Effects of targeted deletion of A1 adenosine receptors on postischemic cardiac function and expression of adenosine receptor subtypes

R. Ray Morrison,1 Bunyen Teng,2 Peter J. Oldenburg,3 Laxmansa C. Katwa,4 Jurgen B. Schnermann,5 and S. Jamal Mustafa2

1Division of Critical Care Medicine, St. Jude Children’s Research Hospital, Memphis, Tennessee; 2Department of Physiology and Pharmacology, West Virginia University, Morgantown, West Virginia; 3Durham Research Center, University of Nebraska Medical Center, Omaha, Nebraska; 4Brody School of Medicine at East Carolina University, Greenville, North Carolina; and 5National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Submitted 16 February 2005; accepted in final form 2 May 2006

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: R. R. Morrison, Division of Critical Care Medicine, St. Jude Children’s Research Hospital, 332 N. Lauderdale St., MS 734, Memphis, TN 38105 (e-mail: ray.morrison@stjude.org).

http://www.ajpheart.org

First published May 5, 2006; doi:10.1152/ajpheart.00158.2005.
to ischemia-reperfusion and to evaluate baseline and postischemic expression of adenosine receptor subtypes in isolated wild-type (WT) and A1KO mouse hearts. We hypothesized that tolerance to ischemia-reperfusion would be impaired in A1KO hearts and that the expression of other adenosine receptor subtypes would demonstrate compensatory changes either at baseline or by induction through ischemia-reperfusion.

**MATERIALS AND METHODS**

**Animals.** All animals were cared for in accordance with a protocol approved by the Animal Care and Use Committee of the Brody School of Medicine at East Carolina University. A1KO mice and their respective WT littermate controls were obtained from a sub colony of the original lines generated by Sun et al. (42) on a background strain mix of 129Sv/J and C57BL/6. Colonies of WT and A1KO mice were bred on site at the Brody School of Medicine, and genotype status was verified by tail-snip PCR. All animals used in a given experiment originated from the same breeding series and were matched for age and weight. Both male and female mice were used, and the representation of each sex was equal within all experimental groups. Standard laboratory food and water were available ad libitum. Housing temperature was held constant at 23 ± 2°C, humidity was 60 ± 10%, and an inverted 12-h:12-h light-dark cycle was used (lights off at 1900). All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (Publication No. 85-25, Revised 1996).

**Langendorff isolated heart model.** Isolated heart experiments were performed as previously described (30–32, 43, 44, 46), using a murine model that has been fully characterized (16, 17). Animals were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), a thoracotomy was performed, and the hearts were excised into ice-cold heparinized (5 U/ml) perfusate. After the removal of lung and surrounding tissue, the aorta was rapidly cannulated with a 20-gauge, heparinized (5 U/ml) perfusate. After the removal of lung and surrounding tissue, the aorta was rapidly cannulated with a 20-gauge, blunt-ended needle, and retrograde coronary perfusion was initiated at a constant pressure of 80 mmHg with modified Krebs-Henseleit buffer containing (in mM) 120 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, 15 glucose, and 0.05 EDTA. The perfusate was equilibrated with 95% O2-5% CO2 at 37°C, giving a pH of 7.4 and PO2 of 550 mmHg. The left atrium was removed, and the left ventricle was venticled with a small polyethylene aipal drain. A fluid-filled balloon constructed of plastic film was inserted into the left ventricle across the mitral valve and connected to a pressure transducer, permitting continuous measurement of left ventricular pressure. Balloon volume was modified through a stopcock attached to the ventricular pressure transducer using a 500-μl glass syringe (Hamilton; Reno, NV) to maintain a left ventricular diastolic pressure of 2–5 mmHg. Tungsten wires were connected to the stainless steel cannula and through the apical myocardium for pacing during reperfusion. Hearts were immersed in a water-jacketed perfusate bath maintained at 37°C, and the bath was rinsed once at the onset of perfusion to clear blood from the bathing environment. Coronary flow was continuously monitored via a Doppler flow probe (Transonic Systems; Ithaca, NY) located in the aortic perfusion line. Coronary flow, aortic pressure, and left ventricular pressure were all recorded on a PowerLab multi-channel data acquisition system (ADInstruments; Castle Hill, Australia) connected to a Macintosh G4 computer. The ventricular pressure signal was digitally processed (using PowerLab Chart software version 5.0.1, ADInstruments) to yield diastolic and systolic pressures, heart rate, and positive and negative first derivative of pressure (+dP/dt and −dP/dt, respectively).

