Targeted deletion of A3 adenosine receptors improves tolerance to ischemia-reperfusion injury in mouse myocardium

RACHAEL J. CERNIWAY,1 ZEQUAN YANG,2 MARLENE A. JACOBSON,3 JOEL LINDEN,1 AND G. PAUL MATHERNE1
1Department of Pediatrics and the Cardiovascular Research Center, 2Department of Biomedical Engineering, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908; and 3Department of Pharmacology, Merck Research Laboratories, West Point, Pennsylvania 19486

Received 24 January 2001; accepted in final form 5 July 2001

Cerniway, Rachael J., Zequan Yang, Marlene A. Jacobson, Joel Linden, and G. Paul Matherne. Targeted deletion of A3 adenosine receptors improves tolerance to ischemia-reperfusion injury in mouse myocardium. Am J Physiol Heart Circ Physiol 281: H1751–H1758, 2001.—A3 adenosine receptors (A3Rs) have been implicated in regulating mast cell function and in cardioprotection during ischemia-reperfusion injury. The physiological role of A3Rs is unclear due to the lack of widely available selective antagonists. Therefore, we examined mice with targeted gene deletion of the A3AR together with pharmacological studies to determine the role of A3Rs in myocardial ischemia-reperfusion injury. We evaluated the functional response to 15-min global ischemia and 30-min reperfusion in isovolumic Langendorff hearts from A3AR−/− and wild-type (A3AR+/+) mice. Loss of contractile function during ischemia was unchanged, but recovery of developed pressure in hearts after reperfusion was improved in A3AR−/− compared with wild-type hearts (89 ± 3 vs. 51 ± 3% at 30 min). Tissue viability assessed by efflux of lactate dehydrogenase was also improved in A3AR−/− hearts (4.5 ± 1 vs. 7.5 ± 1 U/g). The adenosine receptor antagonist BW-A1433 (50 μM) decreased functional recovery following ischemia in A3AR−/− but not in wild-type hearts. We also examined myocardial infarct size using an intact model with 30-min left anterior descending coronary artery occlusion and 24-h reperfusion. Infarct size was reduced by over 60% in A3AR−/− hearts. In summary, targeted deletion of the A3AR improved functional recovery and tissue viability during reperfusion following ischemia. These data suggest that activation of A3Rs contributes to myocardial injury in this setting in the rodent. Since A3Rs are thought to be present on resident mast cells in the rodent myocardium, we speculate that A3Rs may have proinflammatory actions that mediate the deleterious effects of A3AR activation during ischemia-reperfusion injury.

knockout; myocardial infarction; inflammation; cardiac protection

ADENOSINE IS RAPIDLY RELEASED in large amounts during cardiac ischemia and is a potent mediator of endogenous protective functions in the heart (6, 16, 45). Previous studies have shown that adenosine or adenosine receptor (AR) agonists can delay the onset of contracture (17), improve postischemic contractile function, reduce the rate of ATP catabolism (16), attenuate myocardial stunning (30), and reduce infarct size (3, 24, 42–44). Adenosine exerts its function via G protein-coupled receptors classified into at least four receptor subtypes: A1, A2A, A2B, and A3. The A3AR is the most recently identified subtype and has been cloned in multiple species, including rats (27, 49), sheep (21), humans (35), rabbits (11), and dogs (2).

Evaluation of the physiological role of A3ARs in the heart and other target tissues has been hampered by 1) multiple AR subtypes in the same target tissue, 2) lack of selective antagonists, and 3) diverse receptor affinities (particularly to xanthine antagonists) across species. Activation of A3ARs in a variety of models has been shown to elicit mast cell degranulation and subsequent release of inflammatory mediators (8, 12, 32, 37) to either induce (14, 38) or reduce apoptosis (9, 47) and to exert preconditioning (1, 24, 33, 39). Myocardial ischemia-reperfusion has also been shown to elicited degranulation of cardiac mast cells that may contribute to myocardial dysfunction and preconditioning (for a review, see Ref. 19). A3ARs have more recently been implicated in mediating cardioprotection from myocardial ischemia-reperfusion injury (3, 14, 43, 44). Pretreatment with selective A3 agonists has resulted in protection from ischemia-reperfusion injury in isolated hearts (43), attenuation of myocardial stunning and infarct size in conscious rabbits (3), and attenuation of postischemic dysfunction through ATP-sensitive K+ channels (KATP) (40, 44). Furthermore, Jordan et al. (14) recently reported that activation of A3Rs with 2-chloro-N6-(3-iodobenzyl)adenosine-5′-N-methyluronamide attenuated reperfusion injury by decreasing neutrophil function in isolated rabbit hearts.

