CD39/Ectonucleoside Triphosphate Diphosphohydrolase 1 Provides Myocardial Protection During Cardiac Ischemia/Reperfusion Injury

David Köhler, PhD*; Tobias Ecke, MD*; Marion Faigle, BS; Almut Grenz, MD; Michel Mittelbronn, MD; Stefanie Laucher, BS; Melanie L. Hart, PhD; Simon C. Robson, MD; Christa E. Müller, PhD; Holger K. Eltzschig, MD, PhD

Background—Extracellular adenosine, generated from extracellular nucleotides via ectonucleotidases, binds to specific receptors and provides cardioprotection from ischemia and reperfusion. In the present study, we studied ecto-enzymatic ATP/ADP-phosphohydrolysis by select members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family during myocardial ischemia.

Methods and Results—As a first step, we used a murine model of myocardial ischemia and in situ preconditioning and performed pharmacological studies with polyoxometalate 1, a potent E-NTPDase inhibitor (Na6[H2W12O40]). Polyoxometalate 1 treatment increased infarct sizes and abolished beneficial effects of preconditioning. To define relative contributions of distinct E-NTPDases, we investigated transcriptional responses of E-NTPDases 1 to 3 and 8 to preconditioning. We noted robust and selective induction of E-NTPDase 1 (CD39) transcript and protein. Histological analysis of preconditioned myocardium localized CD39 induction to endothelia and myocytes. Cd39+/− mice exhibited larger infarct sizes with ischemia (cd39+/− 43.0±3.3% versus cd39−/− 52%±1.8; P<0.05), and cardioprotection was abrogated by preconditioning (cd39+/− 13.3%±1.5 versus cd39−/− 50.5%±2.8; P<0.01). Heightened levels of injury after myocardial ischemia and negligible preconditioning benefits in cd39−/− mice were corrected by infusion of the metabolic product (AMP) or apyrase. Moreover, apyrase treatment of wild-type mice resulted in 43±4.2% infarct size reduction (P<0.01).

Conclusions—Taken together, these studies reveal E-NTPDase 1 in cardioprotection and suggest apyrase in the treatment of myocardial ischemia. (Circulation. 2007;116:1784-1794.)

Key Words: adenosine ■ endothelium ■ enzymes ■ myocardial infarction ■ reperfusion

Several studies have found a pivotal role of extracellular adenosine signaling in tissue protection, particularly during conditions of limited oxygen availability.1–4 During hypoxia, extracellular adenosine stems mainly from increased phosphohydrolysis of precursor nucleotides (ATP/ADP/AMP) and contributes to cardioprotection from ischemia and reperfusion injury. Ischemic preconditioning (IP)5 and postconditioning events6 involve protective cellular adaptive phenomena in the heart associated with protein kinase C activation, a component of the mitochondrial K(ATP) signaling cascade that combats ischemic stress. In such pathophysiological settings, the myocardial cellular phenotype alters to become more resistant to subsequent ischemia and tissue injury. This polygenic response involves mediators including adenosine,7 bradykinin,8 opioids,9 erythropoietin,10 adrenergics, and muscarinics.11

Clinical Perspective p 1794

Our group has been interested in the increases in extracellular adenosine that are critical in cardioprotection during IP.12 For example, studies measuring interstitial adenosine concentrations in swine or perfused rabbit hearts via micro-

Received January 12, 2007; accepted August 17, 2007.
From the Department of Anesthesiology and Intensive Care Medicine (D.K., T.E., M.F., S.L., H.K.E.), Department of Pharmacology and Toxicology (A.G.), and Institute of Brain Research (M.M.), University Hospital, Tübingen, Germany; Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women’s Hospital (M.L.H.), and Liver and Transplant Centers (S.C.R.), Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Mass; Pharmaceutical Institute, Pharmaceutical Sciences Bonn, University of Bonn, Bonn, Germany (C.E.M.); and Mucosal Inflammation Program, Department of Anesthesiology, University of Colorado Health Science Center, Denver (H.K.E.).

*The first 2 authors contributed equally to this work.

Guest Editor for this article was Thomas F. Lüscher, MD.

