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A role for the A3 adenosine receptor in determining tissue levels of cAMP and blood pressure: studies in knock-out mice

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Abstract

Adenosine administration has been reported to lower blood pressure by activating specific membrane receptors. The rat and human heart and aorta have been previously found to express both A2-type adenosine receptors, which activate adenylyl cyclase, and A3 adenosine receptors (A3AR), which inhibit adenylyl cyclase. In the current study, we used A3 adenosine receptor (A3AR) knock-out mice to examine the hypothesis that the relative levels of the A2-type adenosine receptors and A3AR determine the steady-state levels of cAMP in the cells and may affect blood pressure. We found that the A3AR knock-out mice express normal levels of the A1- and A2-type adenosine receptors. In situ hybridization demonstrated that the level of A3AR is high in the vascular smooth muscle layer of aortas derived from wild-type mice, but is not detectable in the knock-out mice. The steady-state level of cAMP is elevated in the aorta and heart of knock-out mice, as compared to wild-type mice, but is not altered in platelets, where A3AR is not expressed naturally. A3AR knock-out mice possess a blood pressure comparable to this in wild-type mice. However, when challenged with adenosine, the knock-out mice display a further increase in cAMP levels in the heart and vascular smooth muscle and a significant decrease in blood pressure, as compared to wild-type mice. In contrast, the effect of adenosine on ADP-induced platelet aggregation is similar in both types of mice. These studies indicate that the A3AR affects the steady-state level of cAMP in the tissues where it is naturally expressed, and that it influences the blood pressure in response to adenosine.

1. Introduction

Adenosine, a metabolite of adenine nucleotides, is released by facilitated diffusion from metabolically active cells and is generated by degradation of released ATP [1]. In addition to its biological role in cellular metabolism, it has become clear that adenosine plays an important physiological role in the cardiovascular system. Adenosine receptors, members of the superfamily of the G protein-coupled receptors, have been classified into subtypes based on: (a) their primary structure; (b) the secondary messenger systems to which they are coupled; and (c) the differential affinities of a number of adenosine receptor agonists and antagonists [2,3]. Based on these criteria, four adenosine receptor (AR) subtypes have been described: A1AR, A2aAR, A2bAR and A3AR. These subtypes have been confirmed extensively by cloning and functional characterization of rat, human, bovine and canine receptors [4]. The A3AR has been detected in a number of tissues including

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the lung, heart and brain. Interestingly, striking differences have been reported in tissue localization and levels of A3AR transcripts between species [4]. In humans, abundant transcripts have been found in the liver, with moderate levels measured in heart, aorta, kidney, lung and placenta [5].

The various physiological effects of adenosine are mediated through its activation of the above-described specific cell surface receptors. Adenosine binding to the A1 or A3 adenosine receptors is coupled to inhibition of adenylyl cyclase, while binding of this ligand to the A2-type (A2a or A2b) adenosine receptors is coupled to the activation of adenylyl cyclase [6,7]. Adenosine plays a major role in regulating the balance of myocardial oxygen supply-demand through its effects on vascular tone [8]. During myocardial ischemia, adenosine is released in large amounts and can exert potent, protective effects in the cardiovascular system [9–11]. For example, vasoconstriction is attributed to A1 receptor activation [12], which also mediates the inhibition of renin secretion [13]. Activation of A2 receptors is associated with renal vasodilation. Adenosine acting via A2 receptors is also a potent coronary vasodilator [14]. In some vascular beds, such as rabbit aorta or canine femoral artery, adenosine exerts its effects in the absence of the endothelial layer [15], indicating the importance of the direct smooth muscle response. In accordance, administration to rats of an adenosine analog which binds to A2 adenosine receptors also lowers blood pressure and heart rate [14].

