjected to 15 seconds of total flow occlusion to elicit hyperemia. Catalase (Sigma Chemical Co.; St. Louis, MO) was delivered into the aortic perfusion line using a microinjection pump (Harvard Apparatus; Holliston, MA) as 1% of coronary flow to achieve a final concentration of 1250 U/ml (57, 60).
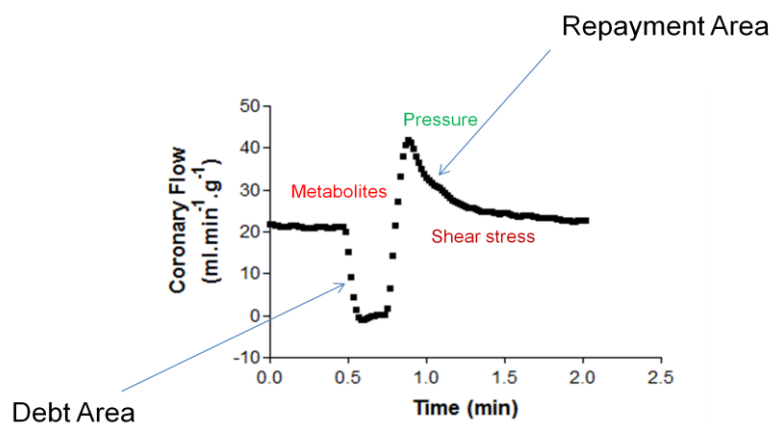
Patch clamp. Single smooth muscle cells were enzymatically isolated from the aortas of WT and A_{2A}/A_{2B} knockout mice. For cell isolation, a simple HEPES-buffered saline was used and contained (mmol/L) 135 NaCl, 5 KCl, 1 $MgCl_2$, 0.36 $CaCl_2$, 10 glucose, 10 HEPES, and 5 Tris; pH 7.4. The aorta was placed in this solution plus (mg/ml) 2 collagenase, 1 elastase, 2 bovine serum albumin, and 1 soybean trypsin inhibitor for 15 min at 37°C. The tissue was passed through the tip of a fire-polished Pasteur pipette to liberate single cells. Smooth muscle cells were pelleted at 0.5 g, resuspended in enzyme-free buffer, placed on ice, and used within 8 hr. Cells were placed in a recording chamber atop an inverted microscope and perfused with a HEPES-buffered solution (as above) containing 140 mmol/L K^+. Patch pipettes (3-5 MOhms) were filled with this 140 mmol/L K^+ solution supplemented with 5 mmol/L Mg-ATP and 0.1 mM Na-GTP;

pH 7.1. Inward K_{ATP} currents were recorded in the conventional whole-cell mode (with Nernst equilibrium potential for K^+ set to 0 mV). The membrane potential was held at -80 and ramped or stepped to potentials between -100 and +100 mV. Currents were low-pass filtered a 1 kHz and digitized at 5 kHz. Series resistance and whole-cell capacitance were compensated as completely as possible using circuitry of the amplifier (PC-505, Warner Instruments; Hamden, CT). This amplifier was interfaced to a computer with pClamp 9 software for data acquisition and analysis (Molecular Devices; Sunnyvale, CA).

Statistical analysis. Statistical analyses were made with *t*-test and one- or two-way analysis of variance (ANOVA) with post hoc tests as indicated. Results were considered significant when $P < 0.05$. Values are means ± SEM from *n* number of animals.

Results

Involvement of A_{2A}ARs in coronary reactive hyperemia. We used WT and A_{2A}KO mice in order to test the involvement of A_{2A}ARs in coronary RH. The flow debt-to-repayment ratio was significantly reduced in A_{2A}KO (0.9±0.1, n=8) compared with WT (1.3±0.1, n=6) mice (Figure 1B) while, the peak flow was not different between the two groups (WT: 42.7±2 ml/min/g, n=6 *vs.* A_{2A} KO: 42.9±1.7 ml/min/g, n=8, Figure 1C). The resting flow of 19.4± 1.6 ml/min/g (n=8) in A_{2A}KO was similar to WT (19.8 ± 0.9 ml/min/g, n=6, Table 1).

A_{2B}ARs may not be involved in coronary reactive hyperemia. In order to characterize the role of A_{2B}ARs in coronary RH, we performed our RH experiments in WT and A_{2B}KO mice. The flow debt-to-repayment ratio (Figure 2B) and peak flow (Figure 2C) in A_{2B}KO, 1.2±0.1 and 44.2±0.5 ml/min/g (n=4), respectively, were not different compared with WT, 1.3±0.1 and 42.7±2 ml/min/g (n=6), respectively. Additionally, the resting flow of A_{2B}KO, 20.3±1.1 ml/min/g was not different compared with WT, 19.8±0.9 ml/min/g, respectively (Table 1).

Involvement of only A_2ARs in coronary reactive hyperemia. Since we have previously shown that only A_{2A} and A_{2B}ARs, out of four ARs, are involved in the regulation of CF (48), we

tested the involvement of other ARs than A_2ARs in coronary RH using WT and $A_{2A/2B}DKO$ mice. The flow debt-to-repayment ratio was significantly higher in WT (1.3 ± 0.1, n=6) compared with $A_{2A/2B}DKO$ (1 ± 0.1, n=8) mice (Figure 3B), which may be only due to the absence of $A_{2A}ARs$, nevertheless, the peak flow was not different between the two groups, 42.7 ± 2 ml/min/g and 41.6 ± 1.2 ml/min/g, respectively (Figure 3C). Additionally, the resting flow of $A_{2A/2B}DKO$, 18.6 ± 0.6 ml/min/g (n=8) was not different compared with WT (Table 1).

Adenosine activates K_{ATP} channels. It is known that K_{ATP} channels play a role in coronary RH (2, 62). We have also previously reported that both A_{2A} and A_{2B} ARs induce coronary vasodilation, and hence an increase in CF, through activation of K_{ATP} channels (48). We established this finding by using our isolated heart system (48). However, in this study we wanted to investigate this finding on a molecular level using patch clamp. Since both A_{2A} and A_{2B} ARs were shown to activate K_{ATP} channels, in this study, we used $A_{2A/2B}DKO$ mice in order to be able to distinguish the currents activated due to activation of ARs. Adenosine (10µmol/L) activated a conductance (nS/pF) sensitive to the K_{ATP} channel blocker, glibenclamide (10µmol/L), in smooth muscle cells from WT (0.048 ± 0.01) but not $A_{2A}/A_{2B}DKO$ (0.016 ± 0.007) mice. Also, pinacidil (10 µmol/L; a K_{ATP} channel opener) activated the same conductance similarly in cells from both WT and $A_{2A}/A_{2B}DKO$ mice (0.013 ± 0.034 vs. 0.012 ± 0.04 nS/pF) (Figure 4).

Involvement of H_2O_2 in adenosine-induced increase in coronary flow. To test if H_2O_2 induces coronary vasodilation, we performed exogenous H_2O_2 concentration-curve. Exogenous H_2O_2 significantly increased the coronary flow from baseline (from 11.3 to 38.1 ml/min/g, data not shown). We used catalase (1250 u/ml), an enzyme which breaks down hydrogen peroxide into water and oxygen, to test its effect on adenosine-induced increase in CF in WT mice. Catalase significantly decreased adenosine-induced increase in CF in WT (from 30 ± 4.4 to 8.4 ± 1 ml/min.g, n=4), $A_{2A}KO$ (from 33.2 ± 2.3 to 9.9 ± 1 ml/min/g, n=4), and $A_{2B}KO$ (from 35.2 ± 1.5 to 13.4 ± 0.8 ml/min/g, n=4) mice (Figure 5A). Catalase also significantly decreased adenosine-induced changes in LVDP in WT (from 122.4 ± 11 to 78 ± 7 mmHg), $A_{2A}KO$ (from 119.2 ± 4.1

to 74.6 ± 4 mmHg), and $A_{2B}KO$ (from 139.1 ± 6 to 81 ± 10.1 mmHg) mice while it had no effect on HR (Figures 5 B-C).

Involvement of H_2O_2 in coronary reactive hyperemia. Since we showed that adenosine is involved in coronary RH and that it induces coronary vasodilation through release of H_2O_2, we wanted to test if H_2O_2 itself is also involved in coronary RH. Therefore, we used catalase (1250 u/ml) in WT mice. Catalase significantly decreased the flow debt-to-repayment ratio in WT compared with its control, 0.8±0.1 (n=6) *vs.* 1.3±0.1 (n=6), respectively (Figure 6B). However, the resting flow was similar to WT in the presence of catalase (21.4±2.1 ml/min/g, n=6) compared to its control (19.8±0.9 ml/min/g, n=6) (Table 1). Nevertheless, the peak flow in WT mice, in the presence of catalase, (38.6±2.4 ml/min/g, n=6) was also significantly attenuated compared to its control (42.7±2 ml/min/g, n=6) (Figure 6C).

Involvement of H_2O_2 in A_2AR-mediatesd coronary reactive hyperemia. We used catalase (1250 u/ml) in $A_{2A}KO$ mice in order to investigate the role of H_2O_2 in $A_{2A}AR$-induced effects in coronary RH. Catalase significantly decreased the flow debt-to-repayment ratio in $A_{2A}KO$ (0.9±0.1, n=6) (Figure 7B) compared with WT in the absence of catalase (1.3±0.1, n=5) (Figures 1,2,3, and 6 B) while, it was not different compared to WT (0.8±0.1, n=6) in the presence of catalase. Also, there was no difference between flow debt-to-repayment ratio in $A_{2A}KO$ in presence of catalase (0.9±0.1, n=6) compared with $A_{2A}KO$ (0.9±0.1, n=8) (Figures 7B). Further resting flow of $A_{2A}KO$ in the presence of catalase (16.8±0.9 ml/min/g) was significantly lower compared with WT (19.8±0.9 ml/min/g) and $A_{2A}KO$ (19.4±1.6 ml/min/g) in the absence of catalase, while there was no difference between the two latter groups (Table 1). The peak flow in $A_{2A}KO$, in the presence of catalase, (36.3±1.4 ml/min/g, n=6) (Figure 7C) was significantly lower compared with WT (42.7±2 ml/min/g, n=6) (Figure 1C) and $A_{2A}KO$ (42.9±1.7 ml/min/g, n=8) in the absence of catalase (Figure 7C); this is when the WT and $A_{2A}KO$ peak flow, in the presence of catalase, were similar (Figure 1C). These findings may suggest that $A_{2A}ARs$ may, at least in part, play their role in coronary RH through release of H_2O_2.