The online-only Data Supplement, consisting of Methods and tables, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.690180/DC1.

Correspondence to Holger K. Eltzschig, MD, PhD, Mucosal Inflammation Program, Department of Anesthesiology and Perioperative Medicine, University of Colorado Health Sciences Center, 4200 E Ninth Ave, Campus Box B113, Denver, CO 80262. E-mail holger.eltzschig@uchsc.edu

© 2007 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.107.690180
dialysis noted a 6- or 12-fold increase in extracellular adenosine with IP, respectively.7,13 Other studies in mice gene targeted for individual adenosine receptors also provide convincing evidence for adenosine signaling in cardioprotection by IP.12 Increases in extracellular adenosine predominantly reflect enhanced extracellular adenosine generation from nucleotides. For example, studies of renal IP found dramatic increases of adenosine levels with IP treatment in the kidneys that were blunted in gene-targeted mice for cd73 (conversion of AMP to adenosine)14 or cd39 (conversion of AMP to adenosine). Similarly, cardiac studies have shown a critical role of CD73 in the elevation of cardiac adenosine levels during IP.12,16,17 On the basis of these studies and the fact that extracellular levels of ATP and ADP are increased dramatically during preconditioning,18 we presumed a contribution of extracellular ATP/ADP phosphohydrolysis in cardioprotection from myocardial ischemia and reperfusion injury.

Extracellular ATP/ADP-phosphohydrolysis is mainly achieved enzymatically by ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), a recently described family of ubiquitously expressed membrane-bound enzymes.19,20 The catalytic sites of plasma membrane–expressed E-NTPDases 1 to 3 and 8 are exposed to the extracellular milieu, and the others are intracellular.20 The presumptive biological role of plasma membrane–bound E-NTPDases (E-NTPDases 1 to 3 and 8) is to fine-tune extracellular nucleotide levels. For example, E-NTPDase 1 (CD39) plays an important role in vascular endothelial function by blocking platelet aggregation via the phosphohydrolysis of ATP and ADP from the blood to maintain vascular integrity.21,22 At the same time, E-NTPDase 1 is also important in the maintenance of platelet functionality by preventing platelet P2Y1-receptor desensitization. As such, mice gene targeted for E-NTPDase 1 (cd39−/− mice) show prolonged bleeding time with minimally perturbed coagulation parameters.23 Of significant physiological relevance to the present study is the fact that the E-NTPDase end product, AMP, serves as the major metabolic substrate for CD73-dependent generation of extracellular adenosine.1 Thus, E-NTPDase expression and function are key regulators of extracellular adenosine signaling. Therefore, we addressed the role of ATP/ADP-nucleotide phosphohydrolysis in cardioprotection by applying a murine model of myocardial ischemia and in situ IP. This model uses a hanging-weight system for coronary artery occlusion, thus eliminating the necessity of intermittently occluding the coronary artery with a knotted suture.24

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 University Hospital Tübingen and the Regierungspräsidium Tübingen. C57BL/6j and C57BL/6x129SvJ mice were obtained from Charles River (Sulzfeld, Germany). Mice deficient in cd39 on the C57BL/6j129SvJ strain were generated, validated, and characterized as described previously.23

Murine Model of Myocardial Ischemia and IP

C57BL/6x129SvJ strain, cd39−/− mice, or littermate controls were matched in age, gender, and weight. Cardiac IP was performed with the use of a hanging-weight system as described previously (see Methods in the online-only Data Supplement).24

Transcriptional Analysis

To assess the influence of IP on cd39 transcript level, IP was performed, and the area at risk was delineated by Evan’s blue staining and excised at indicated time periods, followed by isolation of RNA and quantification of transcript levels by real-time reverse transcription polymerase chain reaction (iCycler; Bio-Rad Laboratories, Munich, Germany), as described previously.12

Western Blots for CD39

C57BL/6x129SvJ mice were euthanized, cardiac IP was performed, and the area at risk was excised at 30, 60, 90, and 120 minutes after IP and immediately frozen at −80°C (remaining blood was removed before). In subsets of experiments, we determined CD39 protein content from the area at risk, as described previously.12

