Cardioprotection by Ecto-5′-Nucleotidase (CD73) and A2B Adenosine Receptors

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Background—Ecto-5′-nucleotidase (CD73)—dependent adenosine generation has been implicated in tissue protection during acute injury. Once generated, adenosine can activate cell-surface adenosine receptors (A1AR, A2AAR, A2BAR, A3AR). In the present study, we define the contribution of adenosine to cardioprotection by ischemic preconditioning.

Methods and Results—On the basis of observations of CD73 induction by ischemic preconditioning, we found that inhibition or targeted gene deletion of cd73 abolished infarct size-limiting effects. Moreover, 5′-nucleotidase treatment reconstituted cd73−/− mice and attenuated infarct sizes in wild-type mice. Transcriptional profiling of adenosine receptors suggested a contribution of A2BAR because it was selectively induced by ischemic preconditioning. Specifically, in situ ischemic preconditioning conferred cardioprotection in A2AR−/−, A3AR−/−, or AAR−/− mice but not in A2BAR−/− mice or in wild-type mice after inhibition of the A2BAR. Moreover, A2BAR agonist treatment significantly reduced infarct sizes after ischemia.

Conclusions—Taken together, pharmacological and genetic evidence demonstrate the importance of CD73-dependent adenosine generation and signaling through A2BAR for cardioprotection by ischemic preconditioning and suggests 5′-nucleotidase or A2BAR agonists as therapy for myocardial ischemia. (Circulation. 2007;115:1581-1590.)

Key Words: adenosine ■ infarction ■ ischemia ■ reperfusion ■ nucleotidase

Myocardial ischemia represents a major health problem in Western countries. Current therapeutic interventions focus mainly on early and persistent coronary reperfusion, and additional pharmacological strategies to increase resistance to myocardial ischemia are currently areas of intense investigation. A powerful strategy for cardioprotection would be to recapitulate the consequences of ischemic preconditioning (IP), in which short and repeated episodes of ischemia and reperfusion before myocardial infarction result in attenuation of infarct size. Despite multiple attempts to identify the underlying molecular mechanisms, pharmacological strategies using such pathways have yet to be further defined and introduced into clinical practice.

Clinical Perspective p 1590

Recent studies have implicated extracellular adenosine in the modulation of acute inflammation and tissue protection, particularly during conditions of hypoxia.1-5 Extracellular adenosine is derived mainly via phosphohydrolysis of AMP. Ecto-5′-nucleotidase (CD73), a ubiquitously expressed glycosyl phosphatidylinositol-anchored ectoenzyme, is the pace-maker of this reaction.6 Because of its transcriptional induction by hypoxia,6,7 CD73-dependent adenosine generation is particularly prominent during conditions of limited oxygen availability, as may occur during myocardial ischemia.2 Nevertheless, pharmacological studies on the role of CD73-dependent adenosine generation in cardioprotection during ischemia and reperfusion have yielded conflicting results.8,9

Extracellular adenosine produced by CD73 can signal through any of 4 extracellular adenosine receptors (A1AR, A2AAR, A2BAR, or A3AR). All 4 ARs have been associated with tissue protection in a variety of physiological settings.1,10,11 Although all 4 ARs are expressed in cardiac tissues,12 the contribution of individual receptors to cardioprotection from ischemia and reperfusion remains controversial13,14 and may in part be related to a lack of studies in which all 4 AR gene-targeted mice are subjected to the same IP protocol in parallel.
To elucidate the contribution of CD73-dependent adenosine generation and to clarify the role of individual ARs in cardioprotection during IP, we used a recently described model of murine in situ preconditioning incorporating a hanging weight system for intermittent coronary artery occlusion, thus minimizing the variability associated with knot-based coronary occlusion systems. In the present study, we applied this model in mice gene targeted for cd73 or each individual AR. In addition, we used specific pharmacological adenosine therapeutics to confirm the findings from gene-targeted mice. We found a critical role for CD73-dependent adenosine production and, surprisingly, signaling through the A2B AR for cardioprotection by IP. Consistent with these findings, we observed a significant reduction in infarct size after acute ischemia by treatment with soluble 5′-nucleotidase or a specific A2B AR agonist.