H₂O₂ activates K_{ATP} channels. In smooth muscle cells from WT mice, H_2O_2 (1 mmol/L) activated a glibenclamide-sensitive conductance (0.074 ± 0.02 nS/pF) (Figure 8).

Fig. 4.1 Repayment volume (B), but not resting flow (A) or peak flow (C), is reduced in A$_{2A}$KO (n=8) mice, compared with WT (n=6), in coronary RH. *Significant difference compared with WT, $p<0.05$.
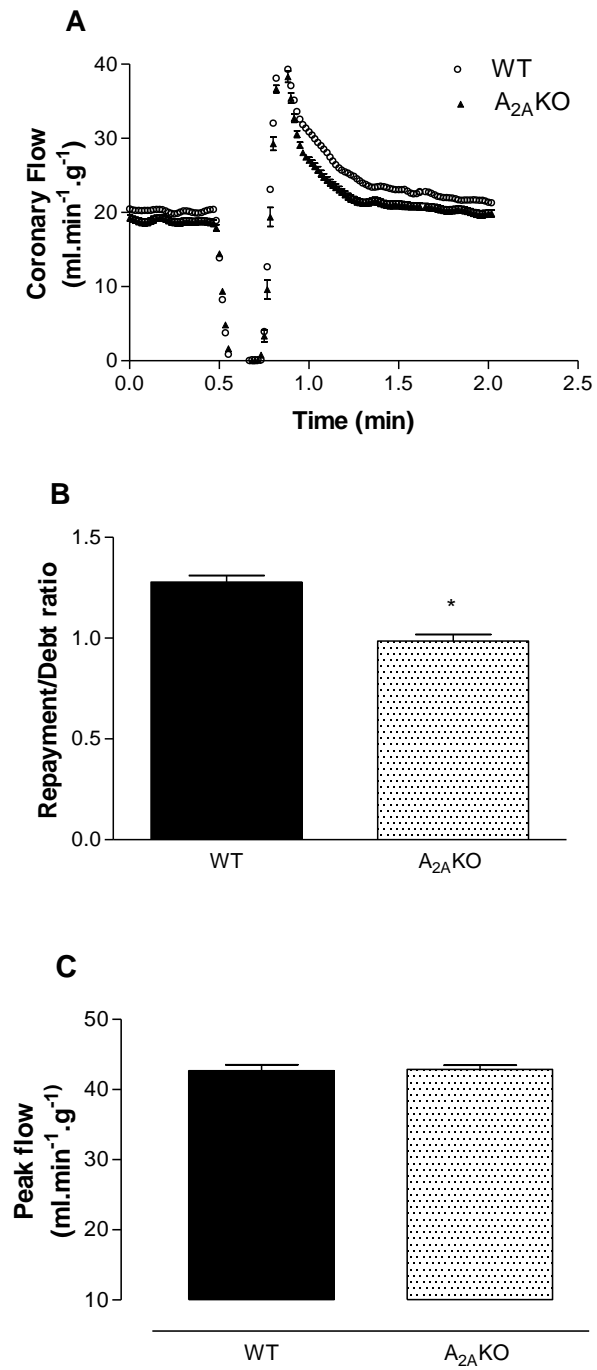
Fig. 4.2 Baseline coronary flow (A), peak flow (B), and flow repayment area (C) of