**Ischemia-reperfusion protocol.** All hearts were equilibrated for 30 min before ischemia-reperfusion. Coronary flow, heart rate, developed pressure (systolic pressure – diastolic pressure), +dP/dt, and −dP/dt were continuously measured in WT and A1KO hearts, and baseline data for these parameters were sampled at the end of equilibration. After equilibration, hearts underwent 20 min of global normothermic ischemia, followed by 40 min of reperfusion. Global ischemia was produced by clamping the aortic cannula inflow and simultaneously bubbling 95% N2-5% CO2 through the organ bath to reduce ambient P02. Reperfusion was achieved by unlclamping the aortic inflow and discontinuing the nitrogen bubbling. Hearts were paced at 7 Hz throughout reperfusion beginning 90 s after unclamping the aortic inflow (16, 30) with the use of a Grass S6 stimulator (Grass Instruments; Quincy, MA) with 2-ms square-wave pulses at 20% above threshold (usually 3–6 V). Functional performance was continuously recorded throughout the ischemia-reperfusion protocol, and data for coronary flow, heart rate, diastolic pressure, developed pressure, and +dP/dt were sampled at 1, 3, and 5 min and at 5-min intervals thereafter during both ischemia and reperfusion. At the end of reperfusion, hearts were weighed and flash frozen at −80°C for determination of adenosine receptor mRNA expression by real-time RT-PCR.

**Coronary efflux of lactate dehydrogenase.** The content of lactate dehydrogenase (LDH) within the coronary venous effluent was examined as a biochemical marker of cardiomyocyte injury. For each heart, a baseline sample of coronary effluent was collected from the superfusate bath overflow during the last minute of equilibration (before the onset of global ischemia). Coronary effluent was then continuously collected throughout the entire reperfusion period. LDH determination was performed using an automated spectrophotometric clinical assay using an ACE Chemistry Analyzer (Alfa Wassermann; West Caldwell, NJ). The total volume of coronary efflux during ischemia-reperfusion was measured at the end of reperfusion, and a 1.5-ml aliquot was stored on ice for assay within 24 h of the experiment. Total efflux of LDH was normalized to wet weight of the heart (in U/g).

**Adenosine receptor expression.** To determine whether targeted deletion of A1 ARs induces compensatory changes in expression of other adenosine receptor subtypes, real-time RT-PCR was performed on uninstrumented hearts (not cannulated or perfused with the Langendorff apparatus) from WT and A1KO mice. Hearts in this subset were extracted, and extraneous tissue was removed, rinsed of blood (using modified Krebs buffer), and flash frozen at −80°C pending analysis of adenosine receptor expression. To determine whether the relative expression of each receptor subtype is altered during ischemia-reperfusion, real-time RT-PCR was performed on subsets of isolated WT and A1KO hearts. Isolated heart experiments were performed as described above, but the ischemia-reperfusion protocol was interrupted at three time points: 1) at the end of equilibration (before ischemia), 2) at the end of ischemia (before reperfusion), and 3) at the end of reperfusion. WT and A1KO hearts from each of these time points were removed from the Langendorff apparatus and flash frozen at −80°C pending analysis of adenosine receptor expression. Whole hearts were homogenized in 3 ml TRIzol reagent (GIBCO, Gaithersburg, MD), and total RNA was isolated according to the manufacturer’s instructions and treated with DNase to remove contamination of DNA. Real-time quantitative RT-PCR was performed in duplicate on a Bio-Rad iCycler Real-Time Detection System (Bio-Rad, Hercules, CA). PCR reactions used the Superscript III one-step RT-PCR system with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The adenosine receptor primers, probe sequences, and reaction protocols used were similar to previously reported methods (2). Primer pairs for all adenosine receptor subtypes were purchased from Invitrogen, and specific probes for each of the receptor subtypes were obtained from Applied Biosystem (Foster City, CA). The reaction mixture contained (in μl) 12.5 2× reaction mix, 1 forward primer, 1 reverse primer, 1 probe (FAM-MGB labeled), 1 SuperScript III, 1 total RNA (500 ng), and 6.4 MgCl2 (50 mM), and water was added to total volume of 25 μl. Fluorescent dye SYBR Green I (Bio-Rad) was used for β-actin quantitation as an internal control (no differences in postischemic expression were observed using either β-actin or 18S RNA as internal controls; data not shown). β-Actin oligonucleotide primers were also purchased from Invitrogen and had
the following configurations: forward 5'-ACC-AAC-TGG-GAC-GAT-ATG-GAG-AA-G-A-3'; reverse 5'-TAC-GAC-CAG-AGG-CAT-ACA-GGG-ACA-A-3' (38). For β-actin quantitation, the reaction mixture used was the same as described above, except 1 μl SYBR Green I was used instead of probes and 10 ng of total RNA were used instead of 500 ng. The RT-PCR reaction consisted of an initial step of 30 min at 50°C (cDNA synthesis) and then 95°C for 15 min (denaturation and inactivation of reverse transcriptase enzyme). This was followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. Gene expression for each adenosine receptor subtype was normalized to β-actin as the internal control (adenosine receptor expression-to-β-actin expression within sample ratio × 100).