**Methods**

**Mice**

All animal protocols were in accordance with the German guidelines for use of living animals and were approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice deficient in cd73, A1AR, or A2AR on the C57BL/6 strain or in A2B AR on the CD1 strain have been described previously. 

**Murine Model for Cardiac IP**

In pharmacological studies, age-, gender- and weight-matched C57BL/6J mice were used. Cardiac IP was performed using a hanging weight system as described previously.

**Heart Enzyme Measurement**

Blood was collected by central venous puncture for troponin I (cTnI) measurements using a quantitative rapid cTnI assay (Life Diagnostics, Inc, West Chester, Pa).

**Transcriptional Analysis**

To assess the influence of IP on cd73, A1AR, A2AAR, A2BAB, and A2B AR transcript levels, 4 cycles of IP (5 minutes of ischemia, 5 minutes of reperfusion) were performed, the area at risk (AAR) was delineated by Evan’s blue staining, and excised and transcript levels were determined (see the expanded Materials and Methods section in the online Data Supplement).

**Immunoblotting Experiments**

In subsets of experiments, we determined CD73 and adenosine A2B AR protein content from the AAR as described previously.

**Ecto-5′-Nucleotidase Enzyme Assays**

Ecto-5′-nucleotidase enzyme activity was evaluated as described previously.

**Adenosine Measurements**

Tissue adenosine and AMP levels were determined via high-performance liquid chromatography as described previously.

**Neutrophil Depletion**

In selected experiments, neutrophil depletion was achieved with an anti-Gr-1 monoclonal antibody (BD Biosciences/Pharmingen, San Jose, Calif) as described previously.

**Data Analysis**

Data were compared by 2-factor ANOVA or Student t test where appropriate. Values are expressed as mean±SD from 4 to 6 animals per condition. For analysis of changes in transcript, data are expressed as mean±SEM, and the Dunnett test was used (P<0.05 was considered statistically significant).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Cardiac CD73 Is Induced by IP**

Because previous studies demonstrated tissue protection by extracellular adenosine generated via hypoxia-inducible CD73, we hypothesized that CD73-dependent adenosine generation also may be critical for cardioprotection during IP. Therefore, we investigated the transcriptional consequences of cardiac IP on CD73 expression and function using a previously described model of cardiac IP (Figure 1A).

Briefly, we performed 4 cycles of intermittent left coronary artery occlusion and reperfusion (5 minutes of ischemia, 5 minutes of reperfusion) in an open-chest in situ model of IP using a hanging weight system, which causes virtually no surgical tissue trauma during IP. The so-called AAR (myocardial tissue supplied by the intermittently occluded coronary artery) was identified using retrograde injection of Evan’s blue and was compared with myocardial tissue from unpreconditioned matched litters. To define the transcriptional effects of IP, preconditioned myocardial tissue was harvested at indicated time points after IP treatment and used for real-time reverse-transcriptase polymerase chain reaction. We found a robust induction of cd73 mRNA (eg, 90 minutes after cardiac IP; 14.5±2.7-fold; P<0.01) (Figure 1B). Similarly, Western blots of the AAR confirmed CD73 protein induction after IP (Figure 1C). Immunohistological staining of the AAR and imaging via confocal laser scanning microscopy confirmed the strong induction of CD73 protein (Figure 1D). Conventional microscopy confirmed CD73 induction on both cardiomyocytes and endothelia within the AAR 90 minutes after IP (Figure 1E), whereas isotype controls were negative (Data Supplement Figure 1). We also demonstrated functional induction of CD73 by IP by measuring ecto-5′-nucleotidase enzyme activity (Figure 1F). Taken together, these data provide strong evidence that CD73 is induced within the AAR by cardiac IP.