Previously, we reported the expression of A3 adenosine receptor mRNA in rat cultured vascular smooth muscle cells (VSMC), and demonstrated that a certain level of expression of the A3 adenosine receptor mRNA is sufficient for attenuating adenosine-induced increases in cAMP [16]. The detection of A3AR mRNA in cultured VSMC corresponds with its detection within total RNA of human aortas [17]. Since activation of A2AR in VSMC has been shown to induce vasodilation (reviewed in [9]), it follows that the regulation of expression of the inhibitory A3AR in these cells is crucial for balanced effects of adenosine on cAMP and potentially on blood pressure. In the current study, we used A3 adenosine receptor (A3AR) knock-out mice to examine the hypothesis that the relative levels of the A2-type adenosine receptors and A3AR determines the steady-state levels of cAMP in the cells and may affect blood pressure. We thus used A3AR knock-out mice to investigate: (1) the expression of the A3AR in smooth muscle of intact aortas; (2) the contribution of A3 adenosine receptors to the steady-state concentration of cAMP in aorta, heart, and platelets; and (3) the effects of adenosine on blood pressure and platelet aggregation. Our data indicate that the expression of A3AR in a tissue does affect the steady-state levels of cAMP in the cells and that there is no linear correlation between changes in cAMP levels in the heart or vascular smooth muscle and blood pressure levels. Our data point, however, to the importance of the A3AR in determining blood pressure response to adenosine administration.

2. Materials and methods

2.1. A3 adenosine receptor knock-out mice

The mouse A3 adenosine receptor gene was subjected to homologous recombination leading to disruption of expression of A3 adenosine receptor mRNA as described by [18]. These knock-out mice, on a mixed background (C57BL/6/B6D2), were made available to us by Dr. Marlene Jacobson at Merck Research Laboratories (West Point, PA). The mice were continuously bred with no difficulty and genotyped as follows: genomic DNA was digested with *Bam*HI and subjected to Southern blot analysis, using as a probe 750 base pairs (bp) of *Xho*I/*Hin*dIII fragment isolated immediate upstream of the short arm of the target construct [18]. The probe hybridizes to a 3.1-kb band in the targeted allele (homozygous knock-out); a 5.5-kb band in the endogenous allele (wild-type) and both bands in heterozygous animals. DNA preparation and Southern blot analyses were performed essentially as we described before [19]. Mice deficient in the A3AR-subtype gene and their wild-type controls were used in our studies. The animals were 8 weeks old, weighing 26.2–32.8 g. They were housed in the animal quarters with a 12-h light/dark cycle and were provided food (Purina, Certified Rodent Chow 5002) and distilled water ad libitum. All experiments were conducted in accor-

dance with the guidelines for the care and use of animals approved by the Boston University Medical Center.

2.2. Organ cultures, platelet preparation and cAMP measurement

Aortas derived from age-matched (8-week-old) and sex-matched (female) mice were isolated, washed and extracted for cAMP determination by radioimmunoassay, using appropriate controls for recovery as we detailed before [16]. cAMP measurements were also performed on platelets isolated from mouse blood, as we described before [19]. When indicated, platelets, at a concentration of 9×10^8/ml, were incubated in a cAMP incubation buffer [16] for 5 min with 1 μM forskolin (Sigma, St. Louis, MO) and/or 50 nM of the A3AR-selective agonist, N^6-(3-iodobenzyl) adenosine-5′-*N*-methylcarboxamide (IB-MECA) (RBI, Natick, MA). IB-MECA had no effect on cAMP levels in platelets in accordance with the notion that these cells only possess the A2-type adenosine receptors.

2.3. Platelet aggregation studies

Platelet aggregation studies in a platelet-rich plasma were performed as described elsewhere [20]. Adenosine-5′- diphosphate (ADP) (2 μM) (BIO/DATA, Horsham, PA) was used to activate platelets in the absence or presence of adenosine, as indicated by changes in light-scattering monitored in a platelet aggregometer (BIO/DATA, Horsham, PA).

2.4. Tail-cuff blood pressure and heart rate measurements

To determine whether disruption of the A3AR gene affects blood pressure (BP) and heart rate (HR) in these animals, tail-cuff systolic BP and HR measurements were obtained in knock-out (A3AR −/−), heterozygotes (A3AR +/−) and wild-type (A3AR +/+) male and female mice. Tail-cuff systolic blood pressure and heart rate measurements were obtained using a computerized tail-cuff system (BP 2000 Visitech Systems, Apex, NC) [21]. The system has the ability to determine systolic blood pressure and heart rate in four mice simultaneously, and uses a photoelectric sensor to detect the cuff pressure at which blood flow to the tail is eliminated. Mice were trained for 5 consecutive days (each session consisting of 20 unrecorded measurements) to familiarize the animals to the tail-cuff apparatus. Subsequently, blood pressure and heart rate measurements were recorded daily for an additional 5 consecutive days. Each session consisted of 20 measurements for each mouse and the mean blood pressure and heart rate for the day were calculated. Thus, the mean control blood pressure and heart rate for each mouse were obtained.

