



Rapid Communication

Targeted Deletion of the A₃ Adenosine Receptor Confers Resistance to Myocardial Ischemic Injury and does not Prevent Early Preconditioning

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Y. GUO, R. BOLLI, W. BAO, W.-J. WU, R. G. BLACK, JR., S. S. MURPHREE, C. A. SALVATORE, M. A. JACOBSON AND J. A. AUCHAMPACH. Targeted Deletion of the A₃ Adenosine Receptor Confers Resistance to Myocardial Ischemic Injury and does not Prevent Early Preconditioning. *Journal of Molecular and Cellular Cardiology* (2001) 33, 825–830. We used mice with genetic disruption of the A₃ adenosine receptor (AR) gene (*A₃AR*^{-/-} mice) to assess the *in vivo* role of the A₃AR in modulating myocardial ischemia/reperfusion injury and preconditioning (PC). Surprisingly, infarct size induced by 30 min of coronary artery occlusion and 24 h of reperfusion was 35% smaller in *A₃AR*^{-/-} compared to wild-type mice (*A₃AR*^{+/+}). The reduction in infarct size was not the result of differences in heart rate, body temperature or increased cardiac expression of A₁ARs. However, neutrophil infiltration within infarcted regions was less in *A₃AR*^{-/-} mice. Furthermore, ischemic PC induced by either a single episode (one 5 min occlusion) or multiple episodes (six 4 min occlusions) of ischemia produced equivalent reductions in infarct size in *A₃AR*^{-/-} and *A₃AR*^{+/+} mice. These results indicate that, in the mouse, (i) A₃ARs play an injurious role during acute myocardial ischemia/reperfusion injury, possibly by exacerbating the inflammatory response, and (ii) A₃ARs are not necessary for the development of the early phase of ischemic PC. © 2001 Academic Press

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Introduction

Recent evidence suggests that pharmacological activation of A₃ adenosine receptors (ARs) prior to an ischemic challenge can induce both early and late preconditioning (PC).^{1–5} However, the functional importance of the A₃AR in modulating injury

during acute myocardial ischemia remains to be determined. Furthermore, it is unclear whether or not ischemia-induced PC is mediated through the A₃AR.^{1,3,6–8} To address these issues, highly selective A₃AR antagonists are required, which are not currently available. Recently, however, genetically engineered mice have been created in which the A₃AR

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gene is selectively disrupted.^{9,10} In this study, we used $A_3AR^{-/-}$ mice to examine the role of the A_3AR in the genesis of ischemia/reperfusion injury and ischemic PC using a physiologically-relevant *in vivo* mouse model of infarction.¹¹ Our results suggest that A_3AR s play an injurious role during acute myocardial infarction and that they are not essential for the development of ischemia-induced early PC.

Materials and Methods

In vivo mouse model

Studies were performed in $A_3AR^{+/+}$ and $A_3AR^{-/-}$ (Sv129/C57B/6/D2) male mice weighing 26–35 g (10–14 weeks old). The open-chest mouse model of regional ischemia and reperfusion has been previously described in detail.¹¹ At the end of the experiments, infarct size was assessed by *ex vivo* staining with triphenyltetrazolium chloride and expressed as a percentage of the area at risk.¹¹ Ischemic PC was elicited by either a single 5 min occlusion/10 min reperfusion cycle (single cycle PC) or a sequence of six 4 min occlusion/4 min reperfusion cycles (multiple cycle PC).

Six groups of mice were studied (Fig. 1). *Groups I* ($A_3AR^{+/+}$) and *II* ($A_3AR^{-/-}$) served as control groups and were subjected to 30 min occlusion and 24 h reperfusion without PC. *Groups III* ($A_3AR^{+/+}$) and *IV* ($A_3AR^{-/-}$) underwent the single cycle PC protocol immediately before a 30 min period of occlusion and 24 h of reperfusion. Finally, *groups V* ($A_3AR^{+/+}$) and *VI* ($A_3AR^{-/-}$) underwent the multiple cycle PC protocol immediately before a 30 min period of occlusion and 24 h of reperfusion.