Immunohistochemistry

In subsets of experiments, we determined CD39 protein content from the area at risk, as described previously.12

Adenosine Measurements

Tissue adenosine and AMP levels were determined via high-performance liquid chromatography, as described previously.12

Measurement of CD39 Enzyme Activity

To measure cardiac CD39 activity, we adopted a previously described technique (for details, see the online-only Data Supplement).1

Data Analysis

For comparison of 2 groups, the nonparametric Mann Whitney test was performed. For comparison of ≥2 groups, the Kruskal-Wallis test with a Dunn posttest was performed. Statistical significance was accepted at a level of P<0.05. All values are expressed as mean±SEM from 6 animals per condition.

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

Pharmacological Inhibition of E-NTPDases Results in Increased Myocardial Infarct Size and Abolished Cardioprotection by IP

On the basis of the hypothesis that ATP/ADP phosphohydrolysis attenuates myocardial ischemia/reperfusion injury and contributes to cardioprotection by IP, we first sought to inhibit extracellular phosphohydrolysis pharmacologically. However, previously proposed E-NTPDase inhibitors are also strong inhibitors of ATP receptors, thus making it impossible to selectively study E-NTPDase–dependent phosphohydrolysis in vivo.25 In contrast, we recently identified polyoxometalates as a novel class of E-NTPDase inhibitors without activity on purinergic receptors.26 A screen of different polyoxometalates revealed polyoxotungstate (Na6[12H2W12O40]2−) (POM-1; Figure 1A) as a highly potent E-NTPDase 1 and E-NTPDase 3 inhibitor with Ki values of 2.58 and 3.26 μmol/L, respectively, and an ≈10-fold lower inhibitory activity for E-NTPDase 2 (Ki≈28.8 μmol/L) (Figure 1B). To demonstrate a biological effect of polyoxometalate 1
on ATP/ADP phosphohydrolysis in vivo, we first measured the effect of intravascular ATP treatment on heart rate changes. Application of an intravascular ATP bolus (50 \( \mu \text{L}, 8 \text{ mg/mL} \)) results in a heart rate reduction from 480 to 120 bpm, lasting for 150 seconds. To confirm that this effect is mediated by adenosine, we subjected previously described mice gene targeted for the adenosine A1 receptor (A1AR/\(^{-/}\)mice) to the same regimen. In contrast to wild-type mice, no change in heart rate was observed in A1AR/\(^{-/}\)mice (data not shown), suggesting that heart rate changes elicited by ATP infusion are mediated by adenosine signaling. After intrarterial POM-1 infusion (3 mg/kg), the observed reduction in heart rate was similar in degree but recovered after a significantly shorter time period (50 seconds; \( P<0.05 \)), suggesting that POM-1 treatment results in decreased extracellular adenosine generation from ATP. POM-1 treatment alone did not alter heart rate. Measurements of cardiac adenosine and AMP levels in POM-1–treated mice revealed attenuated increases in cardiac adenosine and AMP with preconditioning (data not shown).

After having shown attenuation of nucleotide-phosphohydrolysis with POM-1 treatment, we used this pharmacological regimen in a recently described model of in situ myocardial ischemia and preconditioning. We thus subjected mice (C57BL/6x129Svj) to 60 minutes of left coronary artery occlusion followed by 120 minutes of reperfusion with or without prior IP treatment (4 cycles, 5 minutes of ischemia, 5 minutes of reperfusion). All mice survived this experiment. Heart rate and blood pressure did not differ between POM-1–treated and untreated mice (data not shown). As shown in Figure 1D, POM-1 treatment resulted in significantly larger infarct sizes and complete inhibition of cardioprotection by IP. These data provide novel pharmacological evidence for a critical role of E-NTPDase–dependent nucleotide phosphohydrolysis in attenuating myocardial ischemia and cardioprotection by IP.
Cardiac E-NTPDase 1 (CD39) Is Selectively Induced by IP

After having shown that pharmacological inhibition of E-NTPDases results in increased susceptibility to myocardial ischemia and reperfusion injury and abolished cardioprotection by IP, we next sought to define the contribution of individual ecto-NTDPases (E-NTPDases 1 to 3 and 8) to cardioprotection. For this purpose, we studied transcriptional responses of cardiac E-NTPDase expression to IP. We thus performed 4 cycles of intermittent left coronary artery occlusion and reperfusion (5 minutes of ischemia, 5 minutes of reperfusion) and harvested preconditioned myocardial tissues.