**CD73 Inhibition Attenuates Cardioprotection by IP**

We next pursued the functional contribution of CD73 to cardioprotection by IP by treating mice with an intra-arterial infusion of the specific CD73-inhibitor adenosine 5′-(α,β-methylene) diphosphate (APCP; 40 mg · kg⁻¹ · h⁻¹) or vehicle control before cardiac IP and/or ischemia. As shown in Figure 2A, this resulted in a 3-fold reduction in cardiac CD73 enzyme activity. Similarly, AMP-induced bradycardia was significantly attenuated in APCP-treated animals (heart rate reduction from 480 to 360 bpm versus from 480 to 120 bpm in control mice; P<0.0001; Figure 2B). After having shown effective inhibition of cardiac CD73 enzyme activity with APCP, we investigated the role of CD73 in cardioprotection...
by IP by subjecting mice to 60 minutes of left coronary artery occlusion, followed by 2 hours of reperfusion with or without prior IP (4 cycles; 5 minutes of ischemia, 5 of minutes reperfusion) and with or without APCP. All mice survived this experiment. Heart rate and blood pressure did not differ between APCP-treated and untreated mice. To assess myocardial tissue damage, we measured plasma levels of a previously described marker for murine myocardial ischemia, cTnI.\textsuperscript{21} Consistent with previous studies,\textsuperscript{15} plasma cTnI concentrations were attenuated by IP (Figure 2C). However,
APCP treatment abolished the cardioprotective effects of IP. Similarly, measurement of infarct size via 2,3,5-triphenyltetrazolium chloride (TTC) staining confirmed inhibition of cardioprotection by IP after APCP treatment (Figure 2D and 2E). Taken together, these data provide pharmacological evidence for a critical role of CD73 in cardioprotection by IP.

Cardioprotection by IP Is Abolished in cd73−/− Mice

To further demonstrate the importance of CD73 in the cardioprotective effects of IP, we next studied mice with targeted deletion of the cd73 gene.6 We first confirmed the absence of CD73 enzyme activity in the cardiac tissue of these mice (Figure 3A). Similarly, AMP-induced bradycardia was significantly attenuated in these mice (Figure 3A). Similarly, AMP-induced bradycardia was blocked by APCP. Mice were anesthetized and infused with APCP as in a. After 30 minutes, a bolus of AMP (50 μL, 8 mg/mL) was given, and heart rate was measured by surface ECG. Bradycardia is expressed as the percentage change in heart rate. C, APCP treatment prevents IP-induced reduction in troponin I levels. In situ preconditioning with 4 cycles of IP (5 minutes of ischemia, 5 minutes of reperfusion) was performed with and without APCP infusion before 60 minutes of myocardial ischemia. After 2 hours of reperfusion, troponin I plasma levels were measured by ELISA. D, APCP treatment prevents IP-induced decreases in infarct size. In situ preconditioning was performed as in c, and infarct sizes were measured by double staining with Evans’s blue and TTC. Infarct sizes are expressed as the percent of the AAR that underwent infarction (mean ± SD; n=6). E, Representative images of infarcts from the experiment in d are displayed (blue indicates retrograde Evans’s blue staining; red and white, AAR; and white; infarcted tissue).

Afforded by IP persisted for at least 90 minutes after the completion of IP in wild-type mice but was abolished in cd73−/− mice (Data Supplement Figure II). Moreover, cd73−/− mice had significantly bigger infarcts after 60 minutes of ischemia without IP compared with controls.

To suggest that the absence of cardioprotection by IP in cd73−/− mice reflects a lack of extracellular adenosine, we reconstituted extracellular adenosine levels via intra-arterial infusion (200 μL/h, adenosine 8 mg/mL) with a dose we previously determined not to induce hypotension or bradycardia (data not shown). Indeed (Figure 3E), this treatment resulted in partial reconstitution of cardioprotection by IP in cd73−/− mice. Similar treatment of wild-type mice resulted in attenuation of infarct sizes only after IP, suggesting additional mechanisms (eg, increases in AR-mediated signaling by IP). In additional experiments, we reconstituted cd73−/− mice via intra-arterial soluble 5′-nucleotidase (1 U 5′-nucleotidase from Crotalus atrox venom). As shown in Figure 3F, 5′-nucleotidase treatment was associated with an almost complete reconstitution of a wild-type phenotype. Moreover,
5′-nucleotidase treatment was associated with a significant reduction in infarct size in wild-type animals (Figure 3F). Taken together, these data reveal for the first time genetic evidence for CD73-dependent cardioprotection by IP. Furthermore, we show treatment with soluble 5′-nucleotidase as a potential novel therapy during acute myocardial ischemia.