Neutrophil accumulation

$A_3AR^{+/+}$ mice ($n=6$) or $A_3AR^{-/-}$ mice ($n=6$) were subjected to 30 min of coronary artery occlusion and 3 h of reperfusion, after which the hearts were fixed in paraformaldehyde, sectioned and then stained with hematoxylin and eosin. The average number of neutrophils per high power field in midmyocardial zones that showed clear evidence of infarction was counted in three independent fields.

Radioligand binding

A_1AR density in heart membranes from $A_3AR^{+/+}$ and $A_3AR^{-/-}$ mice was determined by

quantification of specific binding of the A_1AR -antagonist 8-cyclopentyl-1,3-³H]dipropylxanthine (³H]CPX).

Statistical analysis

Data are reported as means \pm s.e.m. Heart rate and body temperature were analysed by two-way repeated measures ANOVA (time and group). Infarct size, risk region size and accumulation of neutrophils were analysed with one-way ANOVA followed by unpaired *t*-tests with Bonferroni's correction.

Results

Exclusions

A total of 67 mice (33 $A_3AR^{+/+}$ and 34 $A_3AR^{-/-}$ mice) were used in the studies. Seven mice died in the $A_3AR^{+/+}$ group and eight died in the $A_3AR^{-/-}$ group.

Heart rate and body temperature

There were no significant differences among the different treatment groups in either of these two determinants of infarct size at any time throughout the experiments (Table 1).

Myocardial infarct size

There were no significant differences among the six groups with respect to body weight, left ventricular weight or weight of the region at risk (Table 2). In *group I* ($A_3AR^{+/+}$ mice), infarct size averaged $57.0 \pm 2.9\%$ of the area at risk (Fig. 1 and Table 2). In contrast, infarct size in *group II* ($A_3AR^{-/-}$) was only $36.0 \pm 4.0\%$ of the region at risk ($P < 0.05$ v *group I*; Fig. 1 and Table 2), indicating that the extent of cell death was reduced by $\sim 35\%$ in mice lacking functional A_3AR s.

In *groups III* and *V* ($A_3AR^{+/+}$ mice), PC with either one or six occlusion/reperfusion cycles reduced infarct size to 21.4 ± 5.2 and $17.5 \pm 3.0\%$ of the region at risk, respectively ($P < 0.05$ v *group I*; Fig. 1 and Table 2). A similar PC effect was noted in *groups IV* and *VI* ($A_3AR^{-/-}$ mice), in which infarct size was $11.2 \pm 2.5\%$ and $9.6 \pm 3.5\%$, respectively.

