Genetic Deletion of the A1 Adenosine Receptor Limits Myocardial Ischemic Tolerance

Melissa E. Reichelt,* Laura Willems,* Jose G. Molina, Chun-Xiao Sun, Janci C. Noble, Kevin J. Ashton, Jurgen Schnermann, Michael R. Blackburn, John P. Headrick

Abstract—Adenosine receptors may be important determinants of intrinsic ischemic tolerance. Genetically modified mice were used to examine effects of global A1 adenosine receptor (A1AR) knockout (KO) on function and ischemic tolerance in perfused mouse hearts. Baseline contractile function and heart rate were unaltered by A1AR KO, which was shown to abolish the negative chronotropic effects of 2-chloroadenosine (A1AR-mediated) without altering A2 adenosine receptor–mediated coronary dilation. Tolerance to 25 minutes global normothermic ischemia (followed by 45 minutes reperfusion) was significantly limited by A1AR KO, with impaired contractile recovery (reduced by ≈25%) and enhanced lactate dehydrogenase (LDH) efflux (increased by ≈100%). Functional effects of A1AR KO involved worsened systolic pressure development with little to no change in diastolic dysfunction. In contrast, cardiac specific A1AR overexpression enhanced ischemic tolerance with a primary action on diastolic dysfunction. Nonselective receptor agonism (10 μmol/L 2-chloroadenosine) protected wild-type and also A1AR KO hearts (albeit to a lesser extent), implicating protection via subtypes additional to A1ARs. However, A1AR KO abrogated effects of 2-chloroadenosine on ischemic contracture and diastolic dysfunction. These data are the first demonstrating global deletion of the A1AR limits intrinsic myocardial resistance to ischemia. Data indicate the function of intrinsically activated A1ARs appears primarily to be enhancement of posts ischemic contractility and limitation of cell death. (Circ Res. 2005;96:363-367.)

Key Words: adenosine □ A1 adenosine receptor □ gene knockout □ ischemia □ reperfusion

The heart possesses protective or retaliatory mechanisms providing tolerance to ischemia/reperfusion. These may represent targets for therapeutic manipulation of ischemic tolerance, and conversely, alterations in these mechanisms could underlie changes in outcome with aging and/or disease. We and others have been studying the role of the purine nucleoside adenosine and its receptors in modulating injury during ischemia. Although contentious,2 the AR system may be an integral component of the hearts intrinsic protective arsenal, limiting damage during ischemia and after ischemic challenge.6 Although this is consistent with benefit via AR agonists,1–3,7 effects of AR antagonism (to unmask responses to endogenous adenosine) are equivocal, with studies supporting4–6,8–11 and refuting12–15 a role for endogenous adenosine in dictating ischemic tolerance. Some of this controversy may stem from inherent limitations in pharmacological approaches to abrogating receptor-mediated responses; these can be hampered by potentially poor antagonist selectivity or potency, and/or potentiation of local agonist levels as a result of opening feedback loops linking “signal” (adenosine generation in this case) to tissue “response” (protection of cellular homeostasis).16–18 An alternative approach involves selective gene deletion, which, coupled with complementary analysis of effects of transgenic overexpression, may facilitate assessment of the specific role of a protein in wild-type tissue.19,20 In this study, we document for the first time the ability of genetic removal of A1ARs to modify intrinsic tolerance to ischemia.

Materials and Methods
Investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Experimental Protocol
A1AR knockout (A1AR KO) mice (Jackson Laboratories, Bar Harbor, Me) were generated and genotyped as described previously,21 with genotypes determined via PCR analysis of genomic DNA. All mice were on a mixed 129sv/C57BL/6J background and phenotypic comparisons were performed among littermates. Details of generation

Original received September 10, 2004; revision received January 4, 2005; accepted January 4, 2005.
From the Heart Foundation Research Center (M.E.R., L.W., K.J.A., J.P.H.), Griffith University, Southport, Australia; the Department of Biochemistry and Molecular Biology (J.G.M., C.-X.S., J.C.N., M.R.B.), University of Texas Health Science Center at Houston, Medical School, Houston; and the National Institute of Diabetes and Digestive and Kidney Diseases (J.S.), National Institutes of Health, Bethesda, Md.
*Both authors contributed equally to this study.
Correspondence to John Headrick, Heart Foundation Research Centre, Griffith University, Southport, QLD 4217, Australia. E-mail J.Headrick@griffith.edu.au
© 2005 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000156075.00127.C3
of C57/BL/6J mice selectively overexpressing cardiac A1ARs have been reported previously.22 Hearts for this study were isolated from young (2 month) mice from the following groups: A1AR KO (n=20); wild-type littermates (n=24); A1AR overexpression (n=15); and wild-type C57/BL/6J (n=16).