2.5. Adenosine infusion-catheterization

As adenosine has been shown to modulate several cardiovascular functions, the effect of adenosine administration on BP was investigated in A3AR knock-out mice and their wild-type controls. Arterial and venous catheterization was performed in all age-matched (8-week-old) female mice under anesthesia induced by sodium pentobarbital (50 mg/kg, i.p.) [22]. A modified polyethylene catheter (PE-50) was introduced into the right iliac artery for direct blood pressure (BP) recording and a silastic tubing was placed into the right iliac vein for drug infusion. Both catheters were tunneled subcutaneously and exteriorized at the back of the animal's neck. Subsequently, they were filled with heparin in 0.9% saline solution, sealed with heat and attached to the animal's nape. After the surgery the animals were returned in their cages and allowed an overnight recovery period. On the following day, the arterial catheter was connected to a BP transducer attached to a recorder (model 220S, Gould) for direct BP monitoring. Control BP was recorded for no less than 30 min or until the BP became stable. The venous catheter was connected to a Harvard infusion pump and adenosine was administered at a dose of 600 μg/kg/min for 60 min.

2.6. Statistical analysis

All data are presented as mean ± S.E.M. Student's *t*-test for paired and unpaired data was used, as appropriate. The Mann–Whitney rank sum test was used for non-parametric data. Differences at $P<0.05$ were considered to be significant.

2.7. In situ hybridization

A3AR knock-out and control mice were anesthetized with methoxyfluorane and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Aortas from these mice were fixed for an additional 2 h in paraformaldehyde, followed by dehydration in increasing concentrations of ethanol and embedding in paraffin. Sections of 5 μm were affixed to slides and treated as described before [23]. Briefly, slides were deparaffinized with xylene, washed in ethanol, and rehydrated. Sections were then treated with 20 μg/ml proteinase K, acetylated by incubation with acetic anhydride in 0.1 M triethanolamine, and dehydrated once again. The probe used was derived from the rat heart A3 adenosine receptor cDNA, which we have derived by reverse transcription followed by the polymerase chain reaction (RT-PCR) on heart tissues, as we described before [16]. The cDNA was confirmed by DNA sequencing and comparison to the published clone (GenBank accession number M94152). Sense or antisense A3AR ^{35}S-labeled riboprobes were prepared as described before [24] and applied to the sections, which were subsequently covered with hybrislips and incubated for 16 h at 52°C. Hybridized slides were washed in 50% formamide/2×SSC at 65°C, followed by treatment with RNase A (Sigma, St. Louis, MO). Subsequent washes in 2× and 0.1×SSC at 37°C were followed by dehydration and dipping in autoradiography emulsion (Eastman Kodak, Rochester, NY). Following development of the emulsion, slides were stained with hematoxylin and eosin prior to analysis.

2.8. Northern blot analysis

RNA was extracted from different tissues derived from wild-type and knock-out mice and subjected to Northern blot analysis, as we described before [16,19]. The blots were probed with the mouse A3AR cDNA [25] or rat A2a or A2b or A1 adenosine receptor cDNAs available to us [16]. The rat clones were generated by RT-PCR of rat tissues, followed by subcloning to a PUC-based vector and DNA sequencing [19], to confirm the correct sequence. The sequences were compared to GenBank

