Targeted Disruption of *cd73*/Ecto-5′-Nucleotidase Alters Thromboregulation and Augments Vascular Inflammatory Response

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Abstract—To investigate the role of adenosine formed extracellularly in vascular homeostasis, mice with a targeted deletion of the *cd73*/ecto-5′-nucleotidase were generated. Southern blot, RT-PCR, and Western blot analysis confirmed the constitutive knockout. In vivo analysis of hemodynamic parameters revealed no significant differences in systolic blood pressure, ejection fraction, or cardiac output between strains. However, basal coronary flow measured in the isolated perfused heart was significantly lower (−14%; $P<0.05$) in the mutant. Immunohistochemistry revealed strong CD73 expression on the endothelium of conduit vessels in wild-type (WT) mice. Time to carotid artery occlusion after ferric chloride ($FeCl_3$) was significantly reduced by 20% in $cd73^{-/-}$ mice ($P<0.05$). Bleeding time after tail tip resection tended to be shorter in $cd73^{-/-}$ mice (−35%). In vivo platelet cAMP levels were 0.96±0.46 in WT versus 0.68±0.27 pmol/10^6 cells in $cd73^{-/-}$ mice ($P<0.05$). Under in vitro conditions, platelet aggregation in response to ADP (0.05 to 10 μmol/L) was undistinguishable between the two strains. In the cremaster model of ischemia–reperfusion, the increase in leukocyte attachment to endothelium was significantly higher in $cd73^{-/-}$ compared with WT littermates (WT 98% versus $cd73^{-/-}$ 245%; $P<0.005$). The constitutive adhesion of monocytes in ex vivo–perfused carotid arteries of WT mice was negligible but significantly increased in arteries of $cd73^{-/-}$ mice ($P<0.05$). Thus, our data provide the first evidence that adenosine, extracellularly formed by CD73, can modulate coronary vascular tone, inhibit platelet activation, and play an important role in leukocyte adhesion to the vascular endothelium in vivo. **(*Circ Res*. 2004;95:814-821.)**

Key Words: transgenic mice ■ adenosine ■ ecto-5′-nucleotidase ■ vascular inflammation ■ thrombosis

CD73/ecto-5′-nucleotidase, a 70-kDa glycosylphosphatidylinositol (GPI)-anchored cell surface molecule, is expressed on the vascular endothelium and catalyzes the extracellular conversion of 5′-AMP to adenosine.[1,2] CD73 is the final step of the extracellular nucleotide breakdown cascade that also involves membrane-associated CD39/ATP-diphosphohydrolase.[3] The product of CD73 is adenosine, a purine nucleoside that has been implicated in many physiological and pathophysiological events.[4]

There are four known G-coupled adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3, each of which operates via different intracellular signaling mechanisms and exhibits distinct patterns of tissue distribution.[5] In human neutrophils, adenosine A_1 and A_2 receptor occupancy mediate opposing roles for adenosine in inflammation: A_1 activation is proinflammatory, whereas the A_2 receptor plays an anti-inflammatory role.[6] A_2 receptor activation inhibits the neutrophil oxidative burst, whereas the A_3 receptor inhibits neutrophil degranulation[7] and may play an important role in inflammation by inhibiting eosinophil migration.[8] Recently, deletion of the A_{2A} receptor in transgenic mice revealed that this receptor is critical for the limitation and termination of tissue-specific and systemic inflammatory responses.[9] Similar to neutrophils, platelet A_{2A} receptors are well known to inhibit ADP-induced aggregation[5,10] via a cAMP-mediated process; however, whether and under which conditions this is functionally relevant in vivo has not been explored.

The quantitatively most important source of adenosine in most organs under well-oxygenated conditions is 5′-AMP

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hydrolyzed by cytosolic 5′-nucleotidase N-I.[11,12] It was estimated that the coronary endothelium only contributes ≈5% to total cardiac adenosine production[11] so that >90% of the overall production occurs intracellularly. Thus, it is unclear whether the in vivo production of adenosine by extracellular adenine nucleotide breakdown involving CD73 in a final step is quantitatively sufficient in vivo to mediate the antiaggregatory and anti-inflammatory actions demonstrated previously in pharmacological experiments.

