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−/−, A2AAR−/−, or A3AR−/− 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.

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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. We next pursued the functional contribution of CD73 to the CD73−/−, C57BL/6J mice were used. Cardiac IP was performed using a hanging weight system as described previously.15 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 littermates. 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,
was significantly attenuated in these mice (Figure 3A). Similarly, AMP-induced bradycardia absence of CD73 enzyme activity in the cardiac tissue of cd73 littermates (Figure 3B). We next performed IP in by IP in levels caused by 60 minutes of ischemia were not attenuated with wild-type mice, infarct sizes and increased plasma cTnI mice and littermate controls. In striking contrast to results targeted deletion of the cardioprotective effects of IP, we next studied mice with cd73 gene.6 We first confirmed the 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 cd73/− mice compared with littermates (Figure 3B). We next performed IP in cd73/− mice and littermate controls. In striking contrast to results with wild-type mice, infarct sizes and increased plasma cTnI levels caused by 60 minutes of ischemia were not attenuated by IP in cd73/− mice (Figure 3C and 3D). The protection 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 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
In the present study, we pursued the contribution of extracellular adenosine production and signaling to cardioprotection.
by IP. Transcriptional profiling of preconditioned cardiac tissue revealed a prominent induction of cd73 and A2AR 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 cd73 and A2AR during cardiac IP are related to a transcriptional induction by HIF-1α-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. Similarly, hypoxia exposure of endothelia (HMEC-1) resulted in a selective induction of A2AR mRNA. Further studies examining HIF-1α DNA binding and HIF-1α loss and gain of function confirmed strong dependence of A2AR induction by HIF-1α in vitro and in vivo. Therefore, it is not surprising that repeated episodes of ischemia/hypoxia as used during cardiac IP resulted in coordinated transcriptional induction of cd73 and A2AR mRNA. In addition, mice with normoxic stabilization of HIF-1α by in vivo siRNA knockdown of HIF-1α-prollyl-4-hydroxylase-2 showed cardioprotection from ischemia and reperfusion. Therefore, it is likely that the observed cardioprotective effects of CD73-dependent adenosine production and signaling through the A2AR 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-

tors activates PKC, which in turn is responsible for CD73 activation, production of extracellular adenosine, and subsequent signaling through A2BAR. In vitro studies have indicated a critical role of signaling through the A2BAR in reopening of endothelia during transendothelial migration of neutrophils, particularly during conditions of limited oxygen availability. Moreover, pharmacological inhibition of A2BAR during hypoxia exposure is associated with increased pulmonary edema and vascular leakage. Thus, the findings of the present study are consistent with previous work on adenosine signaling, transcriptional responses of A2BAR after myocardial ischemia, and reperfusion, as well as studies suggesting a cardioprotective role of A2BAR during ischemic postconditioning, but highlight for the first time a pivotal role of A2BAR signaling in cardioprotection by IP.

At present, the source of extracellular adenosine generated during conditions of hypoxia or ischemia remains unknown. Previous reports have indicated that polymorphonuclear neutrophils release ATP during conditions of inflammation or hypoxia. Such extracellular ATP either may signal directly to vascular ATP receptors or may function as a metabolic
A2BAR transcript levels and protein are induced in farct size reduction by activation of A2AAR on T lympho-
addition, the exact cellular location of A2BAR involved in activation of ARs. Besides polymorphonuclear neutrophils, platelets may be an important source for extracellular ATP. In addition, the exact cellular location of A2AR involved in cardioprotection by IP is currently unknown. As we show here, A2AR transcript levels and protein are induced in cardiac tissues by IP, suggesting a role of cardiac A2AR in cardioprotection by IP. However, a very carefully executed study on the role of A2AR signaling during myocardial ischemia suggested infarct size reduction by activation of A2AR on T lymphocytes. Further studies are necessary to reveal which A2AR-expressing cells are responsible for the cardioprotective effects of IP.

In summary, the present study reveals cardioprotection during myocardial ischemia via CD73-dependent generation of extracellular adenosine and signaling through the A2AR. In fact, genetically targeted mice with deficiencies in the major extracellular pathway of adenosine generation (CD73) or in A2AR show increased susceptibility to acute myocardial ischemia and are not protected by IP. In addition, soluble 5'-nucleotidase or selective A2BAR 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, Osuwald, 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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Figure 7. Treatment with an A2AR agonist protects from ischemia. A, B, Chemical structure and EC50 values of BAY 60–6583 on A1AR, A2AAR, and A2BAR receptors measured on Chinese hamster ovary cells overexpressing the indicated ARs. C, An A2AR agonist protects mice from myocardial ischemia. C57BL/6J and matched A2AR mice were subjected to 60 minutes of cardiac ischemia, followed by 2 hours of reperfusion with or without pretreatment with BAY 60–6583 (10 μg/kg IV 40 minutes before ischemia induction). Infarct sizes were measured by double staining of cardiac tissue with Evan's blue and TTC. Infarct sizes are expressed as the percent of the AAR that underwent infarction (mean±SD; n=6). D, Representative images of infarcts from the experiment in A.


**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 A3 receptor (A3AR). Furthermore, pharmacological approaches suggested that adenosine receptor engagement with a specific A3AR 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 A3AR receptor signaling suggest that A3AR 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 transgenic humans, and the pharmacokinetics and potential effects of A3AR agonists on platelet function, blood pressure, and pulmonary function will have to be investigated.
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