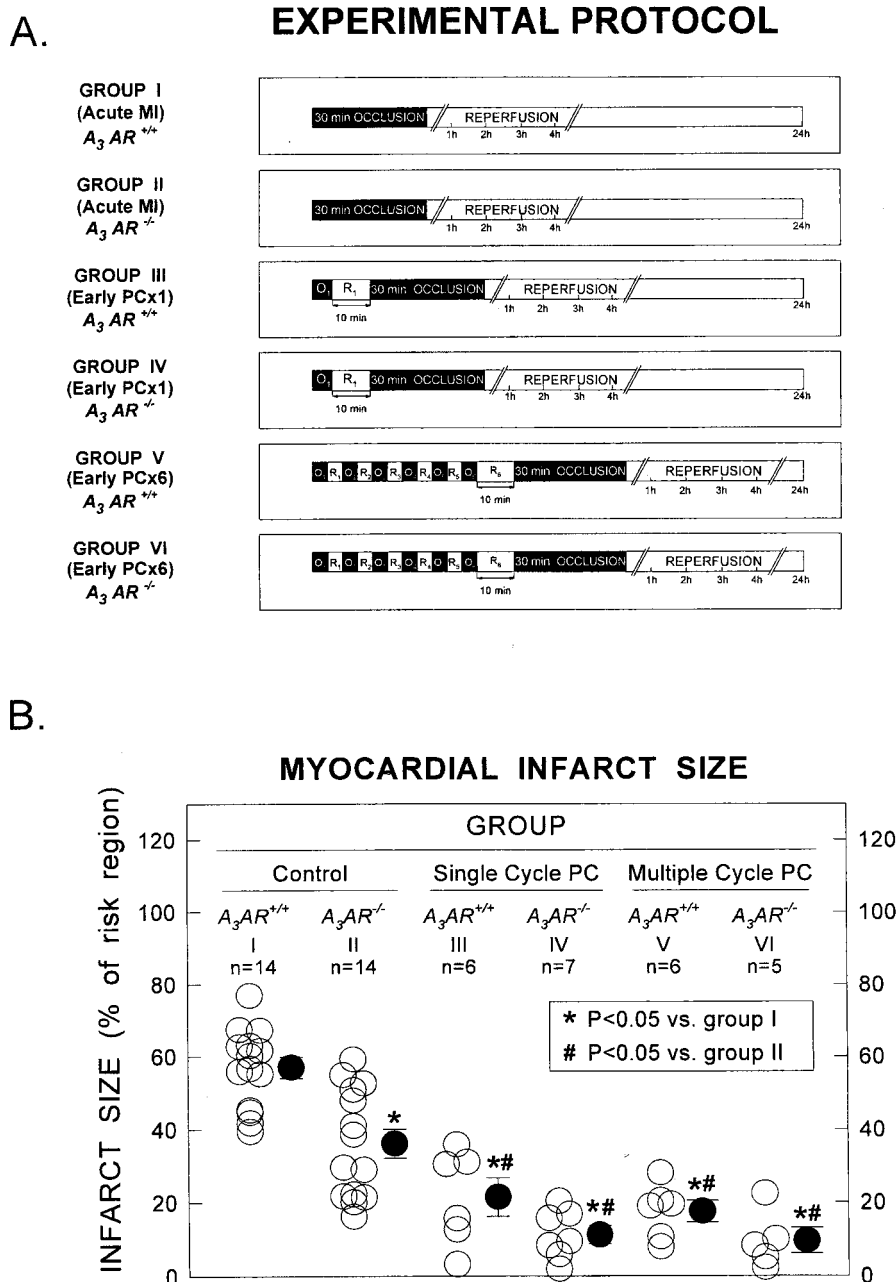


Figure 1 (A) Experimental protocol. (B) Myocardial infarct size in groups I–VI. Infarct size is expressed as a percentage of the area at risk of infarction. Individual mice (○); mean \pm S.E.M. for respective groups (●).

Neutrophil accumulation

The number of neutrophils that accumulated within infarcted regions following 30 min of occlusion and 3 h of reperfusion was significantly less ($P < 0.05$) in A₃AR^{-/-} mice compared to A₃AR^{+/+} mice (5.1 ± 0.4 v 3.0 ± 0.7 neutrophils per high power field, respectively; $n = 6$ /group).

Radioligand binding

Radioligand binding data revealed that the level of expression of A₃ARs was equivalent in membranes prepared from the left ventricles of A₃AR^{+/+} and A₃AR^{-/-} mice. The calculated K_d and B_{max} values were 0.7 ± 0.3 nM and 5 ± 2 fmol/mg protein, respectively ($n = 3$), for A₃AR^{+/+} mice, and

Table 1 Body temperature and heart rate during 30 min coronary occlusion and following reperfusion