Theoretically, changes in the level of extracellular adenosine caused by changes in substrate supply to CD73 may represent an essential mechanism for thromboregulation and inflammation in the vasculature. However, there are other potentially redundant properties of the vascular endothelium that influence the activity of platelets and neutrophils[13] such as NO and prostaglandins, which may compete with CD73-derived adenosine. Therefore, to verify the importance of CD73 in vascular control, we generated mice deficient in CD73 and characterized their hemodynamic, thromboregulatory and inflammatory phenotype.

Materials and Methods

Generation of Mutant Mice

A mouse 129Sv genomic library in a vector Lambda DASH II was constructed in our laboratory and screened using as a probe 1.8 kb *Eco*RI fragment of rat *cd73* gene spanning exon 2.[14] A detailed description of the construction of targeting vector and generation of mutant mice is available in the online data supplement at http://circres.ahajournals.org.

Mice

Mice were bred at the Tierversuchsanlage of the Heinrich-Heine-Universität in Düsseldorf, Germany. Animal experiments were performed in accordance with the national guidelines on animal care. Mice 2 to 3 months of age were used for this study. Animals received care in accordance with the European Convention on Animal Care, and the protocol was approved by the Bezirksregierung Düsseldorf.

RT-PCR Analysis of Mutant Mice

Total RNA was isolated from mouse tissues of the various genotypes using RNeasy Mini Kits (Qiagen). Changes in *cd73* cDNA were identified by PCR using primer 5 (5′-CCATCTGGTTCA-CCGTTTAC-3′), designed from the sequence of exon 1, and primer 6 (5′-GGGCGATGATCTTATTCACAT-3′), from the sequence of exon 3 of the *cd73* gene.

Preparation of Antibodies and Western Blot Analysis

Anti-rat liver ecto-5′-nucleotidase antibody was raised in rabbits by direct injection of the encoding cDNA ligated into the plasmid pcDNA3.[14] Western blot analysis was performed under reducing conditions using the enhanced chemiluminescence system (Amersham Biosciences).

Biochemical Analysis of Ecto-5′-Nucleotidase Activity

Ecto-5′-nucleotidase was extracted from hearts and livers of knockout and wild-type (WT) mice and purified using concanavalin A (ConA)-Sepharose (Sigma) as described previously.[15] Activity of 5′-nucleotidase was determined by analysis of phosphate with 500 μmol/L AMP (Roche Applied Science) as a substrate (pH 7.4).[16] To block alkaline phosphatase (AP) activity, 5 mmol/L levamisole (Sigma) was added.

Enzyme Histochemistry

For localization of ecto-5′-nucleotidase activity, frozen sections and a lead phosphate method (pH 7.4) were applied as described previously.[14] A detailed description is provided in the online data supplement.

Langendorff Heart

Preparation of murine hearts and retrograde perfusion at constant pressure of 100 mm Hg were performed as described.[17] For quantification of AMP hydrolysis in vivo, 50 μmol/L 1,N^6-ethenoadenosine 5′-monophosphate (e-AMP) was infused, and the coronary venous effluent was analyzed on the basis of a protocol published previously.[18]

Cardiac Function In Vivo

Hemodynamic parameters were analyzed as described previously.[17] In brief, mice were anesthetized with urethane, kept at 37°C, and after preparation of the left common carotid artery, a Millar 1.4 French pressure-volume catheter was advanced into the left ventricle.

Tail Bleeding and Time to Occlusion

Tail bleeding times were measured in anesthetized mice (urethane 1.5 g/kg IP) after resection of 0.5 cm of the tail tip. Tails were submerged in 37°C warm saline, and the time to cessation of blood flow was recorded. In a separate set of experiments, the carotid artery model for arterial thrombus formation was used.[19] A vascular lesion was induced by covering the carotid artery with a 7.5% solution of $FeCl_3$ and the cessation of blood flow was measured by an ultrasound flow probe (Transsonics).

Platelet cAMP/cGMP

Fresh citrated blood was obtained by puncturing the inferior vena cava. Blood was immediately resuspended with the unspecific phosphodiesterase inhibitor isopbutylmethylxanthine (IBMX; 0.5 mmol/L). Platelet-rich plasma (300 μL) was deproteinized by addition of 60 μL 25% $HClO_4$ and neutralized with 75 μL 2 mol/L K_2CO_3. cAMP and cGMP were determined by radioimmunoassay.