coronary RH are normal in A$_{2B}$KO (n=4) mice compared with WT (n=6).
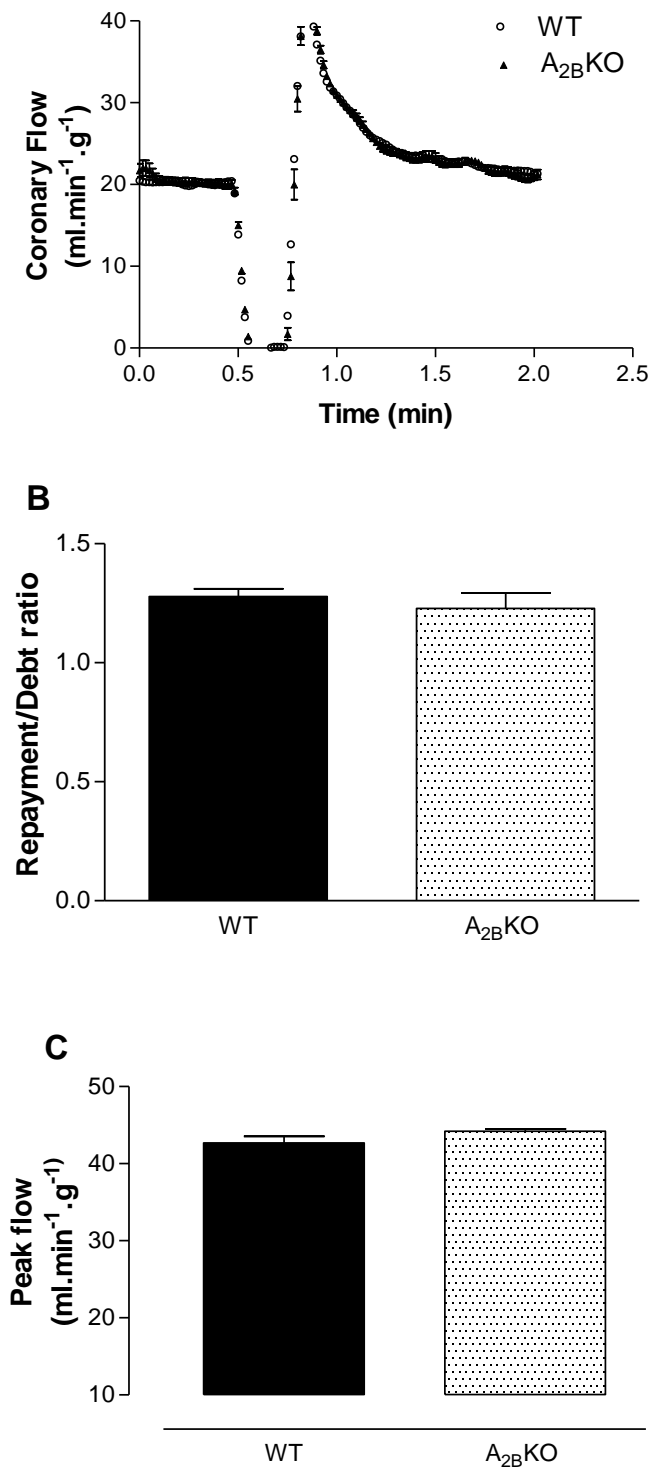
Fig. 4.3 Changes on coronary flow (A), peak flow (B), and flow repayment area (C) in WT (n=6) and A$_{2A/2B}$DKO (n=8) mice during coronary RH. *Significant difference compared with WT, $p<0.05$.
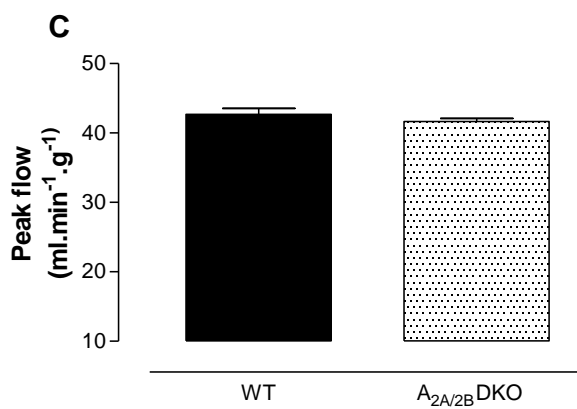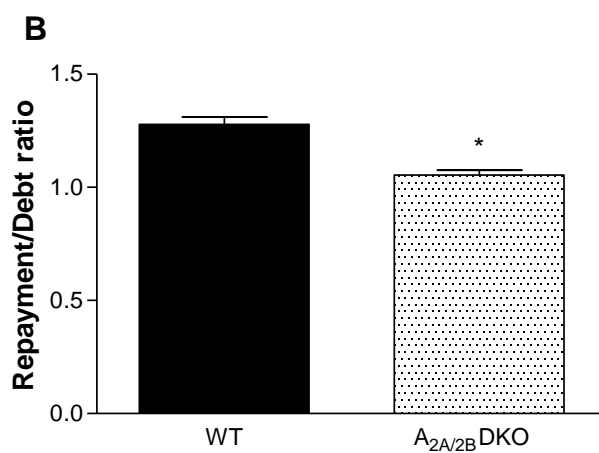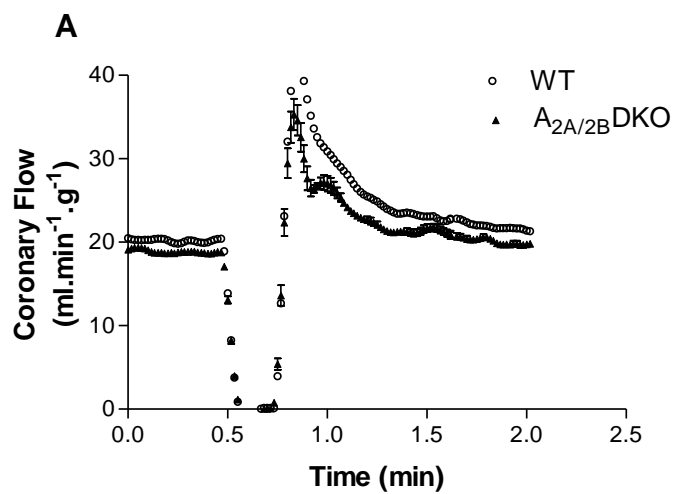
Fig. 4.4 Adenosine-induced KATP current is reduced in smooth muscle cells of A$_{2A/2B}$DKO mice. (A) Glibenclamide-sensitive conductance (nS/pF) in WT and A$_{2A/2B}$DKO mice. (B) Group data showing reduced effect of adenosine in A$_{2A/2B}$DKO mice (n=4), but response to pinacidil were not different from WT (n=5). *Significant difference compared with WT, $p<0.05$.
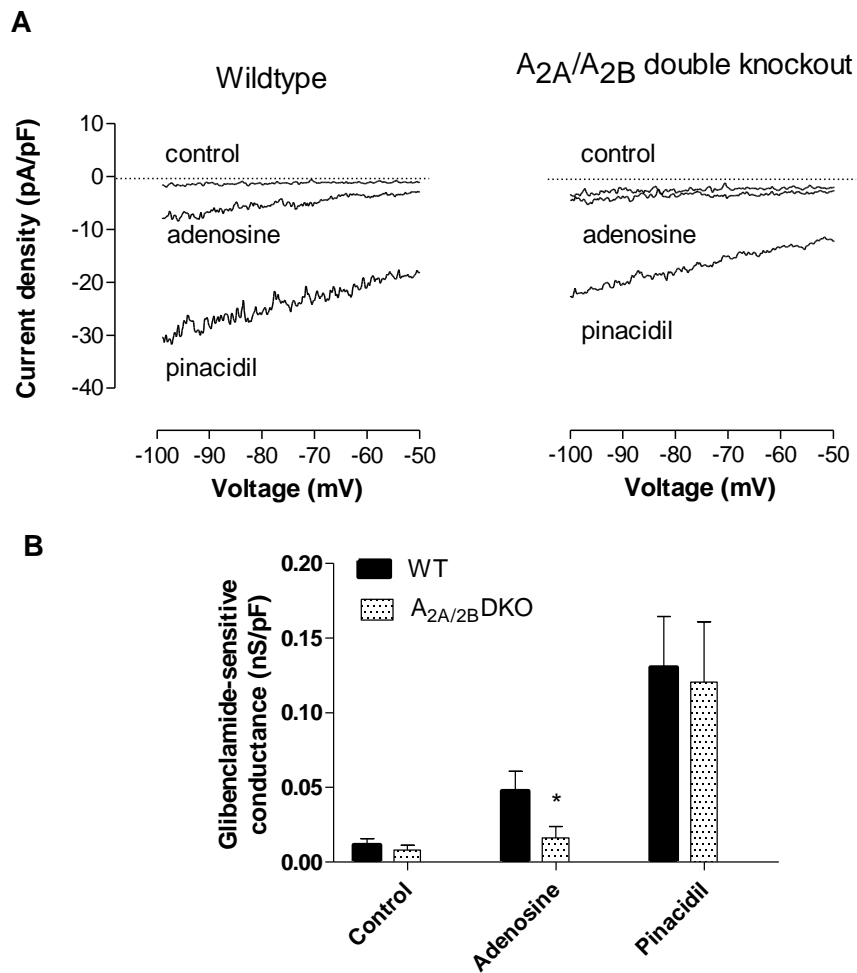
Fig. 4.5 Attenuated adenosine-induced increase in coronary flow (A) and LVDP (C), and normal heart rate (B), in WT (n=4), $A_{2A}KO$ (n=4), and $A_{2B}KO$ (n=4) mice, in the presence of catalase (Cat). *Significant difference compared to corresponding control, $p<0.05$.
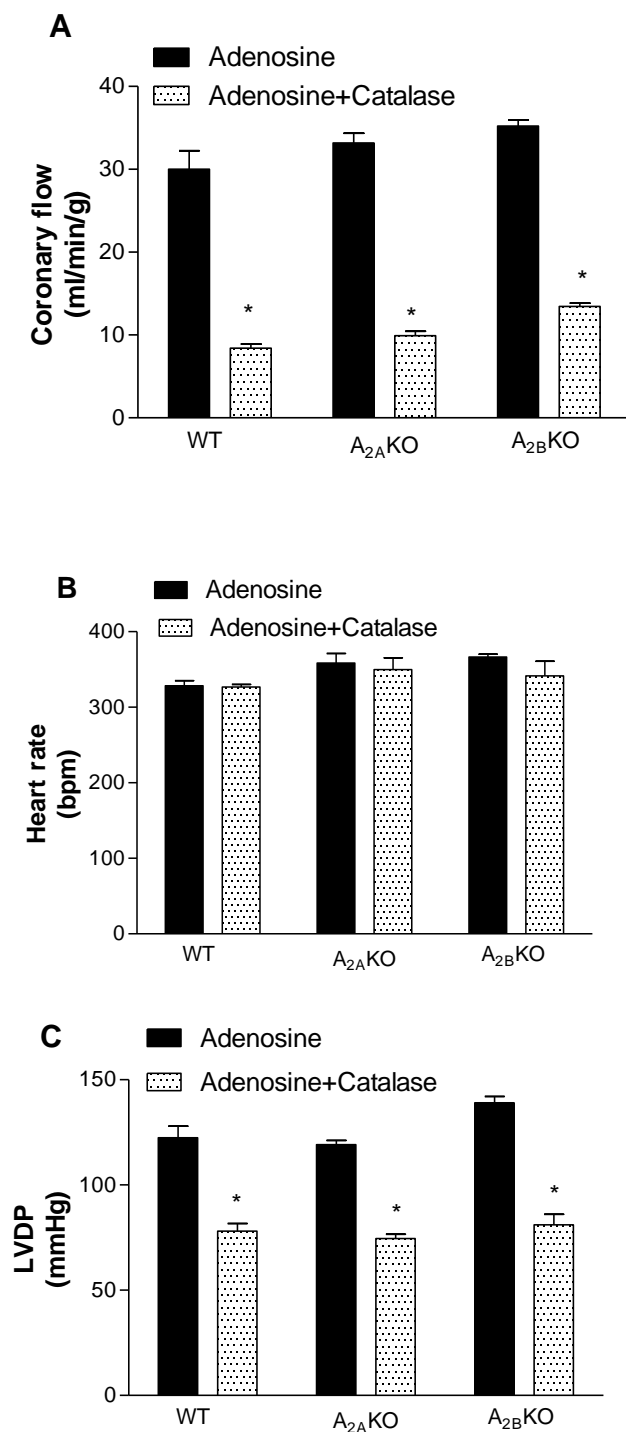
Fig. 4.6 Attenuated peak flow (B) and Repayment area (C), but normal baseline CF (A), in the presence of catalase (Cat) in WT (n=6) mice. *Significant difference compared with WT, *p*<0.05.
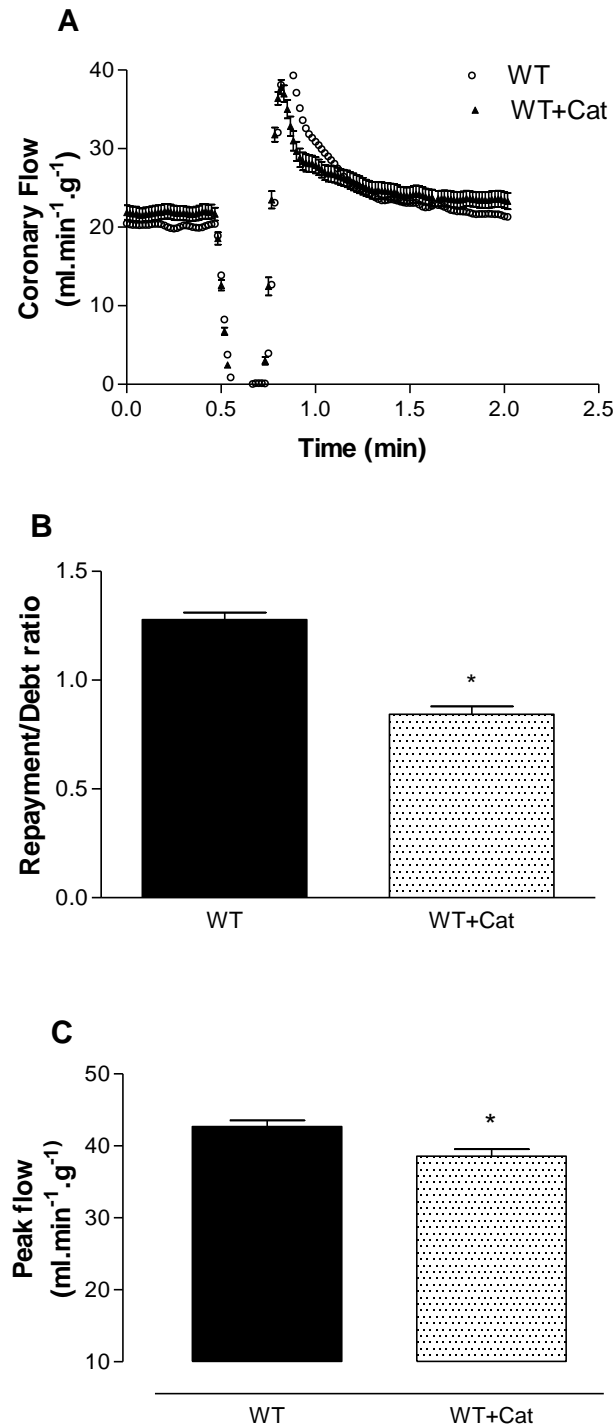
Fig. 4.7 Reactive hyperemia-induced changes on coronary flow (A), peak flow (B), and flow repayment area (C) in the presence (n=6) and absence (n=8) of catalase (Cat) in A_{2A}KO mice.
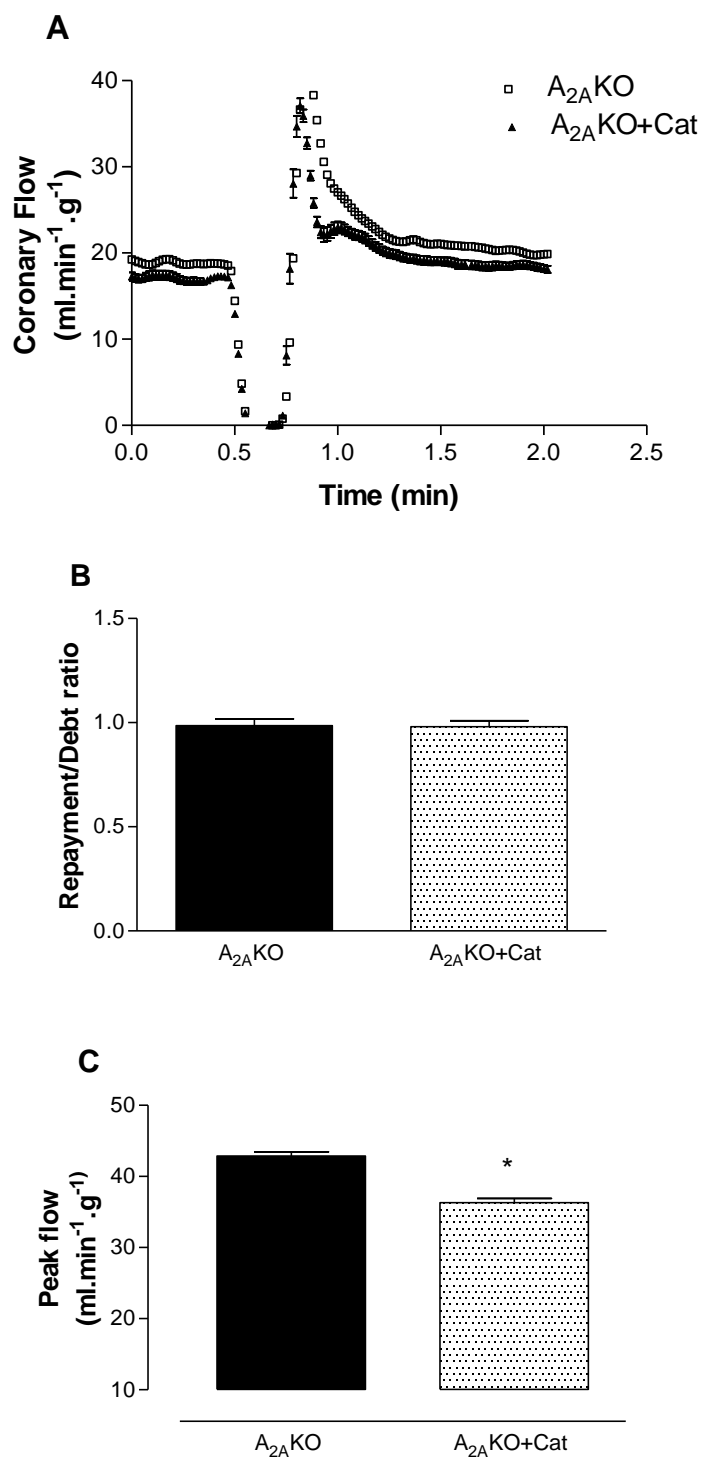
Fig. 4.8 H_2O_2 increases a glibenclamide-sensitive conductance (nS/pF) in WT smooth muscle cells. (A) Representative trace showing effect of H_2O_2 to increase K_{ATP} current. (B) Group data (n=5 mice) illustrate effect of H_2O_2 relative to pinacidil. *Significant difference compared with control, $p<0.05$.
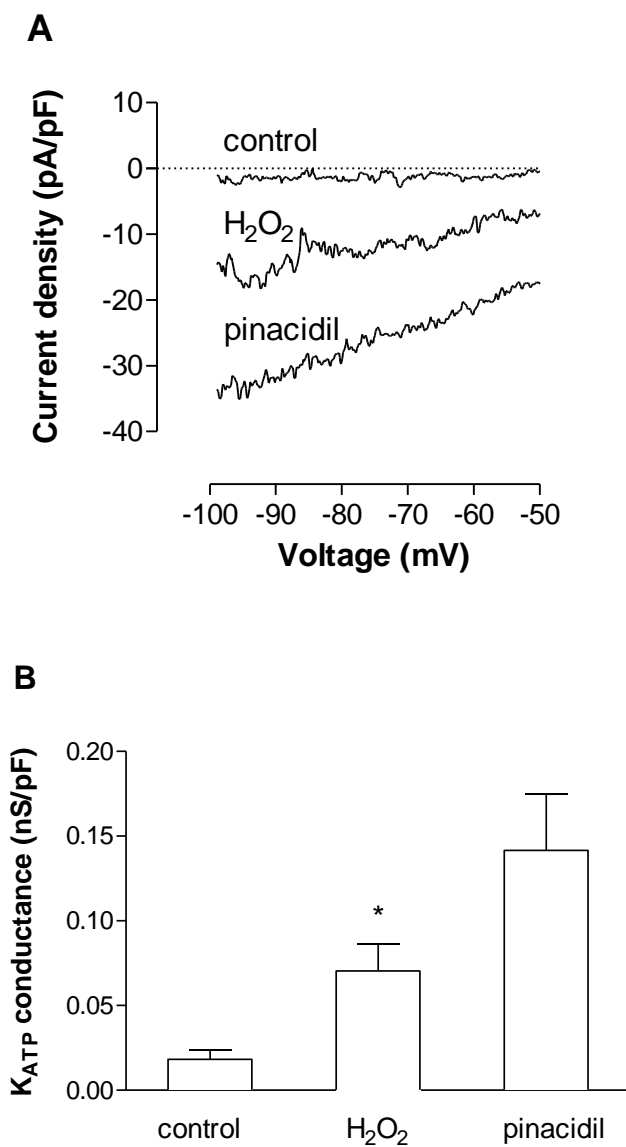
Fig. 4.9 Effect of catalase on adenosine and pinacidil mediated increase in glibenclamide-sensitive conductance (nS/pF) in WT smooth muscle cells (n=5).
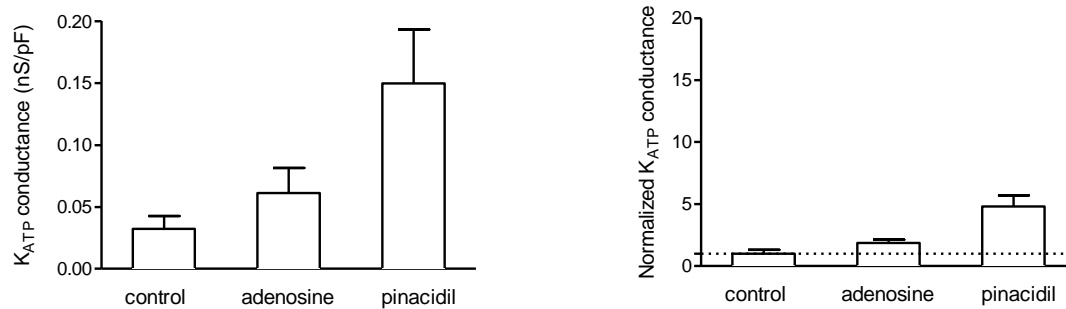
Table 4.1