Statistical analysis. Unpaired Student’s t-tests were used to compare baseline functional data and coronary efflux of LDH between WT and A1KO groups. Functional parameters during ischemia-reperfusion were analyzed by two-way repeated-measures ANOVA, applying Student-Newman-Keuls post hoc test for multiple pairwise comparisons. For isolated hearts, differences in adenosine receptor expression between WT and A1KO groups were analyzed by using two-way ANOVA with Student-Newman-Keuls post hoc test for multiple comparisons. Exact Wilcoxon tests were used to detect differences of adenosine receptor expression between uninstrumented hearts and isolated hearts at the end of equilibration. For all tests, a P value of <0.05 was considered statistically significant.

RESULTS

Baseline function in isolated WT and A1KO hearts. Table 1 summarizes functional parameters after 30 min of equilibration in isolated murine hearts perfused at constant pressure under normothermic aerobic conditions. The average body weight, heart weight, heart-to-body weight ratio, and spontaneous heart rate were similar in WT and A1KO hearts. Baseline functional parameters for both WT and A1KO hearts were within the range of previously reported values from isovolumically contracting isolated murine hearts (16, 31, 35); however, coronary flow, developed pressure, +dP/dt, and −dP/dt were all greater in A1KO than in WT hearts (Table 1).

Functional effects of ischemia-reperfusion. Myocardial function (expressed as the percent change from baseline developed pressure) during ischemia-reperfusion is shown in Fig. 1A. Upon interruption of coronary flow at time 0 (Fig. 1B), all hearts demonstrated a rapid loss of developed pressure and complete asystole within 2–3 min (Fig. 1A). Hearts remained nonfunctional through the 20-min ischemic event, and diastolic pressure rose steadily between 3 and 15 min, indicating the development of ischemic contracture (Fig. 1C). The onset of ischemic contracture, defined as the time to 20% maximum contracture (16), occurred earlier in A1KO hearts than in WT hearts (282 ± 13.1 and 322 ± 10.4 s, respectively; n = 13 both groups, P < 0.05), as illustrated by a significantly higher diastolic pressure at 5 min in A1KO hearts (Fig. 1C).

With the onset of reperfusion (at time 20), all hearts recovered coronary flow (Fig. 1B) and developed pressure (Fig. 1A) and demonstrated a rapid decrease in diastolic pressure (Fig. 1C), although these responses differed between WT and A1KO hearts. Although WT hearts demonstrated an immediate postis-

Table 1. Baseline functional data in wild-type and A1 adenosine receptor knockout mouse hearts