Figure 2. Cardiac CD39 is induced by IP. A, Murine model of cardiac IP. The IP protocol consisted of 4 cycles of ischemia/reperfusion (5 minutes each), followed by indicated times of reperfusion (A indicates anesthesia induction; T, thoracotomy). B, CD39 (E-NTPDase 1) mRNA is selectively induced by IP. After indicated time periods, the area at risk was excised, total RNA was isolated, and E-NTPDases 1 to 3 and E-NTPDase 8 mRNA levels were determined by real-time reverse transcription polymerase chain reaction. Data were calculated relative to an internal housekeeping gene (β-actin) and are expressed as fold change compared with control (no IP) ± SEM at each indicated time (n=6). C, E-NTPDase 1 (CD39) protein is induced by IP. Tissue from the area at risk was excised at the indicated time points, flash frozen, and lysed, and proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with an anti-E-NTPDase 1 antibody. The same blot was probed for β-actin expression as a control for protein loading. One representative experiment of 3 is shown.
at indicated time points after IP treatment for real-time reverse transcription polymerase chain reaction (Figure 2A). Baseline expression levels relative to β-actin revealed lowest expressional rates of E-NTPDase 8, followed by E-NTPDase 3 and E-NTPDase 2, with highest expression of E-NTPDase 1 (data not shown). In addition, we found a robust and selective induction of E-NTPDase 1 (CD39) mRNA (eg, 90 minutes after cardiac IP, 8.0.±1.5-fold; \(P<0.001\); Figure 2B). Analysis of other E-NTPDases (E-NTPDases 2, 3, and 8) revealed no significant induction with IP (Figure 2B). Western blot analysis confirmed that CD39 protein is induced by IP. As shown in Figure 2C, cardiac CD39 protein is significantly induced as early as 90 minutes after IP treatment. To localize CD39 induction by IP to specific cardiac tissues, we performed an immunohistochemical staining of the preconditioned myocardial tissue for CD39. In control tissues without IP, CD39 was expressed mainly on endothelia with hardly any staining of cardiomyocytes (Figure 3A). In contrast, tissues exposed to IP showed dramatic increases in CD39 expression, on both endothelia and myocytes (Figure 3B; isotype controls in Figure 3C and 3D). Taken together, these data provide strong evidence that CD39 is induced by IP on cardiac endothelia and myocytes.

Cardioprotection by IP Is Abolished in cd39−/− Mice

On the basis of the pharmacological evidence of E-NTPDases in cardioprotection and the observation of selective induction of CD39 by IP, we next evaluated functional roles of CD39 in mediating cardioprotection. For this purpose, we used previously described gene-targeted mice for cd39.23 We initially investigated ATP-mediated effects on cardiovascular parameters. As shown in Figure 4A, ATP-induced time of bradycardia was significantly shortened in cd39−/− mice compared with wild-type littermates, suggesting decreased extracellular adenosine generation from ATP in cd39−/− mice. We next performed IP in cd39−/− mice and littermate controls. Consistent with our pharmacological studies with POM-1, infarct sizes caused by 60 minutes of ischemia were significantly increased in cd39−/− mice, and cardioprotection by IP was
abolished in cd39−/− mice (Figure 4B and 4C). In additional studies, we used 240 minutes of reperfusion time, which confirmed results similar to those with 120 minutes of reperfusion (infarct size without and with IP after 120 and 240 minutes of reperfusion, respectively: IP 52 ± 1.8% versus −IP 51 ± 2.0% and +IP 50.5 ± 2.8% versus +IP 51.3 ± 2.3%). Taken together, these studies provide genetic evidence of a cardioprotective role of CD39 in myocardial ischemia.