Resting flow, debt area (DA), peak flow, flow repayment area (RPA), and debt to repayment area

ratio (RPA/DA).

	Resting Flow (ml/min/g)	Debt Area (DA) (ml/g)	Peak (ml/min/g)	Repayment Area (RPA) (ml/g)	Debt to Repayment area RPA/DA
WT	19.8±0.9	4.9±0.2	42.7±2	6.3±0.4	1.3±0.1
$A_{2A}KO$	19.4±1.6	4.9±0.4	42.9±1.7	4.5±0.2*	0.9±0.1*
$A_{2B}KO$	20.3±1.1	5.1±0.3	44.2±0.5	6.1±0.1	1.2±0.1
$A_{2A/2B}KO$	18.6±0.6	4.7±0.1	41.6±1.2	4.9±0.2*	1±0.1*
WT+Cat	21.4±2.1	5.3±0.5	38.6±2.4*	4.4±0.4*	0.8±0.1*
$A_{2A}KO+Cat$	16.8±0.9*	4.2±0.2*	36.3±1.4*	4.1±0.4*	0.9±0.1*

All values are means ± S.E.M. * $p<0.05$ compared with WT

Discussion

The new findings in this study show: 1) a better elucidation of the individual involvement of A_{2A}ARs in RH, 2) a demonstration that H_2O_2 contributes to CF responses in RH, and 3) that H_2O_2 couples adenosine-mediated CF responses in RH to K_{ATP} channel activation. Using A_{2A} and A_{2B}KO and $A_{2A/2B}$DKO mice, we showed that only A_{2A}AR, out of four AR subtypes, is involved in RH. Additionally, we showed for the first time the involvement of H_2O_2 in mouse coronary RH in addition to its involvement in A_{2A}AR-induced K_{ATP} channel activation.

Increased oxygen demand over oxygen supply ratio of the myocardium is exerted through myocardial oxygen tension (pO_2), which is decreased in ischemic conditions. This fall in pO_2 leads to the breakdown of adenine nucleotides leading to the production of adenosine that is known to mediate local metabolic control of CF (14). Previous RH studies investigating the effect of different occlusion periods suggested that metabolic vasodilatory factors are the primary mediators responsible for the increase in flow during RH (7, 17, 38, 39). Further, our laboratory and others have previously shown that activation of A_{2A} and A_{2B} ARs induces an increase in CF (37, 46, 49). Therefore, in this study, we wanted to test the role of adenosine, a well established local metabolite, in CF changes in RH. The involvement of adenosine in coronary RH was recently reported in an *in vivo* dog study, where the AR subtypes involved was not assessed (11). Nevertheless, using an A_{2A}AR selective antagonist (SCH58261), Zatta et al. reported coronary RH responses mediated by A_{2A}ARs with a flow repayment area decrease of up to 30% and no change in peak flow (62). Also, in a previous *in vivo* study in dogs, the role of A_{2B}AR-mediated CF changes in RH was assessed using a non-selective antagonist (alloxazine) which is only about 10 fold more selective towards A_{2B}ARs relative to A_{2A}ARs (2). Therefore, a further study was needed in order to fully elucidate the role of ARs in coronary RH without the effect of pharmacological changes. The availability of gene deleted knockout mice has been an important tool to analyze the physiological and pharmacological pathways for elucidating the role of a single receptor. Therefore, in this study, using A_{2A} and A_{2B}KO isolated mice hearts, we directly

showed that $A_{2A}ARs$ contribute to CF changes in mouse coronary RH by demonstrating a significant decrease in flow repayment time and volume (flow repayment area) in $A_{2A}KO$ mice compared with WT. These results are in accordance with recent studies showing a role for ARs in flow repayment area and not the peak flow (62). However, our results from $A_{2B}KO$ and $A_{2A/2B}DKO$ mice did not suggest any role for $A_{2B}ARs$ in CF changes in RH. Due to its low affinity for adenosine, $A_{2B}ARs$ are suggested to be activated in conditions where a significant increase in adenosine levels is observed (15). For example it was reported that $A_{2B}ARs$ play a role in ischemia/reperfusion and preconditioning (13). Therefore, $A_{2B}ARs$ may be more involved in severe ischemic pathophysiological conditions such as stroke or in protective preconditioning pathways rather than in physiological situations. Indeed, A_2ARs agonists are being investigated as a treatment during percutaneous coronary intervention for the reduction of myocardial reperfusion injury in patients with myocardial infarction, which is well established to be associated with worsening of cardiac injury and function (6, 10).