	Preocclusion	Occlusion 15'	Occlusion 30'	Reperfusion 5'	Reperfusion 15'
	Temperature (°C)				
Group I	37.0 ± 0.1	37.0 ± 0.1	37.1 ± 0.1	37.0 ± 0.0	37.1 ± 0.1
Group II	37.0 ± 0.1	37.1 ± 0.0	37.1 ± 0.1	37.0 ± 0.1	37.0 ± 0.1
Group III	37.1 ± 0.1	37.0 ± 0.1	37.0 ± 0.1	36.9 ± 0.0	36.8 ± 0.1
Group IV	37.0 ± 0.1	37.0 ± 0.0	37.1 ± 0.1	36.9 ± 0.0	36.9 ± 0.1
Group V	37.1 ± 0.1	37.0 ± 0.1	37.1 ± 0.0	37.1 ± 0.1	37.0 ± 0.1
Group VI	36.9 ± 0.1	37.1 ± 0.1	36.9 ± 0.1	37.0 ± 0.1	36.9 ± 0.1
	Heart rate (beats/min)				
Group I	490 ± 14	525 ± 14	520 ± 12	539 ± 13	550 ± 15
Group II	525 ± 13	549 ± 19	559 ± 18	577 ± 19	578 ± 20
Group III	520 ± 19	535 ± 17	549 ± 20	547 ± 25	543 ± 30
Group IV	534 ± 11	545 ± 14	562 ± 17	563 ± 13	570 ± 19
Group V	505 ± 16	511 ± 15	516 ± 11	527 ± 8	536 ± 13
Group VI	543 ± 14	553 ± 12	559 ± 14	573 ± 8	571 ± 11

Data are means ± S.E.M. Measurements of rectal temperature and heart rate were taken before the 30 min coronary occlusion (preocclusion), at 15 and 30 min into the 30 min occlusion, and 5 and 15 min after reperfusion. The experimental protocols for the six mouse groups are specified in the legend to Figure 1.

0.8 ± 0.3 nM and 6 ± 2 fmol/mg protein, respectively ($n = 3$), for $A_3AR^{-/-}$ mice.

Discussion

Recent evidence suggests that A_3AR s are expressed in the heart and that pretreatment with A_3AR agonists induces both the early and late phases of PC.¹⁻⁵ To date, however, it has not been possible to assess the role of the A_3AR during acute myocardial ischemia (as opposed to their role in triggering PC), due to the unavailability of selective A_3AR antagonists. Thus, we used a molecular genetic approach and examined $A_3AR^{-/-}$ mice. We made the surprising observation that infarct size induced by 30 min of coronary occlusion and 24 h of reperfusion is smaller in $A_3AR^{-/-}$ compared to $A_3AR^{+/+}$ mice, suggesting that mice lacking functional A_3AR s are more resistant to the development of irreversible ischemic injury. To the best of our knowledge, this is the first study to assess infarct size in $A_3AR^{-/-}$ mice *in vivo*. Our results support the novel concept that some of the actions of adenosine mediated through the A_3AR are detrimental to the ischemic myocardium.

The mechanisms by which the A_3AR may exacerbate ischemic injury remain to be ascertained. A_3AR s are expressed in resident inflammatory cells such as mast cells^{9,10} where they can stimulate the release of stored mediators including histamine, cytokines, proteolytic enzymes as well as numerous other pro-inflammatory

mediators. Adenosine-induced activation of mast cells has been shown to be responsible for increased vascular permeability and extravasation of serum proteins in mice.¹⁰ Accordingly, it is possible that A_3AR s may increase infarct size by potentiating the inflammatory response. In support of this hypothesis, we observed that the number of neutrophils within infarcted regions in $A_3AR^{+/+}$ mice was significantly greater than in $A_3AR^{-/-}$ mice. It is impossible to discriminate, however, whether the reduced neutrophil accumulation in $A_3AR^{-/-}$ mice was the cause or the result of smaller infarcts. Since A_3AR s have also been suggested to induce apoptosis in cardiomyocytes¹² and to negatively regulate vascular tone,¹³ other potential mechanisms by which A_3AR s may promote the development of ischemic injury include promoting apoptosis or decreasing coronary blood flow. The possibility that the reduction in infarct size in $A_3AR^{-/-}$ mice was due to differences in systemic hemodynamics or body temperature can be ruled out, since these variables were similar throughout the studies (Table 1) and since it has been demonstrated previously that systemic blood pressure is not altered in $A_3AR^{-/-}$ mice.¹³ It must be kept in mind, however, that the reduction in infarct size in $A_3AR^{-/-}$ mice may be the result of compensatory changes which occur due to chronic absence of A_3AR s. Our radioligand binding studies exclude the possibility that expression levels of A_1AR s are altered in $A_3AR^{-/-}$ mice. Additional studies with A_3AR -selective antagonists, once developed, will