**Increases in Cardiac AMP and Adenosine With IP Are Attenuated in cd39−/− Mice**

On the basis of the aforementioned findings of CD39 induction and abolished cardioprotection by IP in cd39−/− mice, we hypothesized that increases in cardiac AMP and adenosine levels with IP are attenuated in cd39−/− mice. Consistent with previous studies,17 cardiac adenosine and AMP concentrations measured immediately after IP (Figure 5A and 5B) were increased, suggesting that AMP is produced during IP treatment. In contrast, cd39−/− mice exhibited attenuated adenosine levels in conjunction with decreased AMP concentrations after IP treatment. Taken together, these studies demonstrate that CD39 functions to increase myocardial AMP/adenosine during preconditioning.

**Cardiac CD39 Activity Is Increased After IP**

Because cardiac CD39 induction of transcript and protein occurs only 90 minutes after IP, these transcriptional effects cannot account for the increased AMP/adenosine production and cardioprotection that occur immediately after IP. To confirm that the increased adenosine/AMP production immediately after IP reflects CD39 enzyme activity, we adopted a previously described technique to measure cardiac CD39 activity by assessing the conversion of etheno-ATP to etheno-AMP.1 As a first step, we measured baseline CD39 activity in hearts from cd39−/− mice or corresponding littermate con-
Reconstitution of cd39−/− Mice
As proof of principle and to demonstrate that the absence of cardioprotection by IP in cd39−/− mice reflects lack of extracellular AMP, we reconstituted extracellular AMP levels via intra-arterial infusion (100 μL/h, AMP 8 mg/mL), a dose we previously determined not to induce hypotension or bradycardia (data not shown). This treatment of cd39−/− mice was associated with decreased infarct sizes in nonpreconditioned animals and with complete reconstitution of cardioprotection by IP in preconditioned mutant mice (Figure 6A). In additional experiments, we reconstituted cd39−/− mice via intraperitoneal application of soluble potato (apyrase) NTPDase (80 U/kg). Similar to the aforementioned results, soluble apyrase treatment was associated with attenuated infarct sizes and reconstitution of cardioprotective effects of IP in cd39−/− mice (Figure 6B). These studies confirm an important role of CD39 in attenuating myocardial ischemia and reperfusion injury after coronary artery occlusion.

Therapeutic Effects of Apyrase or AMP Treatment During Myocardial Ischemia and Reperfusion in Wild-Type Mice
After having demonstrated that pharmacological or genetic inhibition of extracellular nucleotide-phosphohydrolysis is associated with increased myocardial infarct sizes and abolished cardioprotection by IP, we hypothesized that increasing CD39 activity levels in cd39−/− mice would be associated with decreased infarct sizes in nonpreconditioned animals and with complete reconstitution of cardioprotection by IP in preconditioned mutant mice (Figure 6A). In additional experiments, we reconstituted cd39−/− mice via intraperitoneal application of soluble potato (apyrase) NTPDase (80 U/kg). Similar to the aforementioned results, soluble apyrase treatment was associated with attenuated infarct sizes and reconstitution of cardioprotective effects of IP in cd39−/− mice (Figure 6B). These studies confirm an important role of CD39 in attenuating myocardial ischemia and reperfusion injury after coronary artery occlusion.
extracellular AMP generation ab initio by treatment with soluble apyrase or AMP infusion may provide a therapeutic approach. As shown in Figure 7A and 7C, soluble apyrase or AMP treatment of wild-type mice without IP resulted in a 43 ± 4.2% or 41 ± 3.7% reduction of infarct size, respectively. To confirm that 120 minutes of reperfusion would be sufficient to show a therapeutic effect of AMP/apyrase, we also determined infarct sizes after 240 minutes of reperfusion. Consistent with previous studies, no differences between 120 or 240 minutes of reperfusion time were observed (apyrase/AMP-treated mice without IP after 120 minutes and 240 minutes of reperfusion, respectively: 23.1 ± 1.9 versus 22.5 ± 2.1 and 33.1 ± 2.6 versus 34.1 ± 1.3). Figure 7B and 7D depicts representative and corresponding photographs of myocardial tissues after double staining with Evan’s blue and triphenyltetrazolium chloride double staining and expressed as percentage of the area at risk (mean ± SEM; n = 6). D. Representative images of myocardial sections from the experiment procedure in C are displayed (blue/dark, retrograde Evan’s blue staining; red and white, area at risk; white, infarcted tissue).