All mice were anesthetized with 50 mg/kg sodium pentobarbitone administered intraperitoneally, a thoracotomy performed, and hearts excised into ice-cold perfusion fluid for cannulation and perfusion on a Langendorff perfusion system.5,23 Hearts were stabilized at intrinsic rate a further 10 minutes before acquiring concentration-response curves for 2-chloroadenosine–mediated A1AR and A2AAR-dependent bradycardia/coronary dilation. Concentration-response curves were acquired in unpaced normoxic hearts from A1AR KO (n=6), wild-type littermates (n=7), A1AR overexpressing mice (n=7), and wild-type C57/BL/6J mice (n=7), as described previously.24 Chronotropic and vasodilatory responses were scaled as percentage of baseline, and data were analyzed via nonlinear regression to acquire individual pEC50 values, as outlined previously.24,25

In ischemic studies, hearts were stabilized for 20 minutes at intrinsic heart rate before pacing at 420 bpm followed by a further 10 minutes stabilization period.5,23 Baseline measurements were then made, and hearts were subjected to 25 minutes global normothermic ischemia followed by 45 minutes aerobic reperfusion. Coronary venous effluent was collected on ice for enzymatic analysis of lactate dehydrogenase (LDH) activity.23 Total LDH efflux during reperfusion was expressed as international units (IU) per gm wet weight, and has been previously shown to correlate with measures of oncotic injury/infarction in this model.26 Ischemic responses were assessed in A1AR KO (n=7) and wild-type littermate (n=9) mice, and A1AR overexpressing (n=8) and wild-type littermate (n=9) mice.

Effects of adenosinergic cardioprotection with 10 μmol/L of the nonselective agonist 2-chloroadenosine were also assessed in wild-type (n=8) and A1AR KO hearts (n=7) subjected to 25 minutes ischemia and 45 minutes reperfusion. Based on concentration-response data in Figure 1, >3 μmol/L 2-chloroadenosine is required to maximally activate a functional A1AR response (less for an A2AR response). Thus, in an attempt to achieve near-maximal activation of all adenosine receptor subtypes in all murine lines studied, we used a 10 μmol/L agonist concentration. Although it is feasible prolonged treatment with high concentrations of 2-chloroadenosine might induce adenosine receptor-independent actions (because it is a substrate for nucleoside transporters), this is unlikely to be an issue in the current acute studies. The 10 μmol/L concentration is equivalent to or less than functional EC50 values for 2-chloroadenosine activation of adenosine receptor responses in cardiovascular and other cell types.27–31 Recent studies confirm this concentration induces receptor-mediated actions in cardiomyocytes and cardiac fibroblasts (mimicked by selective receptor agonists and/or blocked by adenosine receptor antagonists).32–36 and our preliminary experiments (data not shown) confirmed acute chronotropic and vasodilatory responses to 10 μmol/L 2-chloroadenosine were sensitive to 100 μmol/L of the competitive receptor antagonist 8-sulfophenyltheophylline.

Statistical Analyses
All data are presented as mean±SEM. Baseline data, pEC50 values, final recoveries, and LDH efflux were analyzed via one-way ANOVA. Time course data were compared via two-way ANOVA for repeated measures. When significant differences were detected in ANOVA tests, a Newman-Keuls post hoc test was used for specific comparisons. A value of P<0.05 was considered significant in all tests.