Although there is increasing evidence suggesting a protective role for the A3AR during ischemia-reperfu-
sion injury, a recent report by Guo et al. (10) demonstrated that targeted deletion of A3AR confers resistance to myocardial ischemia injury, but targeted deletion did not prevent early preconditioning. These disparate findings suggest that the role of the cardiac A3AR needs further clarification. Accordingly, the goal of this study was to further determine the role that A3ARs play in protecting the heart from ischemia-reperfusion injury in both isovolumic Langendorff hearts and intact animal myocardial infarction experiments in A3AR−/− hearts and wild-type (A3AR+/+) littermate controls. Although the A3AR has been implicated in preconditioning/cardioprotection, our results and those of Guo et al. (10) indicate that A3AR−/− hearts are protected functionally as well as from infarction associated with ischemia-reperfusion injury.

METHODS

A3AR−/− mouse model. Homozygous A3AR−/− mice and wild-type A3AR+/+ littermate controls were kindly provided by Merck Research Laboratories. Generation and initial characterization of the A3AR−/− mice have been described in detail previously (35).

Langendorff-perfused mouse heart model. Hearts were isolated from 20- to 24-wk-old male and female wild-type (A3AR+/+, 28 ± 2 g body wt, n = 12) and A3AR−/− mice (31 ± 2 g body wt, n = 13) with homozygous deletion of the A3AR. Mice were anesthetized with 50 mg/kg pentobarbital sodium administered intraperitoneally, a thoracotomy was performed, and hearts were rapidly excised into heparinized ice-cold perfusion buffer. The aorta was cannulated, and the hearts were retrogradely perfused at a constant pressure of 80 mmHg with modified Krebs buffer containing (in mM) 118 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, 11 glucose, and 0.6 EDTA. Buffer was equilibrated with 95% O2-5% CO2 at 37°C, giving a pH of 7.4 and a Po2 of ~550 mmHg. The left ventricle was vented with a small transducer by a polyethylene tube permitting continuous measurement of left ventricular pressure. Hearts were immersed in perfusate in a water-jacketed bath maintained at 37°C, and the ventricular balloon was inflated to yield a left ventricular diastolic pressure of 3–5 mmHg. Coronary flow was continuously monitored via a Doppler flow probe (Transonic Systems; Ithaca, NY) located in the aortic perfusion line. Aortic and left ventricular developed pressure were recorded on a MacLab data acquisition system (AD Instruments; Castle Hill, Australia) connected to an Apple 7300/180 computer. The ventricular pressure signal was digitally processed on-line (using MacLab Chart 3.5.6, AD Instruments) to yield diastolic and systolic pressures, heart rate, and the first derivative of pressure over time (dP/dt).

All hearts (A3AR−/− and wild type; n = 6 for both groups) were allowed to stabilize for 30 min at intrinsic heart rate. Baseline diastolic and systolic pressures, heart rate, and coronary flow were recorded, and 15-min normothermic global ischemia was initiated by closing the aortic cannula and simultaneously bubbling the bathing perfusate with 95% N2-5% CO2 to reduce O2. Analysis of the bathing perfusate showed that pH was unchanged and Po2 fell to ~40 mmHg. Following ischemia, reperfusion was initiated by unclamping the aortic cannula and discontinuing the nitrogen bubbling. To verify the role of A3AR activation in this model of ischemia-reperfusion, a separate group of experiments was performed in which we predominately blocked A1- and A3ARs and compared the responses between A3AR−/− and wild-type animals. In the wild-type animals, blocking A1 receptors would result in negative functional effects as seen in our previous work (26). If we also blocked A3 receptors, the overall effect would be worsened recovery (if A3 receptors were beneficial) and a blunted negative effect (if A3 receptors were detrimental). In A3AR−/− hearts, only A1 receptors could be blocked and the result should be detrimental. An antagonist that blocks A1- and A3ARs is BW-A1433, which has been shown to block rat A1 receptors with an inhibitory constant (Ki) of ~15 nM and rat A3 receptors with a Ki of ~24 μM (12, 19). BW-A1433 (50 μM; a gift from Susan Daluge, Glaxo-Wellcome; Research Triangle Park, NC) was infused for 10 min before global ischemia at ~1% of coronary flow and resumed after global ischemia for the duration of reperfusion (n = 7 for A3AR−/− and n = 6 for wild-type hearts).