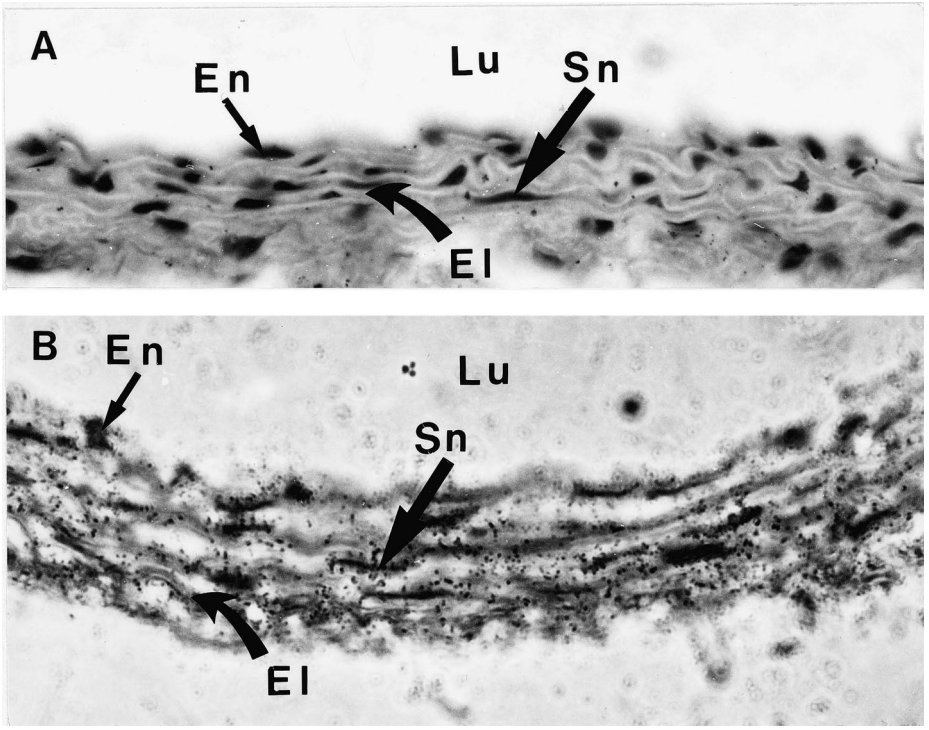

Fig. 1. Expression of the A3AR in mouse aorta. Sections of an aorta derived from an A3AR knock-out mouse (A) or wild-type mouse (B) were subjected to in situ hybridization using the mouse A3AR antisense riboprobe. The arrows point to stained nuclei. En, endothelial cell nucleus; Sn, smooth muscle cell nucleus; El, elastic fiber; Lu, lumen.

(accession numbers: rat A3AR cDNA, M94152; rat A1AR cDNA, M69045; rat A2aAR cDNA, L08102; rat A2bAR cDNA, M91466). In order to determine equal loading of the samples to each lane, the blot was also probed with an 18S ribosomal cDNA.

3. Results

3.1. Expression of A3 adenosine receptor in vascular smooth muscle layer of aorta from wild-type and knock-out mice

Expression of the A3AR in the aorta of rats and humans was reported before [16,17]. However, it was not clear if this receptor is indeed expressed in vascular smooth muscle cells in vivo. The expression of A3AR mRNA in the vascular smooth muscle layer of the mouse aorta was demonstrated by in-situ hybridization with an A3AR antisense riboprobe (Fig. 1). Interestingly, some A3AR mRNA was also detected in the endothelial layer of the sectioned aorta. In contrast, aortas derived from A3AR knock-out mice completely lacked A3AR mRNA (Fig. 1). As expected, no hybridization was detected with the sense probe (data as in Fig. 1B). Similarly, Northern blot analysis indicated that the A3AR knock-out mice do not express A3AR mRNA in a variety of tissues tested (data not shown), but the level of the A1 or A2a or A2b adenosine receptor mRNA and the pharmacological analyses (radioligand binding) were similar in the knock-out and wild-type mice (the tissues tested were: heart, aorta and liver) (our unpublished data and [18]).