Berne's adenosine hypothesis suggests that increase in myocardial oxygen consumption decreases the myocardial oxygen tension which leads to the release of adenosine from cardiomyocyte (53), where $A_{2A}ARs$ have also been found to be expressed in both human and mouse (4, 24). Furthermore, a role for H_2O_2 in metabolic vasodilation is already established (59). Chilian et al. suggested that H_2O_2 production increases in proportion to cardiac metabolism in order to couple the coronary blood flow to myocardial oxygen tension (45). It is interesting to know that both H_2O_2 and adenosine are released from cardiomyocytes through which they modulate vascular tone (19). Nevertheless, it is also well recognized that hemodynamic changes-induced release of mediators are also involved in RH; a sudden increase in flow after reperfusion activates shear stress-sensitive mechanisms (21, 41, 52, 61), illustrating the release of endothelium-dependent derived factors that may limit myogenic constriction and, therefore, modifying the RH time or flow repayment area. In fact, recent reports have established the contribution of H_2O_2 in flow-induced dilation of isolated human coronary arteries (29) and in

reactive dilation of rat isolated coronary arterioles (20). Therefore, in this study, as EDHF in humans and mice (26, 27, 58), we also investigated the role of H_2O_2 in CF changes during coronary RH using isolated heart (compared with isolated vessels). Additionally, due to the involvement of both adenosine and H_2O_2 as metabolic factors regulating CF, in this study, we also tested the involvement of H_2O_2 in AR-induced effect in CF regulation and RH. We showed for the first time that in the presence of catalase, which breaks down H_2O_2 into water and oxygen, adenosine-induced increase in CF is abolished in WT, $A_{2A}KO$ (where $A_{2B}AR$ is the only AR mediating vasodilation), and $A_{2B}KO$ (where $A_{2A}AR$ is the only AR mediating vasodilation) (Figure 5), showing that H_2O_2 plays a role in A_2AR-mediated signaling pathway when inducing an increase in CF. In this study, we also showed for the first time that the RH repayment time and volume (flow repayment area) in addition to flow debt-to-repayment ratio were significantly decreased in WT isolated mice hearts in the presence of catalase, showing the contribution and release of H_2O_2 during coronary RH. Furthermore, since catalase decreased the flow debt-to-repayment ratio in both $A_{2A}KO$ and WT mice with no difference compared to each other and compared to $A_{2A}KO$ in the absence of catalase, we suggest that H_2O_2 released during coronary RH is, at least partly, mediated through activation of $A_{2A}ARs$. Showing that catalase significantly decreased peak flow in WT and $A_{2A}KO$ mice (while peak flow of $A_{2A}KO$ was not different compared with WT mice in the absence of catalase), may further signify that H_2O_2 may also be released through activation of other pathways. Nevertheless, using patch clamp studies, we showed that H_2O_2 increases glibenclamide (K_{ATP} channels blocker) sensitive currents. Putting together these findings with our previous and current results, showing that adenosine activates K_{ATP} channels by means of its A_2ARs, we are suggesting that, at least partly, H_2O_2 couples the activation of ARs to K_{ATP} channels during coronary RH. Nevertheless, further *in vivo* and *in vitro* studies are needed to fully elucidate the complex mechanisms of action involved in RH. We also have to highlight that the magnitude of hyperemia is also determined by the baseline flow. In this study, we observed that H_2O_2 or knocking out of the $A_{2A}ARs$, solely, do not decrease the baseline

CF; however, synergistically they significantly decrease the CF baseline, which by itself may suggest that there might be a compensatory relationship between $A_{2A}ARs$ and H_2O_2 (Table 1).

Since, in this study, we used isolated heart, the contribution of flow/shear stress- or pressure-dependent coronary mechanisms in the development of RH could not be clearly elucidated or isolated from the effect of local metabolic factors. However, an *ex vivo* isolated heart model certainly provides us with more valuable data closer to the physiological *in vivo* responses compared with *in vitro* isolated vessels, since it reflects the overall end effect of shear/pressure stress- and metabolites-induced effects during coronary RH. Additionally, an *ex vivo* study, such as the current study, eliminates the complexity of the role of neuronal and hormonal effects and, hence, enabling us to better understand the tissue selective responses and mechanisms involved during coronary RH. We also have to mention a second limitation of our study that has to do with performing patch clamp experiments on smooth muscle cells from aorta, as a model to complement functional studies from the coronary arteries. Perhaps a better approach would be to study smooth muscle cells isolated from mouse coronary arteries or arterioles. However, to our knowledge, there are no reports using mouse coronary smooth muscle in patch clamp setting in order to study ion channel currents due to its own limitations such as isolation and experimental procedures required. Therefore, most studies investigating coronary ion channels show currents from aorta as a tool (5, 23, 28). Also, K_{ATP} channels are known to be important regulators of coronary vascular tone in mice (5, 28). Thus, aortic smooth muscle cells appear to be an appropriate substitute for coronary smooth muscle cells in this regard.

The release of H_2O_2, as endothelium-derived hyperpolarizing factor (EDHF) in mice and humans (26, 27), could be induced by various factors. Morikawa et al. suggested that Cu,Zn-SOD plays an important role as an EDHF synthase in mice (31). It is also likely that H_2O_2 is produced from superoxide anions from several sources in endothelium, such as eNOS, lipoxygenase, cytochrome *p*-450 enzymes, and NADPH oxidases (27). Further, it is reported that adenosine-mediated effects are partially endothelium dependent and that adenosine also induces vasodilation

through production of cytochrome *p*-450 metabolites (34, 35), NO (50), and NADPH oxidases (data not shown). Therefore, comparing these signaling pathways, one may speculate that adenosine-induced release of H_2O_2 may involve activation of epoxygenases pathway, eNOS, or NADPH oxidase. However, further studies are needed to identify these signaling pathways.

It is known that H_2O_2 induces vasodilation; however, there are also reports showing that H_2O_2 induces vasoconstriction (8, 16, 30) and that H_2O_2 inhibits vasodilation to adenosine in isolated porcine coronary arteries (51). Nonetheless, it was suggested that the vascular effect of H_2O_2 depends on its concentration (8). To the best of our knowledge there is limited information on the endogenous level of H_2O_2, however, H_2O_2 levels between 2.5-50 µmol/L have been reported in human plasma (9, 22, 54). Exogenous infusion of H_2O_2, up to 6×10^{-6} mol/L (6 µmol/L), caused a significant increase in CF in isolated mouse heart (data not shown) from which point onward (at 10^{-5} mol/L) H_2O_2 started to induce coronary vasoconstriction which was also accompanied by a decrease in left ventricle pressure and +dp/dt (data not shown). It seems that endothelial cells are less vulnerable to H_2O_2 since a relatively high concentration of H_2O_2 >200 µmol/L is generally required to produce irreversible endothelial barrier dysfunction (40, 56). Therefore, H_2O_2-induced decrease in CF at 10^{-5} mol/L concentration may be due to H_2O_2-induced damaging effect on cardiomyocytes. Nevertheless, our study showed a significant increase in CF between 10^{-6}- 6×10^{-6} mol/L. Therefore, the discrepancy between our study and other studies regarding H_2O_2-induced effects may be dependent on the level of H_2O_2 released. For example, Thengchaisri et al. showed that H_2O_2 in concentrations below 10 µmol/L did not modify NO-mediated vasodilatory responses. Such variations may also be dependent on the species, vascular beds, and experimental models such as *ex vivo vs. in vitro*. However, overall, we can only speculate that H_2O_2 concentration released due to coronary RH may be in the range of 10^{-6}- 6×10^{-6} mol/L.

It is well established that activation of K_{ATP} channels plays a major role in coronary RH in addition to its contribution to AR-mediated CF changes (2, 48, 62). However, identification of

the factor(s) that activates these channels was not assessed in previous studies. Recent studies have shown the association of K^+ ions in H_2O_2–induced vasodilation (42, 43, 45). These channels were also shown to mediate, at least in part, H_2O_2-induced dilation of arterioles from skeletal muscle and brain (25, 55). Further, it has been shown that H_2O_2 activates K_{ATP} channels in pancreatic beta cells and cardiac myocytes (18, 33). Nevertheless, direct evidence of this mechanism in smooth muscle and the role of adenosine as the inducer of this mechanism is lacking. In this study, we showed for the first time that K_{ATP} channels on smooth muscle cells are activated by H_2O_2 and, at least partly, adenosine induces the release of H_2O_2 and, hence, activation of K_{ATP} channels. It is worth mentioning that A_2AR-induced increase in CF is through activation of non-mitochondrial K_{ATP} channels (48) and, therefore, one can speculate that at least the source of H_2O_2-induced CF changes in RH due to activation of ARs may not be mitochondria related. However, further experiments are needed to better understand the role of mitochondrial and non-mitochondrial K_{ATP} channels in RH coronary flow responses.

Finally catalase induced a decrease in adenosine-induced increase in LVDP. This inhibition may be due to Gregg's effect since at this concentration adenosine has significantly increased both CF and LVDP. The opening of K_{ATP} channels may also affect the contractility independent of CF changes. It is worth mentioning that adenosine is shown to activate K_{ATP} channels and that glibenclamide, a K_{ATP} channel blocker, is reported to decrease reactive oxygen species (36). Nevertheless, further studies are needed to better understand the role of H_2O_2 in LVDP.

This study is another step in the direction of better understanding of the CF regulation and its heterogeneity that may help us towards development of treatments for various clinical uses such as evaluation of endothelial integrity and reperfusion of coronary arteries after an embolism.

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CHAPTER FIVE

CONCLUSION

The work presented in this dissertation confirms the heterogeneity of coronary flow responses mediated by activation of adenosine receptors (AR). In this dissertation, we established that $A_{2A/B}$ AR subtypes may have differing contributions in the regulation of CF under varying conditions.