Affinity of BW-A1433 for mouse A3AR. To determine the affinity of BW-A1433 for mouse A3 receptors, first, we cloned the mouse A3AR. To do this, we prepared cDNA from RT-PCR from mouse testes. The primers used were as follows: forward, TCCGCTGAAGCTTTTCTGAGAACATGGAAGGC; and reverse, TGGCTCTGTATCTGTCAAGGTAACTGCTCA.

The sequence of the PCR product was checked and the cDNA was transferred to the mammalian expression plasmid CLDN10B. The mouse A3 receptor was introduced into HEK-293 cells with Lipofectin (GIBCO-BRL). Stably transfected cells were identified by growth in 0.5 μg/ml G418 and maintained in 0.25 μg/ml. The affinity of BW-A1433 was assessed by competition for the binding of 125I-labeled N6(2-p-aminobenzyl)adenosine (125I-ABA) to membranes prepared from transfected cells (12).

Determination of myocardial tissue viability via lactate dehydrogenase efflux. To assess degree of cell death in isolated hearts, coronary effluent was collected from the fluid-filled bath surrounding the heart for quantitation of lactate dehydrogenase (LDH) content (28). An initial sample was collected at the end of 30-min equilibration for baseline content. On the onset of reperfusion, coronary effluent was collected and pooled throughout the 30-min period. The total volume of pooled effluent was measured, and duplicates of 1.5-ml aliquots were taken and stored at −20°C until analysis. An LDH enzymatic assay kit (Sigma) was optimized for sensitivity of detection and spectrophotometric analysis to determine the LDH concentration in each sample. Total LDH efflux was calculated by multiplying the enzyme concentration in the sample by the total volume of coronary effluent for each heart. To correct for different heart sizes, these values were normalized for wet heart weight and expressed as units per gram similar to previously reported methods (5, 23, 46).

In vivo mouse model of myocardial infarction. Mice were anesthetized with pentobarbital sodium at an initial dose of 100 mg/kg ip. Animals were placed in a supine position on a heating pad (Fine Science Tools; Foster City, CA), and their paws and tails were taped to the operating table. The upper portion of the trachea was exposed through a midline incision in the neck, and an endotracheal tube (polyethylene tubing, PE-60) was inserted orally into the trachea. Artificial respiration was maintained with a SAR-830/P ventilator (ITLC Life Science, Woodland Hills, CA) with 80% oxygen at a frequency of 100 strokes/min, and a tidal volume of 1.5–2.5 ml. After intubation, the midline skin incision in the neck was extended down to the xiphoid process. The left third and fourth ribs were exposed by bluntly dividing the left pectoris major muscle and the muscles beneath it. A parasternal
incision was made to open the left pleural cavity by cutting the left third and fourth ribs and intercostal muscles with a cautery pen (General Medical, Richmond, VA). A 7-0 silk suture was passed underneath the left anterior descending (LAD) coronary artery at a level just below the left atrium. Myocardial ischemia was induced by tying the suture over a piece of polyethylene (PE)-60 tubing for a 30-min period. Significant electrocardiogram changes, including widening of QRS and elevation of S-T segment (monitored with a MacLab/8S Bridge/Bio Amplifier; AD Instruments), and color changes of the region at risk were used to confirm successful occlusion of the LAD coronary artery. Reperfusion was initiated by untying the suture from the PE-60 tubing and then removing the tubing but leaving the suture in place. A volume of 1–1.5 ml of 5% dextrose was given intraperitoneally to replace fluids lost during the operation. Rectal temperature was monitored throughout the operation with a rectal probe (thermocouple thermometer, Barnant; Barrington, IL) and was maintained between 36.5 and 37.5°C.