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>A1 Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Age, wk</td>
<td>15.5 ± 0.4</td>
<td>16.1 ± 0.5</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>28.9 ± 0.9</td>
<td>28.6 ± 0.7</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>136 ± 5.0</td>
<td>133 ± 4.0</td>
</tr>
<tr>
<td>Heart-to-body weight ratio, %</td>
<td>0.47 ± 0.01</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>380 ± 7.0</td>
<td>396 ± 11</td>
</tr>
<tr>
<td>Coronary flow, ml/min·g⁻¹</td>
<td>17.1 ± 0.6</td>
<td>21.4 ± 1.6*</td>
</tr>
<tr>
<td>Developed pressure, mmHg</td>
<td>122 ± 2.8</td>
<td>142 ± 3.7*</td>
</tr>
<tr>
<td>Positive dP/dt, mmHg/s</td>
<td>4,643 ± 123</td>
<td>5,323 ± 173*</td>
</tr>
<tr>
<td>Negative dP/dt, mmHg/s</td>
<td>3,238 ± 107</td>
<td>3,635 ± 108*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of hearts. Functional parameters were measured after 30 minutes of normothermic aerobic perfusion using a standard Langendorff preparation. dP/dt, first derivative of pressure. *P < 0.05 wild-type vs. A1 knockout hearts.
chemic coronary vasodilation to $156 \pm 8.4\%$ of preischemic values, this response was absent in A1KO hearts ($104.4 \pm 8.2\%; n = 8$ both groups, $P < 0.05$), and coronary flow remained approximately one-third lower in A1KO hearts for the duration of reperfusion (Fig. 1B). Likewise, immediate recovery of developed pressure in A1KO hearts was lower than that of WT hearts ($58.5 \pm 8.9$ and $95.7 \pm 8.9\%$ of baseline, respectively, at 1 min; $n = 8$ both groups, $P < 0.05$), and this poorer functional recovery was maintained from 5 min to the end of reperfusion (Fig. 1A). Diastolic pressure dropped equally upon reperfusion in A1KO and WT hearts ($23.8 \pm 4.3$ and $16.3 \pm 2.1$ mmHg, respectively, at 1 min; $n = 8$ both groups; not significant (NS)); however, A1KO hearts sustained significantly higher diastolic pressure for the remainder of reperfusion (Fig. 1C).

Coronary efflux of LDH. The concentration of LDH in the coronary effluent before and after ischemia is represented in Fig. 2. Preischemic release of LDH was minimal for both WT and A1KO groups ($3.8 \pm 0.09$ and $4.0 \pm 0.7$ U/g, respectively; $n = 8$ both groups; NS) and was comparable with previous reports (16). Ischemia-reperfusion resulted in increased coronary efflux of LDH in both groups (Fig. 2); however, the concentration was significantly higher in A1KO than in WT hearts ($45.5 \pm 6.9$ and $25.0 \pm 1.2$ U/g, respectively; $n = 8$ both groups; $P < 0.05$). As a marker of cellular injury, the greater postischemic efflux of LDH in A1KO hearts correlated with impaired functional recovery compared with WT hearts (Figs. 1A and 2).

Adenosine receptor expression. Relative quantitation of adenosine receptor mRNA by real-time PCR demonstrated the absence of A1 adenosine receptor expression in both uninstrumented (Fig. 3) and isolated A1KO hearts (Fig. 4A). In uninstrumented WT hearts, mRNA expression was highest for A1 and and A2A adenosine receptors with approximately two- to threefold lower message for A2B and A3 receptors. Targeted deletion of the A1 receptor resulted in no compensatory changes in A2A, A2B, or A3 adenosine receptors in A1KO compared with WT hearts (Fig. 3). Although A1AR mRNA in isolated WT hearts was reduced at the end of equilibration compared with uninstrumented hearts ($2.35 \pm 0.43$ vs. $4.15 \pm 0.75\%$ β-actin, respectively; $P < 0.05$; Figs. 3 and 4A), ischemia-reperfusion did not affect A1AR expression (Fig. 4A).

In isolated WT hearts, A2A receptor mRNA was reduced at the end of equilibration compared with uninstrumented hearts ($2.20 \pm 0.21$ vs. $6.42 \pm 1.40\%$ β-actin, respectively; $P < 0.05$), but no decrease in A2A expression was observed between isolated and uninstrumented A1KO hearts ($4.09 \pm 0.66$ vs. $7.03 \pm 1.48\%$ β-actin, respectively; NS; Figs. 3 and 4B). A2A adenosine receptor mRNA increased immediately after ischemia in WT hearts compared with the end of equilibration within this group; however, A2A transcript level was unchanged by ischemia-reperfusion in the A1KO hearts (Fig. 4B). This upregulation of A2A receptor expression in WT hearts at the end of ischemia returned to preischemic levels after reperfusion (Fig. 4B).