3.2. Steady-state levels of cAMP in aorta, heart and platelets

As indicated above, the A3AR was reported before to be expressed, among other tissues, in heart and aorta as well as in cultured rat vascular smooth muscle cells. We recently demonstrated that an inverse correlation exists between the level of A3AR mRNA in rat vascular smooth muscle cells in culture and the intracellular level of cAMP, i.e. under conditions where the expression of the A3 adenosine receptor is reduced by antisense oligomers, a higher level of cAMP is found in the cells [16]. Our current study shows that the steady-state levels of cAMP are significantly different in the aorta and heart from wild-type and A3AR knock-out mice (Fig. 2). The level of cAMP was significantly increased in aorta and heart from A3AR knock-out mice relative to wild-type controls. This relatively high base-line level of cAMP in the A3AR knock-out mice could be further elevated by the addition of adenosine. In aortas of heterozygous A3AR knock-out mice, in which only one allele was knocked out, the level of cAMP was only increased by 10% as compared to wild-type. These changes were subjected to statistical analyses, as performed in Fig. 2, revealing that the change was not statistically significant (not shown). In the rest of the study, we thus focused on the A3AR homozygous knock-out mice.

In platelets of wild-type mice, we found, there are no active A3 adenosine receptors, as nanomolar concentrations of the A3AR selective agonist, IB-MECA, had no inhibitory effects on cAMP levels, either in the absence or presence of 1 μM forskolin (our unpublished data). When analyzed in platelets, cells which express A2-type adenosine receptor [26], there was no significant change in the steady-state levels of cAMP between A3AR knock-out and normal mice. These results suggest a contribution of

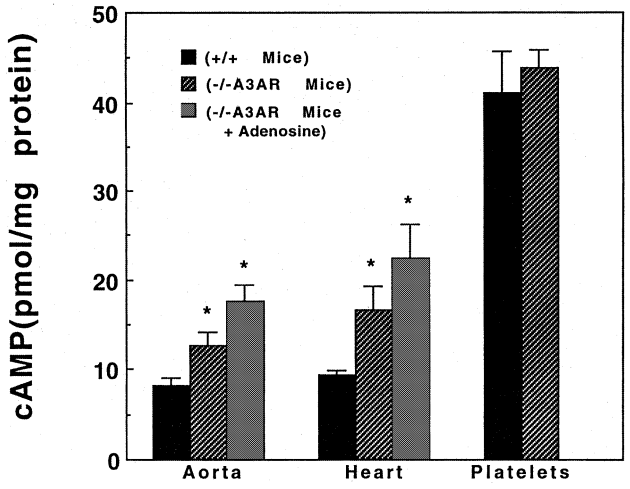

Fig. 2. Levels of cAMP in aorta, heart and platelets of wild-type and A3AR knock-out mice. Tissues from wild-type mice (+/+) or A3AR knock-out mice (−/−) (control) were extracted for cAMP measurements. When indicated, tissues were incubated with 50 μM adenosine for 10 min prior to cAMP determination. Data are averages of four determinations with the indicated standard deviation. The P-value of <0.05 (*) in a group was considered to reflect significant changes.