Intravital Microscopy

Mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg; Ketalar, Parke-Davis), xylazine (0.05 mg/kg), and atropine (0.1 mg/kg; Elkins-Sinn). Animals were kept at 37°C throughout the experiment with a heating pad. The cremaster muscle was externalized over a Plexiglas observation platform and pinned in place as described previously.[20]

Ex Vivo Perfusion of Murine Carotid Arteries

Carotid arteries from *cd73*$^{-/-}$ mice and WT mice were isolated for ex vivo perfusion as described.[21,22] As an established model, monocytic Mono Mac 6 cells (10^6/mL) labeled with calcein-acetoxymethyl (Molecular Probes) were perfused at 7 μL per minute, and adhesive interactions with the vessel wall (arrest, rolling) were recorded using stroboscopic epifluorescence illumination (Drelloscop 250; Drello). Firmly adherent monocytes were counted after 10 minutes of perfusion.

Results

The *cd73* gene of the mouse was disrupted by homologous recombination and activation of the Cre-loxP system (Figure 1). WT, heterozygous, and homozygous offspring from interbreeding heterozygote mice followed approximately Mendelian frequencies of 0.22: 0.54: 0.23 (n=175). There were no abnormalities in development and reproductive capacity in *cd73*$^{-/-}$ mice.

Southern blot analysis of the genomic structures of mice of all three *cd73* genotypes of the F2 offspring revealed that the

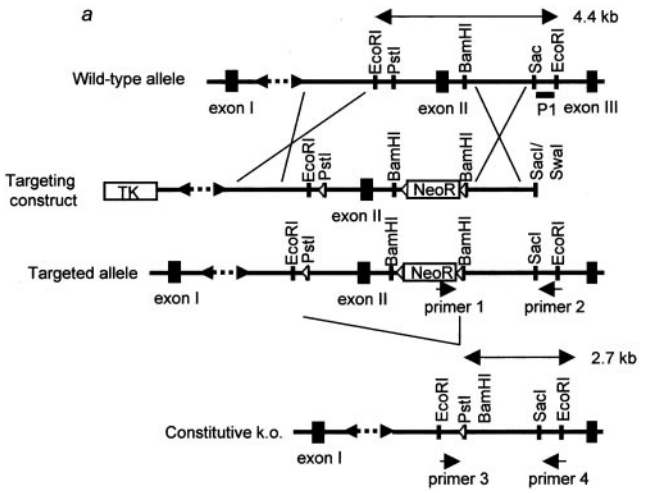

Figure 1. Construction of *cd73*-deficient mice. a, Targeting strategy for *cd73* gene: homologous recombination of the targeting vector and generation of the *cd73* knockout by activation of the Cre-loxP system. Thin vertical lines indicate restriction sites; small open triangles, loxP sites; TK, thymidine kinase; Neo, neomycin resistance gene; thick horizontal bar, probe P1 (used for Southern blot analysis); double-sided arrow, fragments detected with P1 probe, sizes indicated to the right; small arrowheads, PCR primers used for the confirmation of homologous recombination or constitutive knockout; b, Southern blot analysis with the P1 probe to confirm activation of the Cre-loxP system and generation of *cd73* knockout. P1 hybridized with 4.4 kb and 2.7 kb *Eco*RI fragments from the WT or knocked out alleles, respectively.

cd73 locus was properly targeted (Figure 1). RT-PCR analysis of the CD73 transcripts showed that deletion of exon 2 resulted in a shortened mRNA expression at similar levels as the WT transcript (Figure 2a). Because of a frame shift at the new exon 1 to 3 junction, translation terminates after the first exon, which leads to the loss of most the amino acid sequence, including the active site of the protein and its C-terminal domain needed for GPI–anchor linkage to the membrane.