Results
There were no differences in baseline contractile function or coronary flow between groups (Table). However, intrinsic heart rate was reduced in A1AR-overexpressing hearts. Concentration-response analysis confirmed A1AR KO abrogates A1AR-mediated bradycardia without altering sensitivity of A2AAR-mediated vasodilation (Figure 1). Conversely, A1AR overexpression increased the sensitivity of A1AR-mediated bradycardia without altering coronary responses. The pEC50 values for the different responses are provided in Table.

Deletion of A1ARs significantly reduced ischemic tolerance. Recovery profiles for hearts subjected to 25 minutes ischemia and 45 minutes reperfusion are depicted in Figure 2. Effects of A1AR KO were evident in terms of reduced systolic pressure with little change in diastolic dysfunction (Figure 2). Thus, left ventricular pressure development was depressed (Figures 2 and 3). Deletion of the A1AR did not modify the rate of contracture development during ischemia (time to reach 20 mm Hg diastolic pressure)23 (Figure 3B), but significantly worsened cellular damage indicated by postischemic efflux of LDH (Figure 3C). Conversely, A1AR overexpression enhanced ischemic tolerance (Figures 2 and 3), with the primary contractile effect being reduced diastolic dysfunction (Figures 2A and 3A). Overexpression of A1ARs only improved systolic function during the initial minutes of reperfusion (Figure 2B). Ischemic contracture development was also reduced by A1AR overexpression (Figure 3B), in contrast to lack of effect of A1AR KO on this parameter.

Treatment of wild-type and A1AR KO hearts with the nonselective agonist 2-chloroadenosine improved postischemic outcomes in both groups (Figure 3). However, the
were abrogated by A1AR KO (Figure 3B).

Values are mean±SEM. Function was measured after 30 minutes stabilization before ischemia, except for heart rate (measured after 20 minutes stabilization, before pacing). 

The role of endogenous adenosine and adenosine receptors in determining intrinsic tolerance to ischemic insult remains controversial. Many studies do not observe effects of adenosine receptor antagonists on ischemic outcome in various species.12–15 Moreover, there is even evidence A1AR blockade actually improves outcome from ischemia.36,37 In contrast, there is some support for a role for endogenously generated adenosine in protection of ischemic myocardium4–6,8–11 and modulation of processes impinging on recovery from ischemia.38,39 Several explanations may account for varied findings with antagonists, the most likely involving mixed selectivity and potency of agents used and the fact that an antagonist applied to any system in which the signal is coupled to the response (as with adenosine) will likely generate an elevation in the signal (ie, opening the feedback loop). This has been verified in prior work.16–18 On the other hand, it is also important to note that generally observed cardioprotection with adenosine agonists1–3,8,10,26,40 indicates the intrinsic adenosine response must normally be submaximally (if at all) engaged. In assessing potential roles of adenosine receptors, an alternate and relatively selective approach involves gene deletion of receptor protein. In this study, we provide the first evidence that genetic deletion of the A1AR significantly limits the ability of mouse myocardium to withstand injury during ischemia/reperfusion (Figures 2 and 3). Conversely, cardiac A1AR overexpression confers enhanced tolerance, as documented previously.22 These data collectively provide strong support for a role of A1ARs in determining intrinsic tolerance to ischemia/reperfusion.

Effects of A1AR KO are evident in terms of reduced systolic dysfunction and oncotic injury, with little effect on diastolic dysfunction (Figures 2 and 3). This contrasts effects of A1AR overexpression, which are manifest as reduced diastolic contracture with little change in systolic pressure (except during initial reperfusion). Thus, effects of receptor deletion do not mirror effects of receptor overexpression. Rather, data suggest responses mediated by a highly overexpressed receptor may be abnormal or “supraphysiological” and/or that functional effects of A1ARs vary with the level of activation during insult. This is consistent with effects of 2-chloroadenosine, which did reduce postischemic diastolic dysfunction, an action ablated by A1AR KO (Figure 3). These data indicate the A1AR is responsible for “adenosinergic” reductions in diastolic dysfunction, but that the response is evident only with enhanced levels of agonism. Selective
actions of A1ARs on systolic versus diastolic function are consistent with prior observations regarding A1AR antagonism, revealing that postischemic A1AR activation improves systolic force without altering diastolic dysfunction, whereas intraischemic A1AR activation limits diastolic dysfunction in addition to improving systolic force. Extent (and timing) of A1AR engagement likely dictates relative effects on diastolic versus systolic dysfunction.