We suggested that out of all four AR subtypes, A_{2A}ARs and A_{2B}ARs induce coronary artery (CA) vasodilation, since exogenous adenosine did not induce any increase in CF in $A_{2A/2B}$AR double knockout mice. Additionally, each of these two AR subtypes further compensate for the deletion of the other subtype; indeed A_{2A}ARs expression was up-regulated in A_{2B}KO mice. We established the presence of such compensatory mechanism via the use of selective and non-selective agonists and antagonists. Furthermore, since some inconsistencies are reported between the results obtained from pharmacological and molecular (knockout mice) approaches, we also used AR knockout mice to further confirm our findings. The differing expression of $A_{2A/2B}$ ARs is reported in many cardiovascular diseases and, therefore, the knowledge of the presence of such compensatory mechanisms between A_{2A} and A_{2B} ARs may contribute to the future development of new treatment approaches. In fact, A_2ARs are being investigated as a treatment during percutaneous coronary intervention for the reduction of myocardial reperfusion injury in patients with myocardial infarction, which is well established to be associated with worsening of cardiac injury and function.

The next step was to determine the effector, located on SMCs, causing the final effect of coronary vasodilation mediated by activation of A_2ARs. We not only learned that both A_{2A}AR- and A_{2B}AR-mediated CF effects are via activation of non-mitochondrial K_{ATP} channels but, we suggest that, no matter what the A_2AR-induced coronary vasodilation signaling pathway may be, it may be through activation of K_{ATP}

channels since both $A_{2A}AR$- and $A_{2B}AR$-meditaed CF effects were abolished in the presence of glibenclamide (Figure 3.7).

Whereas we showed that activation of both $A_{2A}ARs$ and $A_{2B}ARs$ induces CF vasodilation, in this dissertation we established that only $A_{2A}ARs$ contribute to the regulation of CF in reactive hyperemia, where the metabolic factors are known to be mainly involved. It is interesting to mention that $A_{2A}ARs$ induce the sustained coronary vasodilation during reactive hyperemia through the release of H_2O_2. H_2O_2, depending on the vascular bed and its concentration, acts as a vasorelaxing as well as a vasoconstricting factor. Using isolated mouse hearts, we showed that exogenous H_2O_2 may have a tissue-protective role by inducing coronary vasodilation. In addition, we established that H_2O_2, released due to activation of $A_{2A}ARs$, also activates K_{ATP} channels on smooth muscle cells, which further confirms our findings that K_{ATP} channels may be the end effector in A_2AR-mediated effect on CF (Figure 5.1).

The availability of single and double knockout ARs is a valuable tool to better understand the role of each AR or a subclass of AR subtype. In addition to measurement of baseline parameters, such as baseline CF, heart rate, and left ventricle developed pressure, we also showed that the recently available $A_{2B}KO$ mice have a functional endothelium by showing that bradykinin response was not different in $A_{2B}KO$ mice compared with wild type mice.

In summary, both A_{2A} and A_{2B} ARs are capable of inducing coronary vasodilation, but they each are activated endogenously under different conditions. Needless to say, the role of A_2ARs in the regulation of CF is much more complicated than what has been described in this thesis. It is possible that there might also be other interactions present between this class of GPCRs and β adrenoceptors, in view of the fact

that NECA-mediated increase in CF is ameliorated in β_1/β_2 double knockout mice (Appendix A). These findings require more detailed investigation to better understand the interaction between ARs and β adrenoceptors.

Figure 5.1 Suggested signaling pathways for A$_{2A}$- and A$_{2B}$ ARs-mediated CF regulation
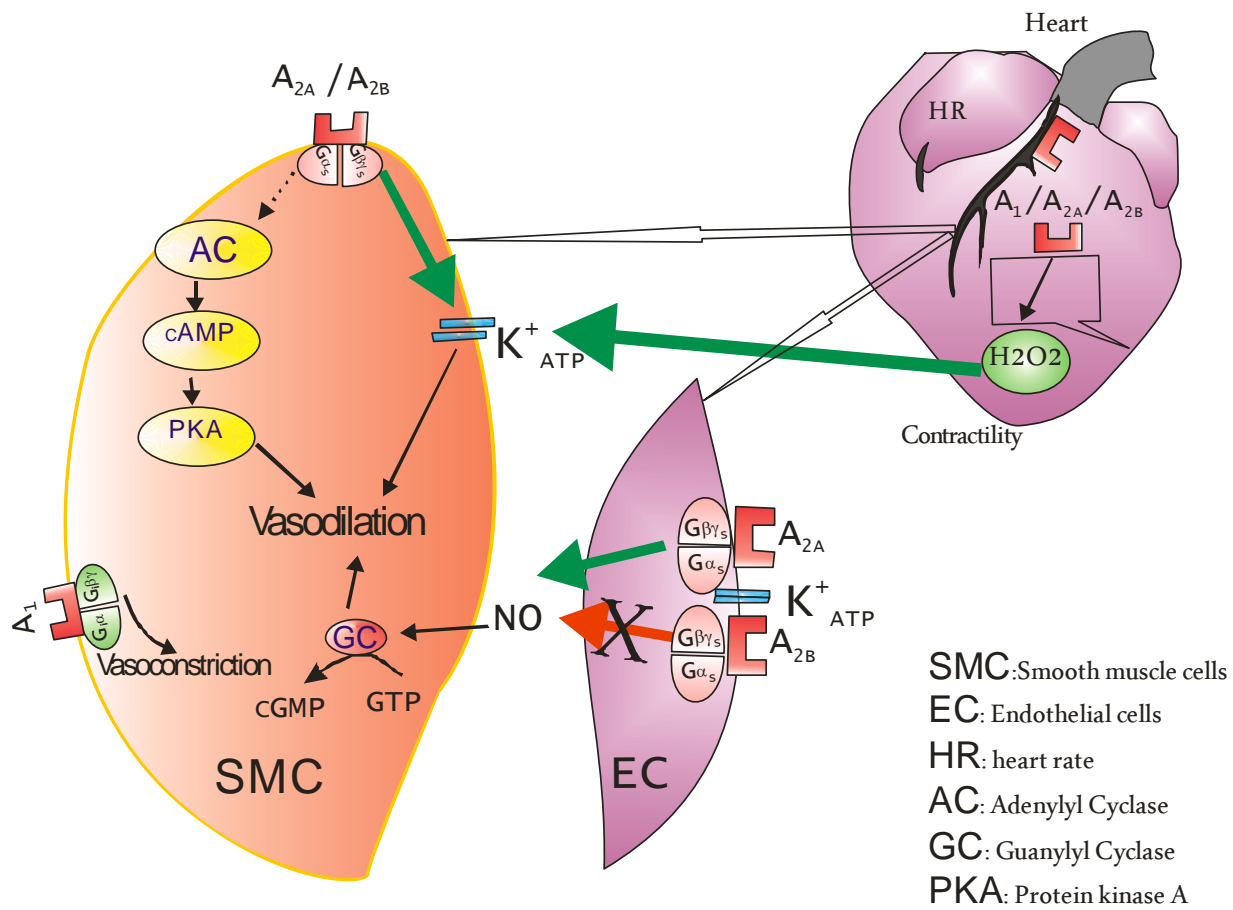
Future Directions

The next step for this project would be to identify the source of H_2O_2; H_2O_2 may be released from the myocardium or the vascular bed itself, in which case, the knowledge of whether the source is the endothelium or SMCs may help us to identify the mechanism behind reduced adenosine coronary response in patients with cardiovascular disease. Furthermore, it would be interesting to identify the factor(s) responsible for the release of superoxide, which subsequently leads to the formation of H_2O_2; among the possible factors, NADPH oxidases and mitochondria are the primary candidate sources of superoxides (Figure 5.2).