Consistently, Western blot analysis demonstrated the absence of protein within ConA-purified protein fractions derived from membrane fractions of heart and liver tissue (Figure 2b). The biochemical activity of AMP hydrolysis as a consequence of gene deletion was tested in the ConA-purified protein fractions and showed a >50-fold decrease in the liver and 10-fold decrease in the heart of *cd73*$^{-/-}$ mice compared with their WT littermates (Figure 2c). The small residual activity within the heart of *cd73*$^{-/-}$ mice was further reduced to background activity in the presence of levamisole, an inhibitor of AP[23] (Figure 2c).

Activity of CD73 at the cellular level was identified using histochemical methods. In WT animals, a strong reaction product for AMP hydrolysis was identified in the endothelium of the carotid (Figure 3a, left) and coronary artery (Figure 3a, right). In *cd73*$^{-/-}$ mice, the enzyme reaction was completely abolished. The same result was obtained for the aorta and the femoral artery and vein (data not shown). As has been described earlier,[24] CD73 was only partially associated with the microcirculatory bed of the myocardium. In WT mice, strong AMP hydrolysis was associated with myocardial arterioles and capillaries (Figure 3b, top left). In *cd73*$^{-/-}$ mice, AMP hydrolysis was reduced but still associated with microvasculature (Figure 3b, top right), which is attributable to the presence of the nonspecific form of AP. Accordingly, in the presence of levamisole, enzyme histochemical staining was reduced but not eliminated in WT mice (Figure 3b, bottom left). In *cd73*$^{-/-}$ mice, levamisole abolished microvascular AMPase activity (Figure 3b, bottom right). In addition, myocardial background staining was eliminated in *cd73*$^{-/-}$ mice in the presence of levamisole.

Intensified staining using the azo dye method and alkaline pH (8.7) revealed that only part of the myocardial capillaries expresses AP. This is clearly demonstrated using double labeling for the endothelial marker IB4 (Figure 3c, left), which often reveals discontinuation of AP activity on the identical microvessel. In addition, double histochemical staining for CD73, pH 7.4, and AP (pH 8.7) in WT animals revealed a separate expression of the two enzymes in the microvasculature of the heart muscle (Figure 3c, right). In few cases, a transition of CD73 to AP-positive microvessels could be observed (data not shown).

In vivo analysis of contractile and hemodynamic parameters in mice using a conductance catheter that was advanced through the carotid artery to the heart revealed no significant differences in systolic blood pressure, ejection fraction, or cardiac output between WT and *cd73*$^{-/-}$ mice (Table). Also,

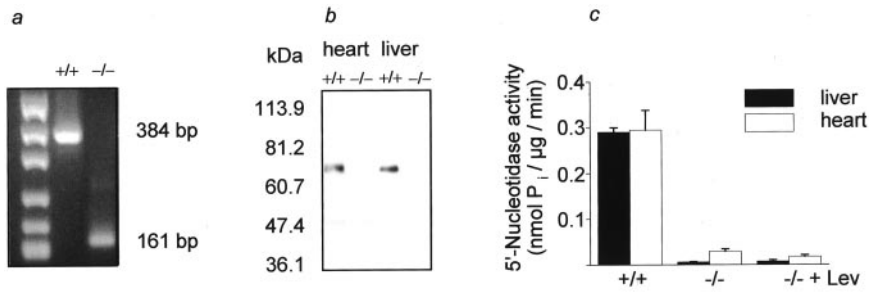

Figure 2. Analysis of mouse *cd73* at transcriptional, translational, and activity level. a, RT-PCR analysis of mouse kidney tissue using different primers generating a 384-bp mRNA product for WT allele and a smaller fragment with 161 bp for the knockout allele. b, Western blot analysis for CD73 of membrane proteins isolated by affinity binding to ConA. c, AMPase activity in membrane fractions from liver (two animals each) and heart (eight animals each) in the absence and presence of levamisole (Lev; 5 mmol/L) an inhibitor of AP (triplicate determinations).