Determination of myocardial infarct size via triphenyltetrazolium chloride staining. After excision, hearts were cannulated through the ascending aorta and sequentially perfused with 2–3 ml of 0.9% sodium chloride solution and 3–4 ml of 1.0% triphenyltetrazolium chloride (TPC) in phosphate buffer (pH 7.4, 37°C). After TPC staining, the LAD coronary artery was reocluded by tightening the suture left in the myocardium after infarction. Hearts were then perfused with 2–3 ml of 10% Pthalo blue to delineate the nonischemic tissue. Hearts were frozen and trimmed free of right ventricle and atria, and the left ventricle was cut into 5–7 transverse slices. Slices were then fixed in 10% neutral buffered formalin solution. Each slice was weighed and photographed from both sides under a low magnification microscope (Olympus SZX12; Olympus Optical) mounted with a high-resolution digital camera (DVC-1300 RGB Color; DVC, Austin, TX). The imaging system was controlled by a computer running Image Pro-Plus (version 4.0, Media Cybernetics, Silver Spring, MD). The images were then transferred to PhotoShop (Adobe), and the borders of the infarction, ischemic area (risk region), and nonischemic area were traced for both sides of each tissue slice. The sizes of the nonischemic area, the risk region, and the infarction area of each slice were calculated as a percentage of corresponding total area multiplied by the total weight of the slice.

Statistical analysis. All results are expressed as means ± SE. Functional parameters during ischemia-reperfusion were analyzed by multivariate ANOVA for repeated measures with Bonferroni's correction for multiple comparisons and Tukey's post hoc test. Comparisons between groups for total LDH efflux and myocardial infarct size was made using Student's t-test. Comparison between groups for final recovery of developed pressure was made using a two-way ANOVA with Bonferroni's correction for multiple comparisons and Tukey's post hoc test. Statistical significance was accepted for P < 0.05.

RESULTS

Baseline function. Baseline functional data for wild-type (heart wt 138 ± 16 mg, n = 12) and A3AR−/− isolated hearts (heart wt 148 ± 14 mg, n = 13) are shown in Table 1. No differences in preischemic functional parameters or coronary flow rates were observed between wild-type and A3AR−/− hearts at baseline (Table 1).

<table>
<thead>
<tr>
<th>Wild Type</th>
<th>A3AR−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl (n = 6)</td>
<td>BW-A1433 (n = 6)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>362 ± 21</td>
</tr>
<tr>
<td>CF, ml·min−1·g−1</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>110 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of mice. Functional parameters were measured after 30 min of normothermic aerobic perfusion using standard Langendorff preparation. Ctrl, no treatment; BW-A1433, treated with 50 μM of the adenosine receptor (AR) antagonist; HR, heart rate; CF, coronary flow; EDP, end-diastolic pressure; LVDP, left ventricular developed pressure. Differences in groups were not significant.

Ischemia-reperfusion in wild-type and A3AR−/− isolated hearts. Global normothermic ischemia immediately reduced heart rate and contractile function, with full arrest within 5 min. Time to ischemic contracture was 13.0 ± 1.9 min in wild-type hearts and 13.1 ± 2.2 min in A3AR−/− hearts. Diastolic pressure increased during ischemia in both wild-type and A3AR−/− hearts to peaks of 40 ± 7 and 38 ± 10 mmHg, respectively. Heart rate and coronary flow were comparable among all the study groups (Fig. 1, A and B). Reactive hyperemia was initially observed on reperfusion in all groups, which then returned to baseline by the end of reperfusion. (Fig. 1B). Myocardial function (expressed as the percent change from baseline developed pressure) during ischemia-reperfusion is shown in Fig. 2. With the onset of reperfusion, hearts resumed spontaneous contraction within 30–60 s and exhibited a marked recovery of left ventricular developed pressure. After an initial increased recovery, there was a decline in function followed by a progressive recovery of developed pressure for the remainder of reperfusion. Deletion of the A2A (A2AR−/−) resulted in a better recovery of left ventricular developed pressure compared with wild-type hearts at all time points during reperfusion. These differences were particularly evident when final recovery of left ventricular developed pressure was examined, with A2A−/− hearts demonstrating significantly better recovery of function compared with wild-type hearts (67 ± 5 vs. 45 ± 2% of baseline, P < 0.05, Figs. 2 and 3). Cell death was estimated by total LDH efflux in isolated hearts. Total LDH efflux was calculated to be 7.5 ± 0.9 U/g in wild-type hearts, whereas A2A−/− significantly reduced total efflux of LDH by ~40% to 4.5 ± 1.0 U/g (P < 0.05), providing evidence for improved tissue viability along with the preserved function in A2A−/− hearts.