The NADPH oxidase-enzyme complex consists of two membrane-bound components (gp91phox [also known as NADPH oxidase (Nox)-2] and p22phox) and several cytosolic regulatory subunits, including p47phox, p67phox, and the small GTPase Rac (Rac1 or Rac2). When NADPH oxidase becomes activated, the cytoplasmic subunits translocate to the cell membrane and the resulting complex transfers electrons from NADPH to molecular oxygen in order to form superoxide. In fact, NADPH oxidase is identified as a major source of superoxide in ECs, and, therefore, the important determinants of the oxidation-reduction (redox) state of the endothelium. Investigators have also identified NADPH oxidase complex as the major source of ROS in VSMCs. Whereas both p22phox and p47phox appear to be essential for NADPH oxidase activity in VSMCs, targeted-deletion of the gp91phox gene in mice had no effect on superoxide production (3). Additionally, two homologues of gp91phox, termed Nox1 and Nox4, are suggested to be mainly present on vascular beds. Therefore, the association of the NADPH oxidase homologue involved in adenosine-mediated release of H_2O_2, and hence coronary vasodilation, needs to be clarified.
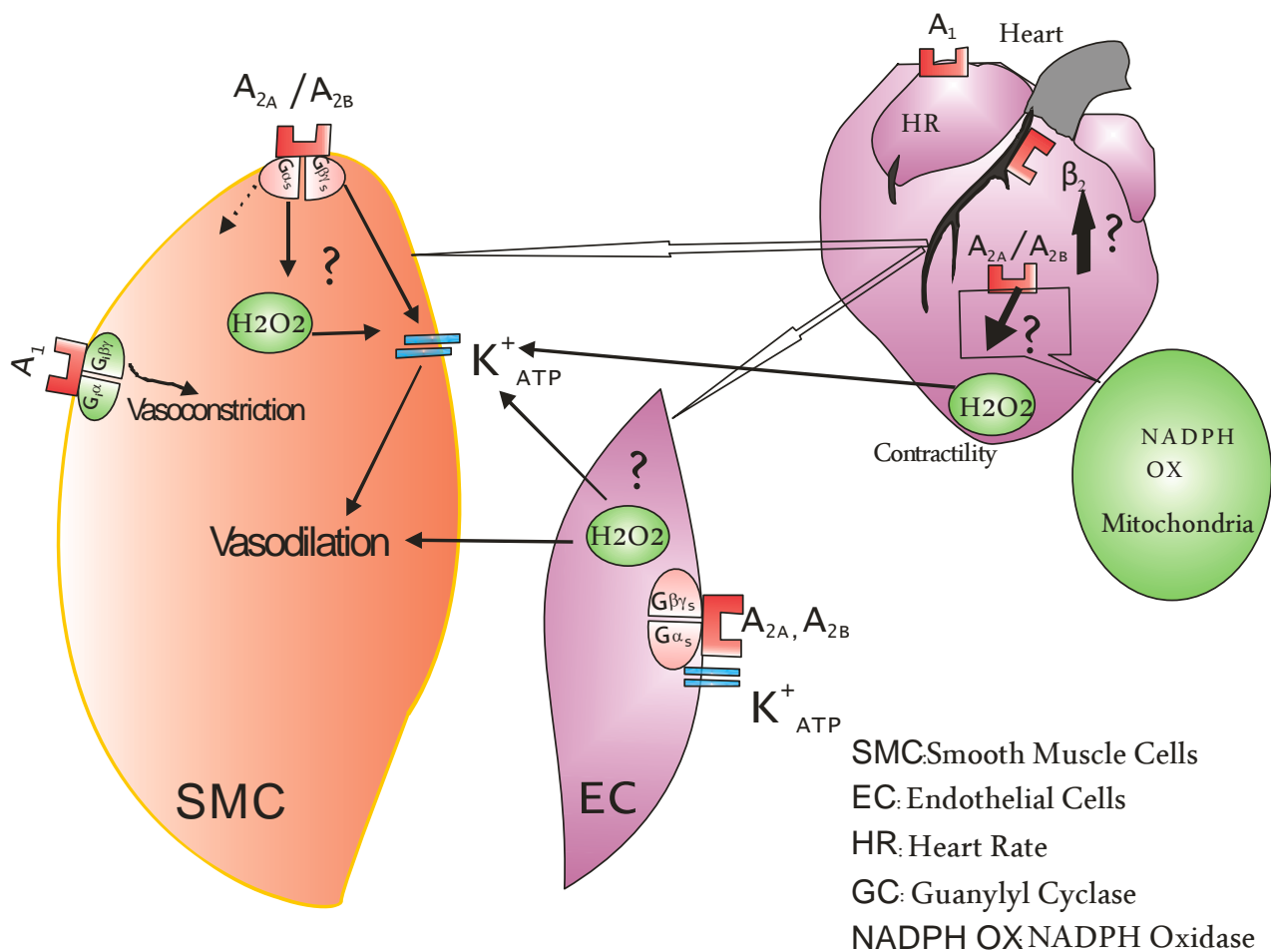
In many cells, the majority of superoxide is generated from mitochondria via cellular respiration and specifically during ubisemiquinone autoxidation. Ubisemiquinone can serve as an electron donor to O_2 to produce superoxide. Electron flow through the mitochondrial electron transfer chain occurs by four inner membrane–associated enzyme complexes, with cytochrome c and the mobile carrier ubiquinone. NADH derived from both cytosol and mitochondria donate electrons to NADH:ubiquinone oxidoreductase (complex I) which then transfers its electrons to succinate:ubiquinone oxidoreductase (complex II). Electrons from reduced ubiquinone are then transferred to ubiquinol:cytochrome c oxidoreductase (complex III). Electron transport then proceeds through cytochrome c, cytochrome c oxidase (complex IV), and finally, molecular oxygen (5-6). Nevertheless, there are two major mitochondrial electron transfer chain regions where ROS are produced: complex I and complex III. Therefore, in order to identify the mitochondrial complex involved in H_2O_2 production, we can use rotenone, a complex I inhibitor, myxothiazol, which inhibits oxidation of ubiquinol to ubisemiquinone in complex III, and cyanide, a complex IV inhibitor, to detect if the trapped radical species originates from the electron transport chain of the mitochondria.

Cardioprotective effect of carvedilol (βADR blocker) was shown to be through an adenosine-dependent mechanism. Additionally, it was reported that βADR-mediated inotropic responses were attenuated by adenosine (7). Our preliminary data also establishes a relationship between βADRs and A_2ARs since the effect of NECA is attenuated in $β_1/β_2DKO$ mice. Therefore, a better understanding of the relationship between ARs and βADRs in CF regulation and the possible signaling pathways involved, would open a new window to better understand the heterogeneity CF regulation in relation to ARs. Our preliminary data show that, like any complex system, regulation of

CF may further involve an interaction between ARs and βADRs. Nevertheless, this investigation requires further experiments to confirm the presence of such a relationship between these two classes of GPCRs.

Finally, our data show that A_{2B}ARs are not involved in RH and may play a role in severe ischemic conditions, where adenosine levels are significantly higher. Therefore, in order to better clarify the role of A_{2B}ARs in CF regulation, further experiments such as ischemia/reperfusion experiments are needed. Indeed, is has been reported that A_{2B}ARs play a cardioprotective role during ischemia/reperfusion (1-2, 4). Therefore, taken together, elucidation of CF regulation and cardioprotection in light of A_{2B}ARs and βADRs, and, ultimately, understanding the signaling pathways involved, would lead to uncovering of the translational mechanisms of cardiac reperfusion subsequent to a heart attack, in addition to finding of better and complete ways of cardiac imaging evaluation for the diagnosis of coronary artery disease (Figure 5.2).

Figure 5.2 Future directions for identifications of A$_{2A}$ARs and A$_{2B}$ARs signaling pathways in the regulation of CF

SMC: Smooth Muscle Cells
EC: Endothelial Cells
HR: Heart Rate
GC: Guanylyl Cyclase
NADPH OX: NADPH Oxidase

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PUBLICATIONS

I. Dovenia S. Ponnoth, **Maryam Sharifi Sanjani**, Catherine Ledent, Kevin Roush, Thomas Krahn and S. Jamal Mustafa. Absence of adenosine-mediated aortic relaxation in A_{2A} adenosine receptor knockout mice. Am J Physiol Heart Circ Physiol. 2009 Nov; 297(5):H1655-60. PMID: 19749167

II. **Maryam Sharifi Sanjani**, Bunyen Teng, Thomas Krahn, Stephen Tilley, Catherine Ledent and S. Jamal Mustafa. Contributions of A_{2A} and A_{2B} adenosine receptors in coronary flow responses in relation to K_{ATP} channel using A_{2B} and $A_{2A/2B}$ double knockout mice. Am J Physiol Heart Circ Physiol. 2011 Sep; PMID: 21949117

III. El-Awady MS, **Sharifi MS**, Mustafa SJ. "Adenosine receptors and the heart: role in regulation of coronary blood flow" Proceedings of the "9th International Congress on Coronary Artery Disease" Pub: Medimond, pages 9-16, 2011.

IV. **Maryam Sharifi Sanjani**, Shinichi Asano, Gregory Dick, Bunyen Teng, Stephen Tilley, Catherine Ledent and S. Jamal Mustafa. Interaction of adenosine A_{2A} receptors, hydrogen peroxide, and ATP-dependent K^+ channels in coronary reactive hyperemia (Under Review)

Abstracts and Presentations

Oral
1. **Maryam Sharifi Sanjani,** Bunyen Teng, Stephen Tilley, S. Jamal Mustafa. Understanding the role of A_{2B} adenosine receptor using knockout in the regulation of coronary flow. American Heart Association (AHA) Fellows Research Day: University of Pittsburgh Medical Center (February 2009)

Poster
1. **Maryam Sharifi Sanjani**, Bunyen Teng, Thomas Krahn, Stephen Tilley, Catherine Ledent and S. Jamal Mustafa. Relationship between H_2O_2 and A_2 adenosine receptor in reactive hyperemia in isolated mouse heart. Experimental Biology, Washington D.C. (April, 2011)

2. **Maryam Sharifi Sanjani**, Bunyen Teng, Thomas Krahn, Stephen Tilley, Catherine Ledent and S. Jamal Mustafa. Relationship between H_2O_2 and A_2 adenosine receptor in reactive hyperemia in isolated mouse heart. Van Lier Research Day West Virginia University (March, 2011)

3. **Maryam Sharifi Sanjani**, Bunyen Teng, Thomas Krahn, Stephen Tilley, Catherine Ledent and S. Jamal Mustafa. Involvement of H_2O_2 in adenosine-mediated regulation of coronary flow (IUPHAR 2010, Paper No. 2222). WorldPharma 2010, Copenhagen, Denmark (July 2010)

4. **Maryam Sharifi Sanjani**, Dovenia Ponnoth, Kevin Roush, Steve Tilley, S. Jamal Mustafa. Evidence for the role of A_{2B} adenosine receptor in the regulation of vascular tone using A_{2B} KO mice (FASEB J. 2009 23:1032.2). Experimental Biology, Anaheim, 2010 (April, 2010)

5. **Maryam Sharifi Sanjani**, Dovenia Ponnoth, Kevin Roush, Steve Tilley, S. Jamal Mustafa. Evidence for the role of A_{2B} adenosine receptor in the regulation of vascular tone using A_{2B} KO mice. Van Lier Research Day West Virginia University (April 2010)

6. **Maryam Sharifi Sanjani**, Stephen Tilley, Bunyen Teng, S. Jamal Mustafa. Understanding the role of A_{2B} adenosine receptor using knockout in the regulation of coronary flow (FASEB J. 2010 24:958.2) Experimental Biology, New Orleans, 2009 (April, 2009)

7. **Maryam Sharifi Sanjani**, Stephen Tilley, Bunyen Teng, S. Jamal Mustafa. Role of A_{2B} adenosine receptor in the regulation of coronary flow. Van Lier Research Day West Virginia University (April 2009)