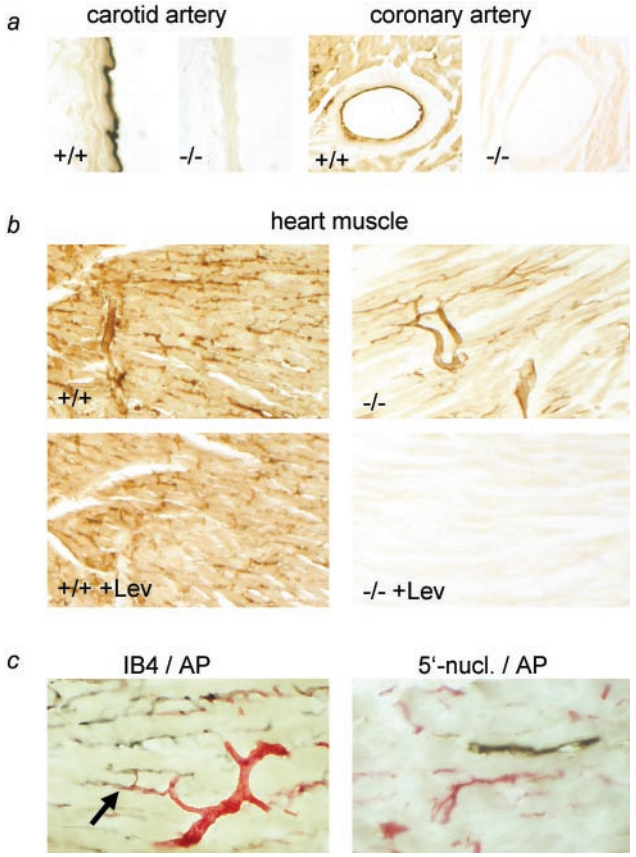

Figure 3. Histochemical analysis of mouse CD73. a, AMP hydrolysis in large conduit vessels. In WT animals, strong reaction product for AMP hydrolysis is identified in the endothelium of the carotid (left) and coronary arteries (right). In *cd73*$^{-/-}$ mice, the enzyme reaction is completely abolished. b, AMP hydrolysis in the micorcirculation. In WT mice, strong AMP hydrolysis is associated with myocardial arterioles and capillaries (top left). In *cd73*$^{-/-}$ mice, AMP hydrolysis is reduced but still associated with microvessles (top right). Accordingly, in the presence of levamisole (Lev), enzyme histochemical staining is reduced but not eliminated in WT mice (bottom left). In *cd73*$^{-/-}$ mice, levamisole abolishes microvascular AMPase activity (bottom right). In addition, myocardial background staining is eliminated in *cd73*$^{-/-}$ mice in the presence of levamisole. c, Expression pattern of AP and CD73 in the microcirculation. Only part of the myocardial capillaries express AP. This is demonstrated using double labeling for the endothelial marker IB4 (left; black), which often reveals discontinuation of AP activity (red) on the identical microvessel (arrow). In addition, double histochemical staining for CD73 (pH 7.4; black) and AP (pH 8.7; red) in WT animals revealed a separate expression of the two enzymes in the microvasculature of the heart muscle (right).

animal weight and heart-to-body weight index showed no differences (data not shown). However, we found basal coronary flow in the isolated perfused heart to be significantly lower (−14%) in the mutant compared with the WT (Table).

Hemodynamic Parameter of WT and *cd73* Mutants

Parameter	*cd73*$^{+/+}$	*cd73*$^{-/-}$
Systolic blood pressure (mm Hg)	94.00 ± 6.00	96.00 ± 7.00
Heart rate (bpm)	615 ± 48	584 ± 44
Stroke volume (μL)	17.30 ± 4.90	15.60 ± 3.00
Coronary flow (mL · min^{-1} · g^{-1})	12.96 ± 1.69	11.16 ± 1.72*

Values are mean±SD (*P<0.05; n=12).