Affinity of BW-A1433 to mouse A3ARs. Binding of 125I-ABA and BW-A1433 to recombinant mouse A3ARs is shown in Fig. 4. Each triplicate assay contained membrane protein, radioligand, and the indicated concentration of BW-A1433. Figure 4, inset, shows equi-
librium specific binding of $^{125}$I-ABA to the same membrane proteins. The maximum number of bound receptors ($B_{\text{max}}$) of $^{125}$I-ABA was calculated to be 178 fmol/mg of protein and the dissociation constant ($K_d$) was calculated to be 6.9 nM. The $K_i$ for BW-A1433 at mouse A3 receptors was calculated to be 12.5 $\pm$ 4.5 $\mu$M. At a concentration of 50 $\mu$M, BW-A1433 is predicted to occupy >80% of mouse A3 receptors and to decrease the potency of adenosine by fivefold.

Adenosine antagonism in wild-type and A3AR$^{-/-}$ hearts. Administration of the nonselective antagonist BW-A1433 (50 $\mu$M) had no significant effect on baseline functional parameters or coronary flow in either wild-type or A3AR$^{-/-}$ isolated hearts (Table 1 and Fig. 1, A and B). The effect of BW-A1433 on the time course and final recovery of left ventricular developed pressure following ischemia-reperfusion is shown in Figs. 2 and 3. Pretreatment with BW-A1433 resulted in no demonstrable change in recovery of left ventricular developed pressure at any time point (Fig. 2), including final recovery of left ventricular developed pressure following ischemia-reperfusion in wild-type hearts (45 $\pm$ 2 vs. 42 $\pm$ 3% in treated hearts, Fig. 3). Conversely, A3AR$^{-/-}$ hearts treated with the AR antagonist demonstrated significantly reduced recovery of left ventricular function to levels that approximated wild-type at all time points (Fig. 2). This was particularly evident when examining final recovery of left ventricular developed pressure (Fig. 3). Values shown are means $\pm$ SE. *$P < 0.05$, A3AR$^{-/-}$ compared with wild-type hearts.

Fig. 1. Time course of heart rate (A) and coronary flow (B) in wild-type ($n = 6$; circles) and A3 adenosine receptor (A3AR)-deficient (A3AR$^{-/-}$) ($n = 6$; squares) isolated hearts subjected to 15 min of ischemia and 30 min of reperfusion. Hearts were either untreated (open symbols; $n = 6$ for both groups) or treated with 50 $\mu$M BW-A1433 (closed symbols; $n = 6$ for wild-type hearts and $n = 7$ for A3AR$^{-/-}$ hearts). Values shown are means $\pm$ SE.

Fig. 2. Time course of left ventricular developed pressure (expressed as a percentage of baseline) in wild-type ($n = 6$; circles) and A3AR$^{-/-}$ ($n = 6$; squares) isolated hearts subjected to 15 min of ischemia and 30 min of reperfusion. Hearts were either untreated (open symbols; $n = 6$ for both groups) or treated with 50 $\mu$M BW-A1433 (closed symbols; $n = 6$ for wild-type hearts and $n = 7$ for A3AR$^{-/-}$ hearts). Values shown are means $\pm$ SE. *$P < 0.05$, A3AR$^{-/-}$ compared with wild-type hearts.