As observed for the expression of A1 and A2A receptors, A2B mRNA in isolated WT hearts appeared lower at the end of equilibration compared with uninstrumented hearts but narrowly failed to meet statistical significance ($1.24 \pm 0.19$ vs. $2.42 \pm 0.50\%$ β-actin, respectively; $P = 0.055$). A2B expression was lower in isolated versus uninstrumented A1KO hearts ($1.69 \pm 0.34$ vs. $2.78 \pm 0.43\%$ β-actin, respectively; $P < 0.05$; Figs. 3 and 4C). When compared with the end of equilibration, A2B adenosine receptor expression increased 2.5-fold after ischemia-reperfusion in both WT and A1KO hearts (Fig. 4C). Moreover, this increase in A2B receptor mRNA was evident only after reperfusion and not at the end of ischemia (Fig. 4C). A3 adenosine receptor transcript levels were not different between WT and A1KO hearts at each time point examined and were unchanged by ischemia-reperfusion (Fig. 4D).

DISCUSSION

The dual purpose of this study was to examine the tolerance to ischemia-reperfusion in isolated A1KO mouse hearts and to determine whether targeted deletion of A1 adenosine receptors alters the expression of other adenosine receptor subtypes either under basal conditions or by induction through ischemia-reperfusion. Baseline contractile function and coronary flow were enhanced in A1KO hearts (compared with WT hearts) during normoxic constant pressure perfusion. A1KO hearts demonstrated impaired tolerance to ischemia-reperfusion as evidenced by poorer postischemic recovery of contractile function, decreased coronary vasodilation in...
reperfusion, and increased tissue injury compared with WT hearts. In uninstrumented WT hearts, receptor expression was highest for A1 and A2A adenosine receptors with two- to threefold lower levels of A2B and A3 mRNA, and targeted deletion of A1 adenosine receptors had no effect on cardiac expression of other adenosine receptor subtypes in A1KO hearts. In isolated WT hearts, A2A adenosine receptor mRNA transiently increased immediately after ischemia, returning to the preischemic level by the end of reperfusion. After ischemia-reperfusion, there was a 2.5-fold increase in A2B adenosine receptor transcript in both WT and A1KO hearts. Taken together, these data affirm the cardioprotective role of A1 adenosine receptors and suggest that induced expression of other adenosine receptor subtypes may participate in the response to ischemia-reperfusion in isolated murine hearts.

Adding to the long-standing recognition that A1AR activation plays a predominant role in protecting the heart from ischemia-reperfusion (7, 13, 24, 35, 37, 48), an expanding body of evidence points to coregulatory involvement of other adenosine receptor subtypes in modifying response(s) to myocardial injury (5, 17, 18, 20, 21, 25, 33, 36, 47). Much of this evidence derives from an increasing array of selective and nonselective adenosine analogs aimed at discerning the cardioprotective contributions of A2A (5, 20, 21, 25) and A3 (18, 36, 47) adenosine receptors across a variety of in vivo, isolated heart and cultured myocyte models from a number of different species (5, 18, 20, 21, 25, 36, 47). Development of selective and potent ligands for the A2B adenosine receptor lags behind that of the other subtypes (8), leaving a paucity of data as to whether A2B receptors modify the cardiac response to ischemia-reperfusion. Thus, although the traditional pharmacological approach yields important data, it is a method with limitations for which gene-modified models of targeted adenosine receptor overexpression (6, 29) and deletion (26, 39, 42) are proving invaluable adjuncts in characterizing the cardiovascular effects of each adenosine receptor subtype. We have successfully applied this combined strategy to examine receptor-specific coronary and aortic vascular responses to adenosine and its pharmacological analogs in A1 (45, 46), A2A (31, 44), and A3 (43) adenosine receptor knockout models and have begun to integrate it in assessing the consequences of ischemia-reperfusion in isolated mouse hearts (32).

Effects of A1AR deletion on baseline cardiac function. The current observation that baseline heart rate was indistinguishable between A1KO and WT hearts is consistent with the initial in vivo characterization of this model (42), as well as recent reports in isolated A1KO hearts (38, 46). It is interesting to note, however, that the similar basal heart rate in A1KO and WT hearts contrasts with the observation of lower heart rates both in vivo (11) and in isolated hearts (11, 29, 30) from transgenic animals with cardiac-specific A1 adenosine receptor overexpression (compared to heart rates of their WT littermates). The absence of a functional A1-mediated negative chronotropic response in A1KO hearts is evidenced by the inability to elicit bradycardia with the selective A1 agonist 2-chloro-N6-cyclopentyladenosine in isolated hearts (10, unpublished observations from our laboratory) or with N6-cyclohexyladenosine in vivo (42). The current findings in A1KO hearts, along with previous observations in transgenic hearts overexpressing A1ARs, indicate that although endogenous activation of A1ARs occurs under basal (normoxic) conditions, the effect on heart rate is negligible in WT hearts but
sufficient to modulate intrinsic rate in the setting of A1AR excess.