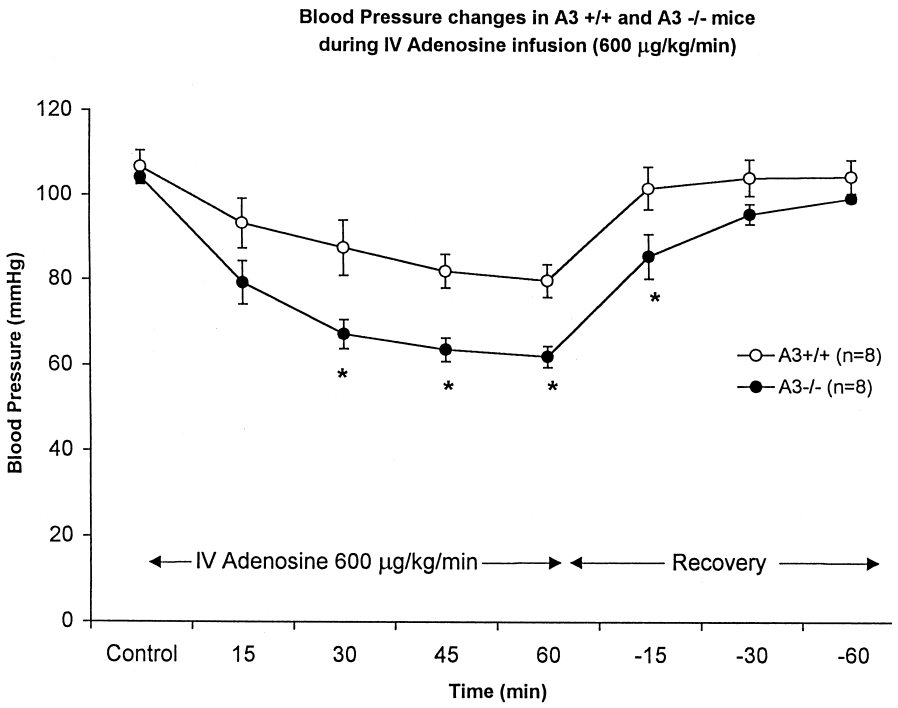

Fig. 3. Blood pressure changes in A3AR knock-out mice after infusion of adenosine intravenus (IV). Adenosine (at the indicated concentration) was infused in wild-type (+/+) or A3AR knock-out (−/−) mice and blood pressure was monitored at the indicated times. Results are averages ± S.E.M. for eight mice.

A3AR to the regulation of the steady-state concentrations of cAMP within a tissue in which it is naturally expressed.

3.3. Tail-cuff measurements of blood pressure

Table 1 summarizes mean tail-cuff blood pressure (BP) and heart rate (HR) measurements in all groups of mice. No difference was found in either the BP or HR between any of the groups studied (wild-type +/+, heterozygotes +/− or knock-out −/− mice), nor was there a difference between male and female mice.

3.4. Changes in blood pressure in response to adenosine

Fig. 3 presents BP changes during adenosine infusion in A3AR +/+ ($n=8$) and A3AR −/− ($n=8$) mice. Control BP was comparable in both groups, averaging 107 ± 3.8 mmHg in A3AR +/+ and 104 ± 1.71 mmHg in A3AR −/− mice. Adenosine administration at a dose of 600 μg/kg/min resulted in a significant BP reduction during the 60-min infusion period in both wild-type (+/+) and knock-out (−/−) animals. However, adenosine infusion resulted in a significantly lower BP in the A3AR −/− mice,

Table 1
Tail-cuff systolic blood pressure and heart rate measurements in A3R +/+, A3R +/− and A3R −/− male and female mice

Group	A3R +/+ ($n=9$)		A3R +/− ($n=7$)		A3R −/− ($n=7$)	
	Male ($n=5$)	Female ($n=4$)	Male ($n=4$)	Female ($n=3$)	Male ($n=4$)	Female ($n=3$)
BP (mmHg)	107.5 ± 3.76	103.8 ± 6.56	103.5 ± 0.96	102.5 ± 4.43	98.4 ± 5.44	107.7 ± 4.42
HR (beats/min)	618.4 ± 30.3	558.3 ± 22.3	606.2 ± 20.2	573.3 ± 23.8	656.8 ± 33.0	589.9 ± 7.6

All data are expressed as mean ± S.E.M. BP, blood pressure; HR, heart rate.

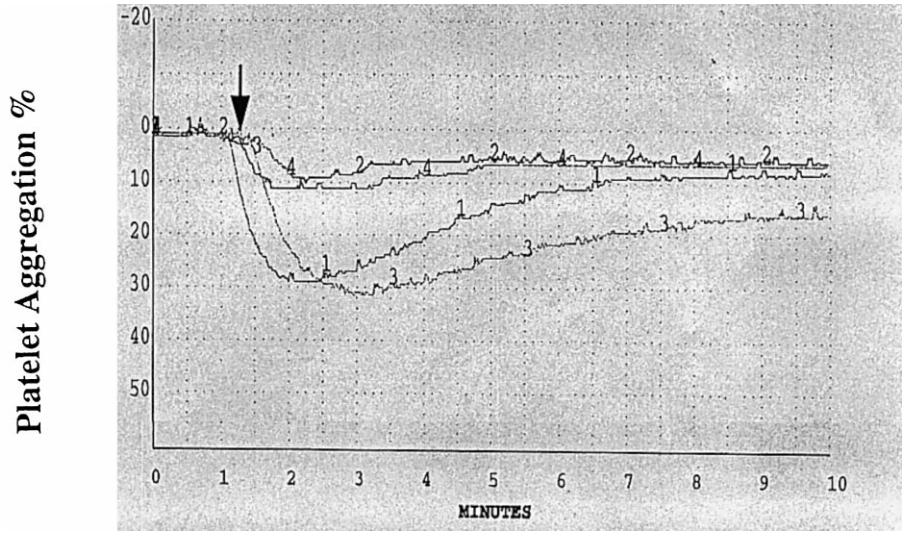

Fig. 4. Adenosine-induced changes in platelet aggregration. Platelet-rich plasma derived from wild-type (1,2) or A3AR knock-out mice (3,4) were induced to aggregate with 2 μM ADP (indicated by an arrow) in the absence (1,3) or presence of 10 μM adenosine (2,4). The data shown are of a representative set out of two different experiments, each with two sets of mice per group.