Fig. 3. Final recovery of left ventricular developed pressure (expressed as a percentage of baseline) in wild-type and A3AR$^{-/-}$ isolated hearts subjected to 15-min ischemia and 30-min reperfusion. Hearts were either untreated (Ctrl; $n = 6$ for both groups) or treated with 50 $\mu$M BW-A1433 ($n = 6$ for wild-type hearts and $n = 7$ for A3AR$^{-/-}$ hearts). Values shown are means $\pm$ SE. *$P < 0.05$, A3AR$^{-/-}$ vs. wild-type hearts; †$P < 0.05$, treated vs. untreated hearts.
Myocardial infarction in wild-type and A3AR<sup>−/−</sup> hearts. To investigate the role of the A3AR in an intact animal model of myocardial infarction, wild-type (n = 8) and A3AR<sup>−/−</sup> (n = 10) mice underwent 30-min occlusion of the LAD coronary artery followed by 24 h of reperfusion. Infarct size (Fig. 5) and the area at risk were determined by TTC staining and Phthalo blue staining, respectively. There was no difference in the area at risk between the two groups (39.6 ± 3.0 vs. 37.5 ± 2.0, P > 0.05). Targeted deletion of the A3AR resulted in a marked reduction in myocardial infarct size (expressed as a percentage of the area at risk) compared with wild-type mice, decreasing over 60% from 45 ± 5% to 13 ± 3% (P < 0.05) (Fig. 5).

DISCUSSION

In this study, we examined the role of the A3AR in providing protection from ischemia-reperfusion injury in the mouse myocardium. For these studies we examined functional responses in Langendorff-perfused hearts and myocardial infarct size in an intact animal model of myocardial infarction. Moreover, we attempted to delineate the role of the A3AR in these studies by using an AR antagonist in a subset of isolated perfused hearts. Here, we present evidence that the A3AR plays a deleterious role during acute ischemia-reperfusion injury on both myocardial function and tissue viability in rodents by a mechanism that supercedes possible A3-mediated cardioprotection. Although a number of studies have examined the role A3ARs play in both ischemia-reperfusion injury and in ischemic preconditioning, the majority of these studies have been in models of either cell culture (cardiomyocytes) or isolated hearts utilizing exogenously applied A3 agonists (1, 14, 24, 40, 43, 44). However, the exact mechanisms associated with the observed cardioprotection afforded by A3ARs, particularly the “end-effector” in these models, are still not clearly defined. This lack of universal agreement on the physiological and functional role of A3ARs in the heart is primarily due to the diversity of both the cellular location and pharmacological profiles of these receptors across species. Although there are a number of specific agonists and antagonists of human, rabbit, and dog A3ARs, there are no known specific antagonists to rodent A3 receptors, with the possible exception of MRS-1523, which may block A3ARs in rats but has not yet been characterized in mice (9). This presents a challenge to the full investigation and comprehensive characterization of the role of the A3 receptor in these species. We therefore utilized a model with heterologous deletion of the A3 receptor in the mouse myocardium to assess its role in acute ischemia-reperfusion injury.

Our initial studies investigated the functional response to ischemia-reperfusion injury in isolated Langendorff-perfused wild-type and A3AR<sup>−/−</sup> hearts. Deletion of A3ARs did not effect baseline heart rate, end diastolic pressure, coronary flow, or left ventricular developed pressure. This is consistent with the recently published characterization of this A3AR<sup>−/−</sup> mouse model (35). Contrary to our original hypothesis, functional recovery from ischemia-reperfusion in A3AR<sup>−/−</sup> hearts was unexpectedly improved compared with wild-type hearts. Recovery of left ventricular developed pressure (percentage of baseline) was significantly increased at all time points throughout reperfusion (Fig. 2), improving by ~50% by the end of 30-min reperfusion. Interestingly, the A3AR<sup>−/−</sup> hearts demonstrated no differences in either time to ischemic contracture or in peak diastolic pressure during ischemia compared with wild-type hearts. Thus the beneficial effects of deleting the A3 receptor in the mouse myocardium appears to only play a significant role during the reperfusion phase of ischemia-reperfusion injury. This effect is consistent with the notion of A3ARs triggering mast cell degranulation (19) and a role for mast cells in reperfusion injury and the inflammatory cascades that follow (8, 32). Total efflux of LDH during reperfusion was reduced in A3AR<sup>−/−</sup> hearts compared with wild-type hearts, which is an indication that these hearts had less cell death and, therefore, demonstrated an improvement in tissue viability.