Cardiac Adenosine Is Increased With Apyrase Treatment

We hypothesized that the cardioprotective effects of apyrase treatment are related to elevation of cardiac adenosine. Therefore, we measured cardiac adenosine after apyrase treatment in wild-type mice with or without prior IP treatment. Apyrase treatment alone was associated with a degree of adenosine elevation similar to that in mice with IP treatment (Figure 8). However, apyrase treatment in mice pretreated with IP was not associated with a “hyperelevation” of adenosine. These findings might explain the fact that apyrase treatment does not provide additional cardioprotecive effects in IP-treated animals (Figure 7A).

**Discussion**

In the present study we pursued the contribution of extracellular ATP/ADP-phosphohydrolysis to myocardial ischemia and cardioprotection by IP. As the first step in these experiments, we made the observation that pharmacological inhibition of E-NTPDases results in decreased myocardial resistance to ischemia and abolished cardioprotection by IP. On the basis of these results, we next pursued transcriptional responses of E-NTPDases by IP. We found that E-NTPDase 1/cd39 mice is selectively induced by preconditioning and that such increases are localized to endothelia and cardiac myocytes. Therefore, we subjected gene-targeted mice for E-NTPDase 1 (cd39−/− mice) to myocardial ischemia.
consistent with our pharmacological studies, we found increased infarct sizes at baseline and abolished cardioprotection by IP. Reconstitution of cd39−/− mice with AMP or soluble NTPDases corrected the deleterious phenotype. Similar treatment in wild-type mice was associated with cardioprotection from ischemia, imitating the cardioprotective effects of IP. Taken together, these studies provide pharmacological and genetic evidence for a contribution of E-NTPDase 1/CD39-dependent ATP/ADP phosphohydrolysis to cardioprotection from ischemia. These studies are consistent with previous work in animal models of cardiac transplantation.28,29 These involved models of cardiac transplantation using cd39−/− mice or other mice overexpressing human CD39. As such, deletion of CD39 rendered mice extremely sensitive to vascular injury, and cardiac xenografts of cd39−/− mice rapidly failed with vascular-type rejection in the setting of enhanced platelet aggregation, increased P-selectin expression, and fibrin(ogen) deposition.23 Conversely, upregulation of CD39 in these transgenic models by somatic gene transfer by recombinant adenoviruses or administration of soluble apyrase had major benefits in prolonging cardiac graft survival and blocking platelet-mediated thrombosis.20,29 Moreover, another study examining the role of CD39 in ischemic brain found increased cerebral infarct volumes and reduced postischemic perfusion in a different cd39−/− mouse line.21 In addition, pharmacological reconstitution with soluble CD39 restored postischemic cerebral perfusion and rescued these mice from cerebral injury.

Although these studies have demonstrated a protective role for CD39 in preventing thrombus formation, it appears unlikely in the present study that the observed protective effects of CD39 during myocardial ischemia are due to a modulation of platelet activation or function. Although extracellular nucleotides (particularly ADP) have been implicated as strong activators of purinergic receptors on platelets, thereby initiating platelet activation and thrombus formation,20 the cd39−/− mice used in the present study had a significantly prolonged bleeding time, resulting in hemorrhagic shock, whereas no substantial differences in plasma P-selectin concentrations could be observed. In addition, cd39−/− mice showed substantial delays in platelet plug formation in an in vivo model of arterial injury and thrombus formation.23 In view of other studies showing potentiation of ischemia/reperfusion injury with platelet activation31 and the known platelet dysfunction and prolonged bleeding time of these cd39−/− mice, it appears unlikely that the present observations of increased myocardial infarction and abolished cardioprotection by IP are caused by the antithrombotic state of the cd39−/− mice. In addition to a thromboregulatory role, E-NTPDase 1–dependent generation of extracellular AMP represents the main substrate for extracellular nucleotides generation by CD73. It is important to note that other studies found that ATPase and ADPase activities were substantially decreased in cardiac tissues cultured from cd39−/− mice, indicating that the contribution of other nucleotidases to cardiac hydrolysis of extracellular adenine nucleotides is minimal.23 These findings are also supported by a recent study demonstrating that gene-targeted mice lacking the major extracellular pathway of ATP/ADP phosphohydrolysis in the kidney (CD39/ENTPdase 1) are not protected from renal ischemia by IP. In this study, IP-elicited improvements of creatinine clearance, urinary flow rate, or histological tissue damage were completely abolished in cd39−/− mice.15