as compared to their wild-type counterparts. This difference in BP reduction was evident within the first minutes of infusion and persisted throughout the 60-min infusion period. Moreover, after the discontinuation of adenosine infusion, A3AR +/+ mice returned to the pre-infusion BP levels faster than their knock-out (−/−) counterparts. It was not technically possible to monitor the HR in these infused mice.

3.5. Platelet activation studies

As indicated above, cAMP levels in platelets of wild-type and A3AR knock-out mice was quite comparable. In accordance, the extent of inhibition by adenosine (used in the range of 1–40 μM) of ADP-induced aggregation was comparable in both types of mice. Fig. 4 shows a result of a representative experiment, using 10 μM adenosine. This suggests that the magnitude of adenosine effect is not altered, as compared to control, in a cell type where the A3AR is not normally expressed.

4. Discussion

In mammalian tissues, adenosine can be formed through de-novo synthesis or from a variety of substrates (reviewed by [27]). The intracellular level of adenosine, determined by the balance of its production and metabolism, is about 15 μM in most cells tested under normal conditions, and can rise to much higher concentrations during stress, such as ischemia or anoxia [28]. The origin of adenosine in the blood can be traced to intracellular adenosine released from cells via a nucleoside transport system, as well as to the activity of ectoenzymes, such as ecto-5′-nucleotidase [28,29]. Aggregating platelets as well as endothelial cells release ADP and ATP, which can be converted to adenosine by ectoenzymes on endothelial or cardiac cells [28,29]. Adenosine exerts its effects via binding to specific adenosine receptors which modulate adenylyl cyclase activity as well as signaling pathways, such as phospholipase C, in the case of A3AR activation. In some tissues, such as heart and aorta, adenylyl cyclase-inhibitory (A1- and A3-types), as well as adenylyl cyclase stimulatory receptors (A2-type) are co-expressed [16,27]. Effects of adenosine in the cardiovascular system may stem from the renal and cardiovascular tissues. The response to adenosine in kidney is biphasic, with an initial transient vasoconstriction being followed by dilation [13]. Vasoconstriction is attributed to A1 receptor activation [12], which also mediates the inhibition of renin secretion [13]. Adenosine also plays a central role in ischemia-reperfusion injury. Under these conditions there is an increase in coronary vessel adenosine levels as a result of ATP degradation [30]. Tissue damage occurs via free radicals and mod-

ulators of the inflammatory response [31]. Local intracoronary infusions of adenosine at the time of reperfusion can reduce myocardial necrosis, endothelial damage, and granulocyte accumulation [32]. Also, adenosine administration lowers blood pressure and heart rate [14,33]. Of the many effects of adenosine in the cardiovascular system, we elected to focus in the current study on exploring the importance of adenosine-mediated activation of A3AR for maintenance of blood pressure, by analysis of A3AR knock-out mice.