Targeted deletion of A1ARs resulted in higher baseline coronary flow, developed pressure, +dP/dt, and −dP/dt compared with WT hearts. These findings are consistent with recent data from Mustafa’s laboratory (46), demonstrating that acute selective A1 antagonism with 1,3-dipropyl-8-cyclopentylxanthine enhances coronary dilation by adenosine and 5′-N-ethyl-carboxamidoadenosine in WT but not in A1KO hearts; that is, deletion of A1ARs eliminates an A1-mediated negative modulatory (vasoconstrictive) effect on coronary regulation by other adenosine receptor subtypes (A2A and A2B). The current data affirm our prior observation that baseline contractility is also enhanced in A1KO compared with WT hearts (46). Our findings in unpaced hearts, however, contrast with those of Reichelt et al. (38) and Lankford et al. (23), who noted similar coronary flow and contractility in isolated A1KO and WT hearts paced at either 420 or 480 beats/min. The causal relationship between higher coronary flow and greater contractile performance in A1KO hearts in the present study is not clear, yet these preischemic differences from WT hearts may have contributed to the increased susceptibility to ischemic injury in A1KO hearts.

Effects of A1AR deletion on ischemic tolerance. Tolerance to ischemia-reperfusion was impaired by targeted deletion of A1ARs, both from the standpoint of functional recovery and degree of tissue oncosis as determined by the concentration of LDH present in coronary effluent. These findings support the established cardioprotective role of A1ARs in ischemia-reperfusion (7, 17, 33), whether it occurs by endogenous activation (35, 48) or exogenously through acute application of high-dose adenosine agonists (7, 24, 33). The decreased tolerance to ischemia-reperfusion in A1KO hearts contrasts with enhanced ischemic tolerance in hearts overexpressing A1ARs (11, 22, 29, 30, 36). In line with recent data (23, 38), the nature of impaired postischemic function in A1KO hearts was multifaceted with lower initial and sustained developed pressure, lower initial and sustained coronary flow, and worsened diastolic dysfunction through all but the initial phase of reperfusion. The effective absence of reactive coronary vasodilation in A1KO hearts, followed by lower than preischemic coronary flow, correlated with diastolic failure during reperfusion (Fig. 1, B and C).

Our current demonstration of impaired ischemic tolerance with A1AR deletion contradicts data by Schulte et al. (41) in isolated hearts from an alternative (concomitantly developed) murine A1KO model (19). They report abrogation of the infarct sparing effect of remote (cerebral) ischemic preconditioning in A1KO hearts, yet their data indicate postischemic functional tolerance was not altered by A1AR deletion (41). The former finding is of interest in that it supports a role for A1ARs in mediating remote preconditioning; however, their latter finding conflicts not only with our current observations and those of Reichelt et al. (38) and Lankford et al. (23) but also with pharmacological data demonstrating that ischemic tolerance is limited by selective A1AR antagonism (24, 35, 48). Indeed, we have previously demonstrated that acute pretreatment with DPCPX reduces postischemic contractile performance by 35% in isolated WT mouse hearts (30). Although reasons for this discrepancy are not readily apparent, it is worth noting that the two A1KO lines differ with regard to background strain and structure of the mutated gene (19, 42). Perhaps more importantly, despite undergoing 40 min of global ischemia (double the ischemic duration reported here), both WT and A1KO hearts in the study of Schulte et al. (41) demonstrated near-complete recovery of developed pressure to 75–90% of preischemic baseline. This unconventionally high functional recovery was immediate and sustained through 60 min of reperfusion, a pattern at odds with well-characterized responses in isolated murine hearts (16, 38).