To confirm that the cardioprotection observed in these A3AR<sup>−/−</sup> mice is due to deletion of the A3AR, we examined the effect of the AR antagonist BW-A1433 (50 μM) administered before ischemia and during reperfusion. We chose this antagonist because high concentrations (>25 μM) have been shown to block rat A<sub>3</sub> receptors and to block A3 receptor-mediated degranulation of rodent mast cells (12). Furthermore, in the current study, we present data from 125I-ABA binding studies at an antagonist concentration of 50 μM, which is predicted to block ~80% of the mouse A3ARs (Fig. 4).
It has been well established by us and others that blocking the adenosine A1 receptor decreases functional recovery in hearts subjected to ischemia-reperfusion injury (17, 28). On the other hand, our current results with A3AR−/− hearts suggest that blocking the A3AR might have a beneficial effect. Therefore, given the known detrimental effect for A1 blockade and the observed protective effect of A3 deletion, blocking both receptors by treating wild-type hearts with BW-A1433 may be expected to have opposing effects, whereas selective blockade of adenosine A1 receptors by treating A3AR−/− hearts with BW-A1433 would be expected to adversely affect myocardial function. Baseline function in the BW-A4133-treated groups was similar to the untreated groups, and they demonstrated similar functional time course profiles (Fig. 2). On examination of final functional recovery of left ventricular developed pressure (Fig. 3), wild-type hearts treated with BW-A1433 showed no difference in functional recovery, whereas A3AR−/− hearts treated with BW-A1433 showed a significant decline in final functional recovery by ~30%. These data are consistent with our proposed notion, that blockade of A1 and A3 receptors may have opposing effects. Furthermore, because BW-A1433 at the concentration used in this study is a nonselective antagonist of all four AR subtypes, we reasoned that the differential effects of BW-A1433 on wild-type and A3AR−/− mice was due to blockade of the A3 receptor in wild-type mice. Another possibility in this regard is whether there is an effect of A3AR−/− on A1AR expression. Salvatore et al. (35) and Zhao et al. (48), in their initial characterization of this mouse, found no changes in expression. Furthermore, Guo et al. (10) recently confirmed this earlier finding: that there is no change to A1R expression in mice with the A3AR deleted. Although these results are challenging to interpret, this study does provide evidence that the increased functional recovery of rodent hearts in response to ischemia-reperfusion injury in this model appears to be a result of deletion of the A3AR.

To further examine this interesting improvement in functional recovery and tissue viability in hearts lacking the A3AR, we examined A3AR−/− mice in an intact animal model of myocardial infarction. Assessment of myocardial infarct size was used as the determinant of recovery (Fig. 5). Myocardial infarct size (expressed as a percentage of the area at risk) in the A3AR−/− mice was markedly reduced by over 60%. These data add to the isolated heart functional data and tissue viability data in providing evidence that activation of the A3AR is deleterious both in vitro and in vivo. The data from this study are consistent with and confirm the recently published data by Guo et al. (10), who reported a decrease of ~35% in infarct size when the A3 receptor was deleted (57.0 ± 2.9% in A3AR+/− mice vs. 36.0 ± 4.0% in A3AR−/−).

Another important finding of this study is that deletion of the A3AR appears to afford its protection during the reperfusion phase of ischemia-reperfusion injury given there was no difference in time to ischemic con-
understanding of the A3 receptor on mast cells in rodents will help to identify the potential pathways, in both rodent and human alike, that are involved in ischemia-reperfusion injury and inflammatory responses.

In summary, the studies reported here are consistent with a role for the A3AR in an inflammatory cascade that is initiated as a result of ischemia-reperfusion injury in the rodent heart. We speculate that A3ARs may be acting deleteriously in both the in vitro and in vivo models of ischemia-reperfusion injury by triggering such an inflammatory pathway either by direct stimulation of mast cell degranulation with subsequent release of injurious mediators or via an as yet unknown mechanism. These results imply an important physiological role in mice for the A3AR in modifying both the functional outcome and tissue viability of the myocardium after ischemia-reperfusion injury. Furthermore, these data suggest that the view that activation of A3ARs is cardioprotective in the rodent heart following ischemia-reperfusion injury needs to be reevaluated.

We thank Melissa Marshall for technical assistance with the mouse A3 cDNA and BW-A1433 affinity data. This work was supported by National Heart, Lung, and Blood Institute Grant RO1-HL-59419. G. P. Matherne was the recipient of an American Heart Association Established Investigator grant.

REFERENCES