8. **Maryam Sharifi Sanjani**, Bunyen Teng, Stephen Tilley, S. Jamal Mustafa. Role of A_{2B} adenosine receptor in the regulation of coronary flow. Van Lier Research Day West Virginia University (April 2008)
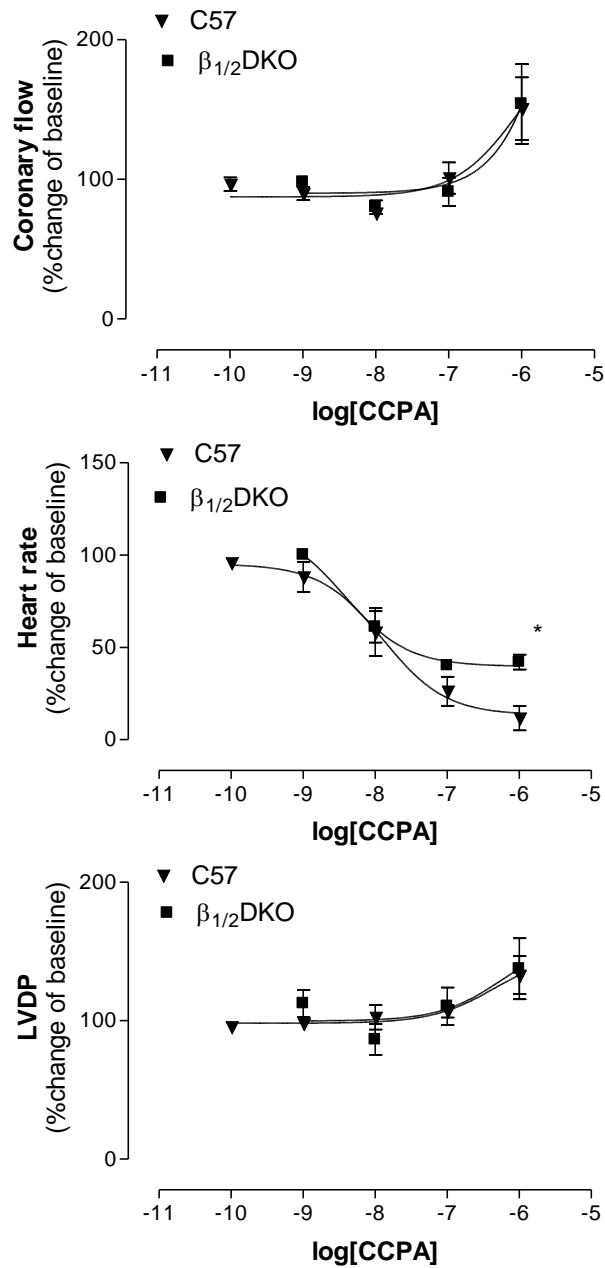
APPENDIX A

Preliminary experiments (relationship between adenosine receptors and β₁ and β₂ adrenoceptors) were carried out. The concentration-response curve for NECA was calculated in WT (FVB) and β₂KO mice isolated hearts. This was done to determine the role of A₂ARs in NECA-induced relaxation in β₂KO. There was no difference between the two groups.
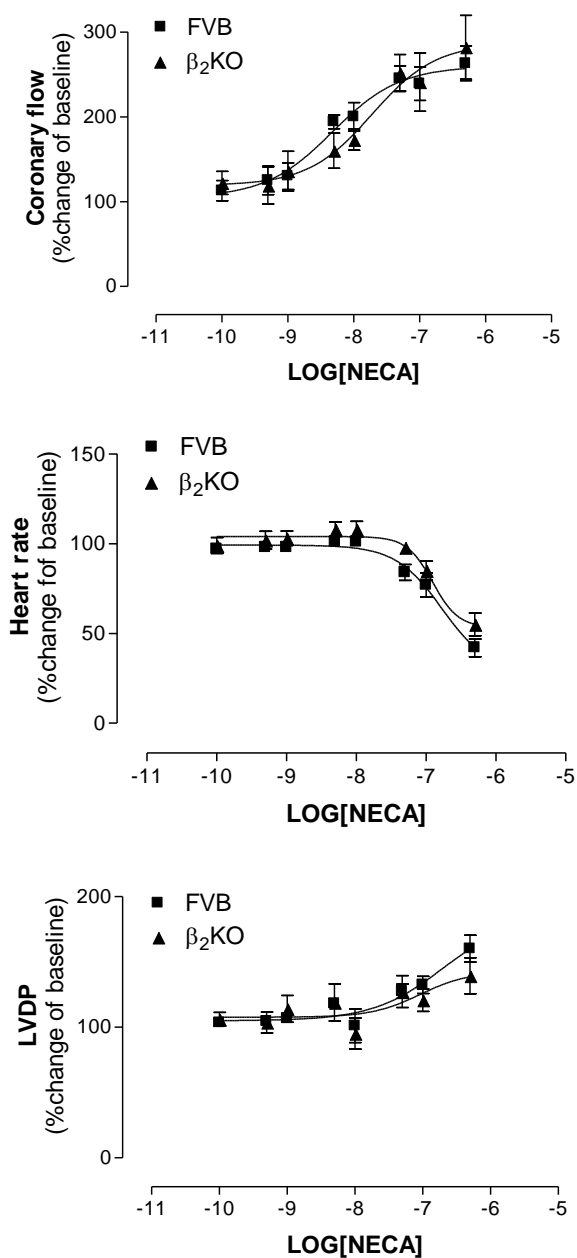
Preliminary experiments (relationship between adenosine receptors and β_1 and β_2 adrenoceptors) were carried out. The concentration-response curve for NECA was calculated in WT (FVB), WT (C57), and $\beta_{1/2}$DKO mice isolated hearts. This was done to determine the role of A_2ARs in NECA-induced relaxation in $\beta_{1/2}$DKO. There was no difference between the two control groups while NECA-mediated effects were significantly diminished in $\beta_{1/2}$DKO compared to both controls.
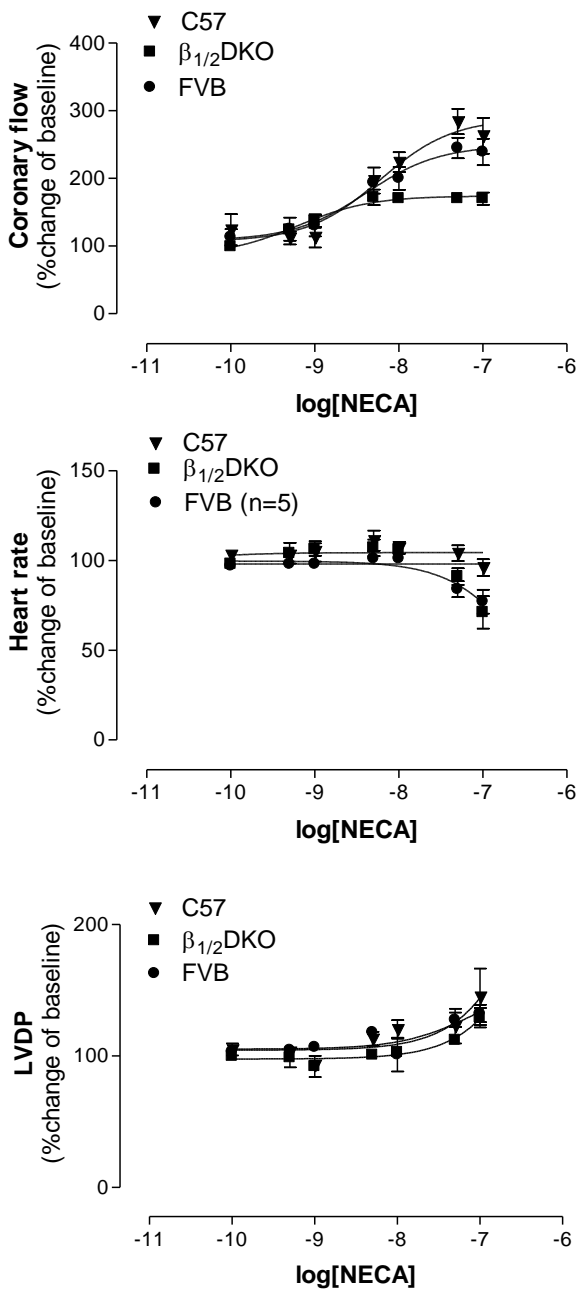
Preliminary experiments (relationship between adenosine receptors and β_1 and β_2 adrenoceptors) were carried out. The concentration-response curve for CCPA was calculated in WT (FVB) and $\beta_{1/2}$DKO mice isolated hearts. This was done to determine the role of A_1AR-mediated effect in $\beta_{1/2}$DKO. There was no difference between the two groups.

APPENDIX B

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From: Mark Goodwin [MGoodwin@The-APS.org]
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To: Kara Hansell Keehan; Penny Ripka
Cc: Masafumi Kitakaze; Joseph Girouard; Sharifi, Maryam
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APPENDIX C

CURRICULUM VITAE

Education

2006-Present	Ph.D. Candidate	Pharmaceutical & Pharmacological Sciences	West Virginia University, Morgantown, WV
2001-2003	M.Sc.	Medicinal Chemistry	University of Sussex, Brighton, U.K.
1996-2000	B.Sc.	Chemistry	Shahid-Beheshti University, Tehran, Iran

Advisor: Dr. S. Jamal Mustafa

Dissertation title: Role and contribution of A_2 adenosine receptors in the regulation of coronary flow

Position

2006-present   Graduate Assistant, Center for Cardiovascular and Respiratory Sciences, Department of Physiology & Pharmacology, West Virginia University, Morgantown, WV

Teaching Experience

2008-Present   I am a facilitator in Pharmacology Additional Learning Exercise (ALE) for medical students conducted by the School of Medicine, West Virginia University.

2009   I have guided and taught methods and approaches to one undergraduate student for a 9-week summer research project.

2011   I have taken Teaching Practicum courses and have lectured Cardiac Physiology.

Grants

1. Recipient of NIH Cardiovascular and Pulmonary Disease training grant (NHLBI-T32 Pre-Doctoral training grant), July 2008-July 2011

2. Applied for American Heart Association Predoctoral Fellowship (2010 Great Rivers Affiliate) (not funded)
   "Interplay between A_2 adenosine receptors, β_2 adrenergic receptors, and H_2O_2 in the regulation of coronary flow"

Professional Honors and Awards

1. Recipient of WorldPharma 2010 (16th IUPHAR WorldCongress of Basic and Clinical Pharmacology) ASPET travel award: March 2010

2. Recipient of Department of Pharmaceutical and Pharmacological Sciences (West Virginia University) travel award: February 2010

3. Recipient of Research Graduate Office (West Virginia University) travel award: January 2010

4. Recipient of American Heart Association (AHA) Fellows Research Day award: February 2009

5. Recipient of Department of Pharmaceutical and Pharmacological Sciences (West Virginia University) travel award: February 2009

6. Recipient of Department of Pharmaceutical and Pharmacological Sciences (West Virginia University) travel award: February 2008

Professional Membership

American Society for Pharmacology and Experimental Therapeutics (ASPET)

The American Physiological Society (APS)

Participation in Scientific Meetings

2011   Experimental Biology, Washington D.C.

2010   American Heart Association, Chicago, IL

2010   WorldPharma 2010 (16th IUPHAR WorldCongress of Basic and   Clinical Pharmacology, Copenhagen, Denmark

2010   Experimental Biology, Anaheim, CA

2009   American Heart Association (AHA) Fellows Research Day, Pittsburgh, PA

2009   Experimental Biology, New Orleans, LA