It is surprising that baseline infarct sizes were increased after inhibition or deletion of CD39. In contrast, prior studies using adenosine receptor antagonists or adenosine destruction by deaminase found no difference in infarct size per se. For example, an elegant study of enflurane-anesthetized pigs found that treatment with adenosine deaminase was not associated with increased myocardial infarct sizes at baseline. In this study, a bradykinin B2 receptor blocker was also used. The authors found that although bradykinin is essential during IP of shorter duration, adenosine appears to be more important during IP of longer duration.8 Similarly, a study on the involvement of endogenous adenosine in IP in swine found that whereas IP was associated with attenuated infarct sizes and increased interstitial adenosine concentrations, adenosine deaminase treatment per se did not alter infarct sizes.7 Although it is currently not clear why the present study found larger baseline infarct sizes with inhibition of E-NTPDases or after genetic deletion of CD39, these differences may reflect details of the anesthetic management or preconditioning protocol or could reflect differences between the species that were studied (eg, murine versus porcine studies). Moreover, it is unclear why in the present study there appears to be no residual cardioprotection with IP after E-NTPDase inhibition or CD39 gene deletion. Given the limitations of any animal model, the authors appreciate that adenosine is not the only mechanism contributing to precon-
ditioning. However, these studies suggest that adenosine contributes significantly to cardioprotection by IP and point toward the extracellular metabolism of ATP/ADP as an important source of adenosine.

The pathophysiological basis underlying the release of nucleotides and nucleosides during ischemia remains unclear. Previous reports have suggested increased nucleotide release (particularly ATP) during conditions of inflammation or hypoxia. In addition, studies using large-animal models could show that after IP treatment, extracellular adenosine levels are increased ~4-fold. In this study, the authors collected blood samples from the coronary veins, thereby confirming extracellular localization of adenosine. Moreover, treatment with the CD73 inhibitor α-β-methylene-diphosphate completely blocked the observed increases in extracellular adenosine. These studies highlight that the major pathway of adenosine generation during cardiac IP is via ectonucleotidase-dependent phosphohydrolysis of precursor nucleotides. This is consistent with the present findings showing increased adenosine concentrations with IP treatment, which are abolished in mice gene targeted for cd39 or cd73. Taken together, these studies confirm that during IP, extracellular adenosine mainly stems from upstream metabolism of nucleotides as opposed to adenosine released from ischemic cells. Increased E-NTPase mRNA and protein expression 90 minutes after IP cannot be causal for the observed protection (reduced infarct size) for simple temporal reasons. However, increased CD39 enzyme activity and elevated adenosine levels immediately after IP suggest that additional, nontranscriptional mechanisms are involved in CD39-dependent cardioprotection. With regard to CD73, the key enzyme of extracellular adenosine generation, a recent study found an immediate increase of enzyme activity after IP. For example, translocation of preformed enzyme to the cell surface could be responsible for the initial increase in enzyme activity.

Taken together, our data indicate an important contribution of CD39 in mediating cardioprotection during myocardial ischemia and reperfusion injury. In addition, the present study indicates a role of soluble apyrase in the treatment of myocardial ischemia. On the basis of the fact that the presented data are all derived from murine studies, further studies will include the translation from mice to humans. In addition, it will be important to address pharmacokinetics and additional effects of apyrase treatment, eg, on coagulation, blood pressure, or pulmonary function, before such studies can be implemented and tested in a clinical setting. Moreover, studies using different time points of treatment (eg, before, during, or after myocardial ischemia) will have to define the therapeutic time window of apyrase treatment in myocardial ischemia.