Increases in Cardiac Adenosine With IP Are Attenuated in cd73−/− Mice
On the basis of the above findings of CD73 induction and abolished cardioprotection by IP in cd73−/− mice, we hypothesized that increases in cardiac adenosine levels with IP are attenuated in cd73−/− mice. Consistent with...
depletion (Figure 5F). Furthermore, staining of cardiac tissues after IP treatment showed only scattered neutrophils (Data Supplement Figure III). Taken together, these data suggest selective and robust induction of A2BAR in cardiac tissues by IP.

**IP Is Abolished in A2AR/−/− Mice**

We next investigated the contribution of individual ARs to cardioprotection by IP using previously described A1AR/−/−,16 A2AR/−/−,18 and A3AR/−/− mice,17 as well as commercially available A2AR/−/− mice. Studies of myocardial A2AR expression and function in these mice confirmed structural and functional deletion of the receptor (see Data Supplement Figure IV). Moreover, transcript levels of other ARs (A1AR, A2AR, A3AR) were unaltered in A2AR/−/− mice compared with controls (data not shown).

As shown in Figures 5G and 6B and 6C, all AR gene-targeted mice showed cardioprotection by IP, except A2AR/−/− mice. Abolished infarct size reduction also was present in A2AR/−/− mice at 90 minutes of reperfusion time after IP treatment (data not shown). The cardioprotective effects of IP as assessed by plasma cTnI levels also were abolished in A2AR/−/− mice (Figure 6D). Because measurements of heart rate and blood pressure did not reveal any differences between gene-targeted mice (Data Supplement Tables I and II), it seems unlikely that abolished cardioprotection could be explained by hemodynamic alterations. Taken together, these data provide the first genetic evidence for a pivotal role of A2AR in cardioprotection by IP. To confirm these findings using a pharmacological approach, we used the highly specific, water-soluble A2AR antagonist PSB1115.22 As shown in Figure 6D, intra-arterial treatment with PSB1115 (5 mg · kg−1 · h−1) abolished infarct size attenuation by IP, thus confirming our findings in A2AR/−/− mice. To demonstrate that PSB1115 does not exhibit its cardioprotective effects through blockade of A1AR, we also treated A1AR/−/− mice with PSB1115. As shown in Figure 6E, cardioprotection by IP in A1AR/−/− mice was attenuated by specific blockade of A2AR. Taken together, these studies provide genetic and pharmacological evidence for a role of A2AR in cardioprotection by IP.

**A2AR Agonist Treatment Results in Smaller Infarcts During Acute Myocardial Ischemia**

Next, we pursued a potential therapeutic role of a novel specific A2AR agonist (BAY 60–6583). The chemical structure and selectivity are displayed in Figure 7A and 7B, and functional in vivo evidence is shown in Data Supplement Figure IV. Wild-type, but not A2AR/−/− mice treated with a single bolus of intra-arterial BAY 60–6583 (10 μg/kg body weight) over 40 minutes before 60 minutes of myocardial ischemia exhibited a significant attenuation of infarct size (Figure 7C and 7D). These studies confirm the specificity of the cardioprotective effects of BAY 60–6583 through A2AR signaling and provide strong rationale for therapeutically targeting A2AR during myocardial ischemia.