We found that the vascular smooth muscle tissue in aortas of wild-type mice express high levels of the A3AR, while as expected, no expression was found in the knock-out mice. Also, Northern blot analysis indicated that A3AR mRNA is not found in a variety of tissues derived from the A3AR knock-out mice, while the levels of the A1, A2a and A2b adenosine receptor mRNAs was similar in heart and aorta of the knock-out and the wild-type mice. This may rule out the possibility that the changes observed in physiological parameters in the A3AR knock-out mice are due to a secondary effect on other adenosine receptors. The blood pressure is similar in the A3AR knock-out and wild-type mice. However, when challenged with adenosine, the knock-out mice display a significant decrease in blood pressure, as compared to wild-type mice. Our findings suggest that under normal adenosine concentrations, the A3-type adenosine receptors are not active in mediating changes in blood pressure and that they are overpowered by A2AR receptors that mediate signals for vasodilation. It is important to note in this regard that the affinity of adenosine to the A2-type receptors is higher by an order of magnitude than the affinity to the A3AR [34]. In a study by Shepherd et al., the vasomotor effects of adenosine were analyzed by following changes in the diameters of microvessels in hamster cheek pouches [35]. This study led to the conclusion that ligands binding to the mast cell A3 adenosine receptors mediate degranulation as well as vasoconstriction. Interestingly, it was also concluded that adenosine initiates multiple conflicting vasomotor signals, as A2 adenosine receptor-mediated dilation was competing with constriction (with the A2AR overpowering the A3AR), and thus adenosine analogs, but not adenosine, were able to induce changes in mast cell activation.

In contrast to the effect of adenosine on blood pressure, the effect of this ligand on ADP-induced platelet aggregation was similar in both the wild-type and A3AR knock-out mice. The effect of adenosine on this cell type was examined since we found that platelets do possess significant levels of an active A3AR. This leads us to speculate that the magnitude and nature of adenosine effect depends on the ability of tissues to co-express the stimulatory and inhibitory adenosine receptors. This hypothesis will await further examination in mice lacking other types of adenosine receptors.

Adenosine actions in different systems are essentially of two types: those that are cAMP dependent, and others that are cAMP independent. Activation of SA, atrial, and AV nodal adenosine receptors results in activation of a specific outward potassium current, which is cAMP independent [36,37]. In ventricular myocytes, adenosine antagonizes the stimulatory actions of catecholamines on I_{Ca} and on the transient inward current [38]. This antagonism by adenosine and its analogs is due to the inhibition of adenylyl cyclase [39,40]. Adenosine and its analogs also inhibit ADP-induced platelet aggregation, dependent on activation of adenylyl cyclase [41,42]. Several pathways have been proposed to explain the mechanism of action of adenosine in various tissues: (1) modulation of adenylyl cyclase activity, as also observed with the adrenergic receptors [43]; (2) modulation of Ca^{2+} channel activity, e.g., adenosine inhibits Ca^{2+} uptake in heart [44]; (3) modulation of K^+ conductance, e.g. in pig atria adenosine causes an increase in potassium conductance, which could explain the shortening of the action potential duration and hyperpolarization caused by adenosine [45]; and (4) modulation of phospholipase C activity which may affect intracellular Ca^{2+} concentrations [46].

In the current study, we focused on changes in cAMP levels in wild-type and in A3AR knock-out mice, as this is one of the immediate change occurring upon A3AR receptor activation. In platelets, in which this receptor is not naturally expressed, there is no change in cAMP levels as compared to wild-type mice or in platelet aggregation in response to adenosine. While several studies have directly linked changes in cAMP levels in aorta and heart to blood pressure values, the increase in cAMP is not directly associated with low blood pressure and vice versa

[47–53]. We found that in the A3AR knock-out mice, the steady-state level of cAMP is elevated in aortas and heart, as compared to wild-type mice, with no change in blood pressure level. Further elevation of cAMP in these tissues from A3AR knock-out mice treated with adenosine was associated with an increase in blood pressure. Our study thus leads to the important conclusions that the presence of A3AR has a significant impact on the steady-state levels of cAMP in the cells, and hence potentially on a variety of cAMP-dependent processes, and that adenosine-mediated effects on blood pressure are not directly correlated with changes in the levels of cAMP. With the lack of a reliable technique to monitor changes in Ca^{2+} in an intact aorta of a mouse, we are forced to postponed these experiments. As described above, based on the studies by Shepherd et al., which were done with adenosine analogs binding to A3AR, one may speculate that adenosine treatment of A3AR knock-out mice will result in a less effective degranulation of mast cells and attenuated release of vasoconstricting substances, a process which may contribute to vascular changes [35]. We could not examine this possibility of attenuation in comparison to wild-type mast cells, as adenosine does not activate these latter cells because of the overpowering effect of the A2AR [35]. The role of mast cells in vasoconstriction and in determining blood pressure will have to be determined in A2AR knock-out mice.

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