Table 2 Size of left ventricle, risk region and infarct

	Age (week)	Body (g)	LV (mg)	Risk region (mg)	Infarct (mg)	Risk region (% of LV)	Infarct (% of risk region)	Infarct (% of LV)
Group I	14.3 ± 1.9	32.1 ± 1.2	121.6 ± 5.2	44.0 ± 3.0	25.2 ± 2.2	34.0 ± 1.9	57.0 ± 2.9	19.3 ± 1.4
Group II	14.3 ± 0.6	30.3 ± 1.3	116.9 ± 5.1	42.4 ± 3.8	14.6 ± 1.5*	36.0 ± 2.1	36.0 ± 4.0*	12.5 ± 1.3*
Group III	11.5 ± 0.8	27.6 ± 0.8	103.8 ± 8.2	38.8 ± 3.3	11.5 ± 1.7*#	37.9 ± 2.6	21.4 ± 5.2*#	7.6 ± 1.5*#
Group IV	12.0 ± 0.7	28.9 ± 0.5	104.1 ± 5.4	39.7 ± 2.8	4.7 ± 1.2*#	38.2 ± 1.8	11.2 ± 2.5*#	4.4 ± 1.0*#
Group V	10.3 ± 0.8	28.0 ± 1.1	118.3 ± 8.2	35.4 ± 1.9	6.4 ± 1.3*#	30.5 ± 2.3	17.5 ± 3.0*#	5.6 ± 1.1*#
Group VI	10.6 ± 0.8	26.5 ± 0.7	110.6 ± 8.3	37.7 ± 3.4	5.2 ± 1.8*#	34.1 ± 2.2	9.6 ± 3.5*#	3.5 ± 1.5*#

LV, left ventricle; Body, body weight. Data are means ± S.E.M. * $P < 0.05$ v group I, # $P < 0.05$ v group II.

be necessary to test whether acute blockade of A₃ARs influences the development of ischemic injury.

Another important finding of the present study is that the early phase of ischemic PC is fully manifest in A₃AR^{-/-} mice. These results provide conclusive evidence that the A₃AR is not necessary for the development of the early phase of ischemic PC. Previously, the role of the A₃AR as a mediator of ischemic PC has been the subject of debate. In isolated rabbit and mouse hearts, Hill and colleagues⁷ and Morrison and colleagues⁸ demonstrated that blockade of A₁ARs abolishes ischemic PC, suggesting that this receptor subtype is the sole mediator of PC. However, Liu and coworkers³ observed in isolated rabbit hearts that the protection induced by ischemia, adenosine, and N⁶-(3-aminophenylethyl)adenosine (APNEA; A₁/A₃AR agonist) could not be blocked by CPX, suggesting that the protection of PC is not mediated exclusively by the A₁AR and may involve the A₃AR. Finally, Armstrong and Ganote^{1,6} proposed that the A₃AR is the sole mediator of PC in isolated rabbit cardiomyocytes, based on the observation that CPX does not block ischemia-induced PC and that the A₁AR-selective agonist (R)-N⁶-phenylisopropyladenosine (R-PIA) is incapable of inducing PC at lower concentrations in this model system. It is likely that these discrepant observations are the result of species differences, differences in the model systems employed, as well as the use of non-selective ligands. Our findings in A₃AR^{-/-} mice are consistent with the hypothesis that either the A₁AR is the sole mediator of PC or that both A₁ and A₃ARs can mediate PC.

In summary, the present study demonstrates that infarct size is smaller in A₃AR^{-/-} mice, suggesting that A₃ARs play an injurious role during myocardial ischemia. Our results further demonstrate that the A₃AR is not essential for the development of the early phase of ischemic PC. Both of these findings have important implications for our understanding of the function of these receptors in modulating ischemia/reperfusion injury in the heart.

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