**Discussion**

In the present study, we pursued the contribution of extracellular adenosine production and signaling to cardioprotection...
Figure 5. A2BAR is selectively induced by IP and is responsible for cardioprotection. A–D, Modulation of AR transcript levels by IP. Age-, weight-, and gender-matched wild-type mice were subjected to in situ IP consisting of 4 cycles of ischemia/reperfusion (5 minutes each). Animals were killed at the indicated time points, and RNA was isolated from the AAR. Transcriptional responses of all 4 ARs were assessed by real-time reverse-transcriptase polymerase chain reaction. Data were calculated relative to β-actin and are expressed as fold change vs sham-operated animals without IP (C; mean ± SD; n = 6; *P < 0.05). E, Comparison of immunoreactivity for A2BAR on cardiomyocytes (arrow) and endothelial cells (*) from within the AAR of controls (−IP) and mice treated with IP (90 minutes, +IP; magnification ×1000). F, Cardiac A2BAR protein is induced by IP. Age-, weight-, and gender-matched C57BL/6 mice were treated with Gr-1 monoclonal antibody 24 hours before the experimental procedure, and neutrophil depletion was confirmed by differential blood counts. Animals were treated with IP; tissue from the AAR was excised, flash-frozen, and lysed; and proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with an anti-A2BAR antibody. One representative experiment of 3 is shown. The same blot was probed for β-actin as a control for protein loading. G, All AR gene-targeted mice preconditioned, except those deficient in A2BAR. Mice gene targeted for each individual AR and matched littermate controls were subjected to in situ IP as above, followed by 60 minutes of ischemia. Mice were killed after 2 hours of reperfusion, and infarct sizes were measured by double staining with Evan’s blue and TTC. Infarct sizes are expressed as percent change from littermate controls without IP (mean ± SD; n = 6).

by IP. Transcriptional profiling of preconditioned cardiac tissue revealed a prominent induction of cd73 and A2BAR mRNA. Pharmacological inhibition or targeted gene deletion of cd73 abolished the cardioprotective effects of in situ IP. Similarly, IP was abrogated in mice gene targeted for A2BAR, whereas mice deficient in each of the other ARs (A1AR, A2AR, A3AR) showed reduced infarct sizes after IP. Moreover, soluble 5’-nucleotidase or A2BAR agonist treatment mimicked cardioprotection by IP because it was associated with significant attenuation of myocardial infarct sizes after ischemia. Taken together, these studies suggest manipulation of CD73 enzyme activity to increase extracellular adenosine concentrations and signaling through A2BAR as therapeutic strategies for the treatment of coronary artery disease.

Previous studies on adaptation to hypoxia have revealed coordinated transcriptional induction of cd73 and A2BAR by hypoxia inducible factor (HIF)-1. In fact, hypoxia exposure of intestinal epithelia (Caco2 cells) or human endothelia (HMEC-1) was associated with robust induction of cd73 transcript, protein, and function.2,7 Furthermore, hypoxia exposure of cd73−/− mice resulted in dramatic increases in vascular leakage, suggesting a functional role of CD73-dependent adenosine production in maintaining vascular barrier function during limited oxygen availability. Moreover, examination of the cd73 gene promoter identified a site for HIF-1α binding, and further studies with promoter constructs and site-directed mutagenesis of the HIF-1α binding site confirmed an HIF-1α-dependent regulatory pathway for cd73 induction.2,7 Similarly, hypoxia exposure of endothelia (HMEC-1) resulted in a selective induction of A2BAR mRNA.2 Further studies examining HIF-1α DNA binding and HIF-1α loss and gain of function confirmed strong dependence of A2BAR induction by HIF-1α in vitro and in vivo.2,3 Therefore, it is not surprising that repeated episodes of ischemia/hypoxia as used during cardiac IP resulted in coordinated transcriptional induction of cd73 and A2BAR mRNA. In addition, mice with normoxic stabilization of HIF-1α by in vivo siRNA knockdown of HIF-1α-prolyl-4-hydroxylase-2 showed cardioprotection from ischemia and reperfusion.2,4 Therefore, it is likely that the observed cardioprotective effects of CD73-dependent adenosine production and signaling through the A2BAR during cardiac IP are related to a transcriptional response coordinated by HIF-1α.

Previous studies on the contribution of AR signaling to cardioprotection during IP have revealed conflicting results
on the role of individual ARs. For example, a very thorough study using 3 different A1AR antagonists [CPX (1,3-dipropyl-8-cyclopentylxanthine), BG 9719, or BG 9928] did not block cardioprotection by IP, suggesting that receptor subtypes other than A1AR may be involved in this phenomenon. In contrast, other studies found an absence of the infarct size-limiting effects of IP in A1AR gene-targeted mice, whereas A1AR-overexpressing mice were protected. Why the results of these latter studies disagree with our own is not clearly understood. Because previous studies suggest that adenosine signaling through the A1AR is responsible for activation of protein kinase C (PKC) and PKC is critically important for activation of CD73, it is conceivable that A1AR signaling may result in PKC activation, which in turn activates CD73. In fact, we found that transcriptional induction of CD73 by IP was abolished after PKC inhibition or in A1AR−/− mice. Similarly, infarct size-limiting effects by IP were attenuated by PKC inhibition (Data Supplement Figure VI). In conjunction with our findings that cardioprotection afforded by IP was less in A1AR−/− than in wild-type mice, it becomes possible that signaling through A1AR or α-adrenergic recep-