**Acknowledgments**

We gratefully acknowledge Stephanie Zug and Matthias Nagel for technical assistance and Jürgen Schnermann and Keiichi Enjoji for kindly providing mice gene targeted for A1AR and cd39, respectively.

**Sources of Funding**

This work was supported by a Fortune grant 1416-0-0, Interdisciplinary Centre for Clinical Research (IZKF) Verbundprojekt 1597-0-0 from the University of Tübingen, and German Research Foundation (DFG) grant EL274/2–2 to Dr Eltzschig and IZKF Nachwuchsgruppe 1605-0-0 to Dr Eckle.

**Disclosures**

M. Faigle, S. Laucher, and Drs Eckle, Grenz, Hart, Köhler, Mittelbronn, and Eltzschig are employees of Tübingen University Hospital. Use of apyrase is currently under consideration for a patent in the treatment of myocardial ischemia by Tübingen University Hospital. The other authors report no conflicts.

**References**

Novel pharmacological approaches to myocardial ischemia are urgently needed. For example, perioperative myocardial infarctions are among the leading causes of morbidity and mortality of surgical patients, despite decades of clinical efforts toward diagnostic and treatment strategies. A very promising approach to myocardial infarct size reduction is ischemic preconditioning, first described in the 1980s, with pretreatment with short time periods of intermittent myocardial ischemia resulting in a robust reduction of infarct sizes. However, it has been difficult to develop pharmacological approaches to utilize such mechanisms and translate the cardioprotection observed in experimental animals into patient treatment. Although previous studies have shown a pivotal role of extracellular adenosine signaling in cardioprotection by ischemic preconditioning, the extracellular source for adenosine remained unclear. In the present study, we pursued the hypothesis that extracellular phosphohydrolysis of ATP or ADP constitutes an important source for adenosine generation in cardioprotection by ischemic preconditioning. In fact, pharmacological and genetic approaches in mice suggest that the key enzyme in this response is CD39, an ectonucleoside-triphosphate diphosphohydrolase, which rapidly converts extracellular ATP or ADP to AMP, which in turn is hydrolyzed to adenosine. In fact, soluble apyrase provides pharmacological activity similar to that of CD39. Thus, pretreatment with soluble apyrase is associated with increased myocardial adenosine levels and a degree of cardioprotection similar to that achieved with experimental ischemic preconditioning. To realize these possibilities, our results will have to be translated from mice to humans, and the pharmacokinetics, time window for treatment, and other effects of apyrase (eg, on platelet function or blood pressure) require further investigation.
CD39/Ectonucleoside Triphosphate Diphosphohydrolase 1 Provides Myocardial Protection During Cardiac Ischemia/Reperfusion Injury

David Köhler, Tobias Eckle, Marion Faigle, Almut Grenz, Michel Mittelbronn, Stefanie Laucher, Melanie L. Hart, Simon C. Robson, Christa E. Müller and Holger K. Eltzschig

*Circulation*. 2007;116:1784-1794; originally published online October 1, 2007; doi: 10.1161/CIRCULATIONAHA.107.690180

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/116/16/1784

An erratum has been published regarding this article. Please see the attached page for:
/content/116/18/e514.full.pdf

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2007/10/01/CIRCULATIONAHA.107.690180.DC1
http://circ.ahajournals.org/content/suppl/2007/10/05/CIRCULATIONAHA.107.690180.DC2

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* 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* is online at:
http://circ.ahajournals.org/subscriptions/
In the version of the article, “CD39/Ectonucleoside Triphosphate Diphosphohydrolase 1 Provides Myocardial Protection During Cardiac Ischemia/Reperfusion Injury,” by Köhler et al that was posted online on October 1, 2007 (DOI: 10.1161/CIRCULATIONAHA.107.690180), an author name was misspelled.

In the byline of the article, “Marion Faiglem, BS” should have been “Marion Faigle, BS.”

The error has been corrected in the final print version of the article in the October 16, 2007, issue of the journal (Circulation. 2007;117:1784–1794) and in the current online version. The publisher regrets the error.

DOI: 10.1161/CIRCULATIONAHA.107.187466