Effects of A1AR deletion on pre- and postischemic expression of adenosine receptor subtypes. Real-time PCR analysis of gene transcript for all four adenosine receptor subtypes demonstrated that targeted deletion of A1ARs did not alter expression of A2A, A2B, or A3 adenosine receptors in uninstrumented hearts. Our data support an apparent downregulation of A1 and A2A receptor mRNA in isolated WT hearts after 30 min of normothermic, normoxic equilibration (compared to uninstrumented hearts), which was not observed in isolated A1KO hearts. Ashton et al. (1) have previously reported downregulation of A1AR transcript in isolated WT hearts after 30 min of ischemia-reperfusion; however, our data raise the possibility that even brief (3–5 min) cold ischemia, which necessarily occurs during dissection and cannulation of the mouse heart, may result in a similar decrease in A1AR mRNA. This unexpected observation warrants further examination to determine whether adenosine receptor protein expression is altered solely by isolating murine hearts. Although mRNA levels may not necessarily reflect actual protein expression (or functional G protein coupling), it is intriguing that at the end of equilibration A2A mRNA was markedly lower in WT hearts, demonstrating lower baseline coronary flow, whereas coronary flow was greater in A1KO hearts expressing almost twice the A2A mRNA. In our review of the literature, this represents the first parallel evaluation of the cardiac phenotype and genotypic adenosine receptor profile resulting from targeted deletion of an adenosine receptor subtype.

Ischemia-reperfusion induced a 2.5-fold induction of A2B receptor transcript in both WT and A1KO hearts. This postischemic induction of A2B receptor expression mirrors findings after hypoxia in human (umbilical vein) endothelial and (bronchial) smooth muscle cells (10) and is particularly interesting given the low affinity of this receptor subtype (8) and its purported role in modulating angiogenesis (9). The induction of A2B expression observed here also compares favorably with data from Ashton et al. (2) in a recent analysis of adenosine receptor transcription after ischemia-reperfusion in isolated murine hearts. The significance of induced A2B receptor expression in WT hearts immediately after ischemia is also not clear, because this increase is not apparent after reperfusion. Nonetheless, the data affirm that ischemia-reperfusion alters the gene expression profile of adenosine receptor subtypes, the most dramatic of which, i.e., A2B expression, is unaffected by targeted A1AR deletion. Given the important modulatory roles of adenosine receptors in inflammation, cardioprotection, and fibroblast growth, postischemic induction of adenosine receptors may have an important impact on the longer term response to ischemic insult. This warrants further investigation.

Study limitations. As with all studies using isolated heart preparations, consideration must be given to the fact that the functional and genotypic evaluation of A1KO hearts may be limited by the absence of humoral and/or cell-mediated factors.
that could modify the response to cardiac ischemia-reperfusion. However, this same limitation may be construed as a potential experimental advantage allowing extraction of the magnitude of these component factors in ischemic injury through a comparison with in vivo models of ischemia-reperfusion using adenosine receptor knockout mice. Importantly, mRNA expression of adenosine receptor subtypes may not actually reflect the relative patterns of protein expression (or G protein coupling), because the efficiency of translation and posttranslational modification and/or trafficking may alter the relationship between mRNA and individual proteins. Also, because the gene expression profile was performed by analysis of whole heart preparations, it is not possible to distinguish differences in adenosine receptor subtypes among cell populations existing therein, including cardiomyocytes, vascular cells, fibroblasts, and any resident inflammatory cells. Finally, a complete analysis of the potential impact of A1AR deletion on other relevant systems (e.g., β-adrenergic) has not been assessed but represents an important component of future work that is needed with this model.

**Conclusions and future directions.** Targeted deletion of A1 ARs results in increased basal coronary flow and contractile performance in isolated murine hearts. A1KO hearts demonstrate impaired tolerance to ischemia-reperfusion as evidenced by worsened postischemic developed and diastolic pressures, decreased coronary vasodilation throughout reperfusion, and increased tissue injury compared with WT hearts. Targeted deletion of A1ARs did not alter expression of A2A, A2B, or A3 adenosine receptors in uninstrumented hearts. The profile of cardiac adenosine receptor expression is changed by ischemia-reperfusion with notable induction of adenosine A2B receptor transcript in both isolated WT and A1KO hearts. Together, these data affirm the cardioprotective role of A1ARs and suggest that expression of other adenosine receptor subtypes participates in the response to ischemia-reperfusion in isolated murine hearts. Ongoing work with this A1KO model is needed to delineate the role of A1ARs in the in vivo response to ischemia-reperfusion. Determining the receptor-specific effects of adenosine within the cardiovascular system remains an essential step in developing adenosinergic therapies for the clinical management of heart disease.

**ACKNOWLEDGMENTS**

The majority of this work was performed at the Brody School of Medicine at East Carolina University. The authors thank WeiXi Qin and Martina Bainbridge for technical support.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grants HL-074001 (to R. R. Morrison), HL-60047 (to L. C. Katwa), and HL-27339 (to S. J. Mustafa).

**REFERENCES**