![Figure 6. IP-induced cardioprotection is abolished in A2BAR−/− mice. A–C, IP is abolished in A2BAR−/− mice. In situ IP was performed in A2BAR−/− mice and matched littermate controls. IP consisted of 4 cycles of 5 minutes of myocardial ischemia and 5 minutes of reperfusion, followed by 60 minutes of ischemia and 2 hours of reperfusion. (white bars, A2BAR+/+ mice; black bars, A2BAR−/− mice), and troponin I plasma levels were measured. In addition, infarct sizes were measured by double staining with Evan’s blue and TTC. Infarct sizes are expressed as the percent of the AAR that underwent infarction (mean±SD; n=6). C, Representative images from the experiment in B. D, E, An A2BAR antagonist abolishes the cardioprotective effects of IP. In situ IP was performed as above in A1AR−/− mice and matched littermate controls in the presence (black bars) and absence (white bars) of the A2BAR antagonist PSB1115 (5 mg · kg−1 · h−1 via carotid artery catheter). Mice were killed after 2 hours of reperfusion, and infarct sizes were measured by double staining with Evan’s blue and TTC. Infarct sizes are expressed as the percent of the AAR that underwent infarction (mean±SD; n=6).]
major extracellular pathway of adenosine generation (CD73) or in $A_2B$AR show increased susceptibility to acute myocardial ischemia and are not protected by IP. In addition, soluble 5'-nucleotidase or selective $A_2B$AR agonist treatment significantly attenuates infarct sizes after ischemia, suggesting possible new strategies to ameliorate the consequences of myocardial infarction. Future challenges include the development of approaches to deal with AR desensitization and delivery of AR agonists to specific anatomic sites.

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Disclosures

D. Köhler and Drs Eckle, Grenz, Mittelbronn, Oswald, Unertl, and Eltzschig are employees of the Tübingen University Hospital. Use of soluble 5'-nucleotidase is currently under consideration for a patent in the treatment of myocardial ischemia by the Tübingen University Hospital. Dr Krah is employee of Bayer HealthCare. Bayer HealthCare has filed patents on the use of BAY 60–6583. The other authors report no conflicts.

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**CLINICAL PERSPECTIVE**

Despite many years of investigation, many molecular aspects of cardioprotection by ischemic preconditioning remain unknown. It has been difficult to translate the cardioprotection observed in experimental animals into patient treatments that affect a reduction in the morbidity and mortality from acute coronary artery occlusion. Recent results of experiments with genetically engineered mice have revived the hope of understanding molecular mechanisms mediating the cardioprotection by ischemic preconditioning. In the present study, we used a gene-targeting approach to study the contributions of extracellular adenosine generation and adenosine receptor signaling to ischemic preconditioning cardioprotection. These studies revealed a pivotal role for the adenosine A2b receptor (A2bAR). Furthermore, pharmacological approaches suggested that adenosine receptor engagement with a specific A2bAR agonist may offer a powerful therapeutic in the treatment of acute myocardial ischemia. In contrast, the treatment of myocardial ischemia with intravenous adenosine has been problematic because adenosine can cause severe bradycardia or hypotension. In addition, nonspecific activation of adenosine receptors often is associated with rapid receptor desensitization. The present study and other work showing a strong antiinflammatory role for A2bAR receptor signaling suggest that A2bAR agonists may represent a new group of therapeutics for patients suffering from coronary artery disease. To realize these possibilities, our results will have to be translated from mice to humans, and the pharmacokinetics and potential effects of A2bAR agonists on platelet function, blood pressure, and pulmonary function will have to be investigated.
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