Development of ischemic contracture was also unaffected by A1AR KO, but was limited by A1AR overexpression and 2-chloroadenosine. The latter response was again abrogated by A1AR KO (Figure 3). Thus, adenosinergic limitation of ischemic contracture is A1AR dependent but evident only with exaggerated receptor agonism or expression. This agrees with early work of Lasley et al demonstrating A1AR agonist-mediated protection against ischemic contracture in rat, and prior data demonstrating negligible effects of A1AR blockade on contracture development in mice.

Although 2-chloroadenosine–mediated protection against contracture and diastolic dysfunction is abrogated by A1AR KO (Figure 3), supporting A1AR-dependent effects of the agonist on diastolic function, the analogue still exerted some beneficial actions in A1AR KO hearts. This is reflected in improved systolic function together with reduced LDH efflux (Figure 3). These effects, refractory to A1AR KO, implicate a protective function for receptor subtypes distinct from A1ARs. Prior evidence that exogenous A1AR but not A2AR agonism is cardioprotective in the model studied here, and that A1AR protection selectively enhances systolic function and reduces cell death, argues for a potential role for this subtype in the remaining protection with 2-chloroadenosine. However, this remains to be directly assessed, and we cannot exclude a potential role for the less well-studied A2BAR.

Two study limitations bear mention before closing. As with all gene deletion studies, adaptations may occur to compensate for life-long absence of a targeted protein. Although it may be fruitful to focus on such adaptations (see for example, Godecke et al and Warth and Barhanin), they also complicate interpretation of phenotypic outcomes. We have assessed, in part, obvious changes in other adenosine receptors, verifying that A1AR deletion selectively abrogates an A1AR response (bradycardia) without modifying A2AR sensitivity. However, we recognize the possibility of undetected compensatory changes contributing to the A1AR KO phenotype. The second limitation relates to the fact that we focus on responses in the isolated buffer-perfused heart. This was deliberate, to assess more directly the myocardial phenotype, because A1AR protection is primarily “direct” and mediated via cardiomyocyte receptors. However, adenosinergic protection in vivo additionally involves modulation of blood cells and related inflammatory responses. We therefore cannot ascertain potential effects of A1AR deletion on these extracardiac responses. However, these responses are predominantly A1AR-dependent and thus not predicted to be substantially modified by A1AR KO. In addition, intrinsic A1AR-dependent protection during ischemia/reperfusion in vivo may involve actions of adenosine generated within neutrophils, platelets, and other blood-borne cells. Thus, this extracardiac component will be absent in the present model, potentially leading to an underestimation of the normal extent of A1AR activation by endogenously generated adenosine.

In summary, the current analysis of the effects of A1AR KO supports an important function of A1ARs in dictating intrinsic myocardial resistance to ischemia/reperfusion. Effects of A1AR deletion suggest these receptors normally play a role in enhancing posts ischemic contractility and limiting cell death, with little effect on abnormalities in diastolic...
function. Finally, reduced yet significant protection via the nonselective agonist 2-chloroadenosine in A1AR KO hearts implicates a significant cardioprotective response mediated via adenosine receptors additional to the A1AR.

Acknowledgments

This work was supported by NIH AI-43572 (to M.R.B.) and National Health and Medical Research Council of Australia grant 231416 (to J.P.H.). J.P.H. was also the recipient of a career development fellowship from the National Heart Foundation of Australia. We are extremely grateful for the provision of mice overexpressing A1ARs by Prof Paul Matherne and for the excellent technical assistance of Kirsten Holmgren.

References

Genetic Deletion of the A₁ Adenosine Receptor Limits Myocardial Ischemic Tolerance
Melissa E. Reichelt, Laura Willems, Jose G. Molina, Chun-Xiao Sun, Janci C. Noble, Kevin J.
Ashton, Jurgen Schnermann, Michael R. Blackburn and John P. Headrick

_Circ Res_. 2005;96:363-367; originally published online January 13, 2005;
doi: 10.1161/01.RES.0000156075.00127.C3
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/96/3/363

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/