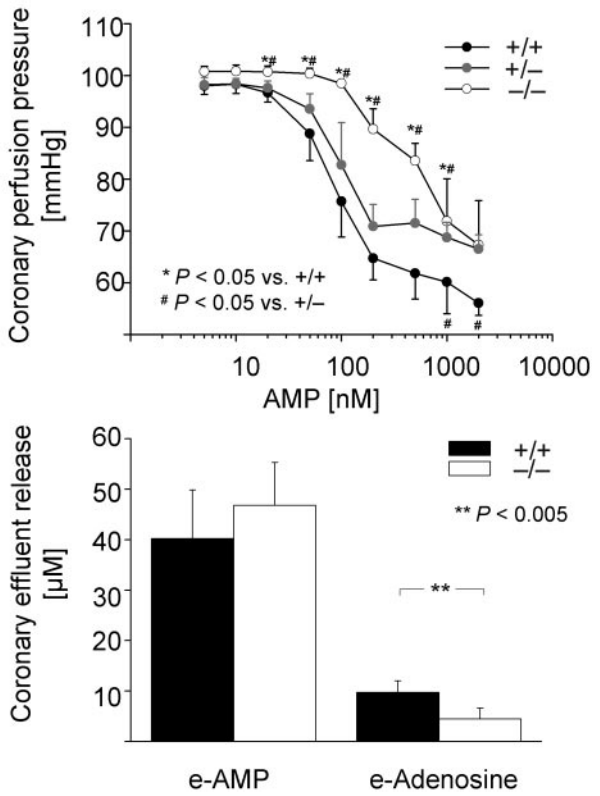

Figure 4. Metabolism of AMP in isolated perfused hearts of *cd73*$^{-/-}$ mice and its effect on coronary perfusion pressure. a, Dose-response curve for AMP (5 nM to 2 μmol/L) is shifted to the right. b, Breakdown of e-AMP to 1,N^6-ethenoadenosine (e-adenosine) after intracoronary infusion of 50 μmol/L. Symbols show means±SD for n=6 hearts.

The reactive hyperemic flow response after 30 seconds of coronary flow occlusion was not different between WT and *cd73*$^{-/-}$ mice (data not shown), indicating a predominant role of factors other than extracellularly formed adenosine. Further functional analysis of the isolated perfused heart revealed that the dose-response curve for adenosine-applied intracoronary (1 to 200 μmol/L) revealed no differences between WT and *cd73*$^{-/-}$ mice (data not shown), suggesting unaltered A_{2A} receptor density/coupling. However, the dose-response curve for AMP was shifted to the right by about one order of magnitude, demonstrating that the dilatory effect of AMP requires hydrolysis to vasoactive adenosine (Figure 4). This result could be confirmed when WT hearts were perfused with 50 μmol/L α,β-methylene ADP, an inhibitor of CD73, leading to a decreased AMP response. Consistent with this finding, perfusion of hearts with fluorescent etheno-AMP resulted in hydrolysis of 24% of this precursor to etheno-adenosine in the WT during single passage through the heart, whereas the respective value in *cd73*$^{-/-}$ mice was only 9% (Figure 4). The residual AMPase activity in *cd73*$^{-/-}$ mice is most likely attributable to AP.

Peripheral blood platelet counts in *cd73*$^{-/-}$ mice (449±95×10^3/μL) were similar to those in WT controls (413±47×10^3/μL). Further evaluation of other relevant he-

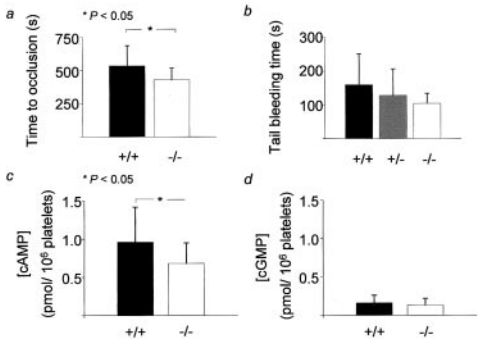

Figure 5. Altered thromboregulation in *cd73*$^{-/-}$ mice. a, The time needed to form an occlusive thrombus within the carotid artery was determined in 13 control and 19 *cd73*-deficient animals after topical application of $FeCl_3$. b, Tail bleeding time as estimated after tail tip resection (n=15 animals for each phenotype). c and d, Measurement of cAMP and cGMP in unstressed mice in venous blood collected with IBMX-stopping solution (n=12 to 14 animals).

matological parameters revealed no abnormalities in mutant mice.

To evaluate the role of CD73 in platelet thrombosis formation in vivo, carotid arteries of WT and *cd73*-deficient mice were subjected to free radical injury using $FeCl_3$. In this model, time to vessel occlusion as measured with a sensitive flow probe was significantly shorter (−20%) in *cd73*$^{-/-}$ mice (Figure 5a). Bleeding time after tail tip resection was reduced

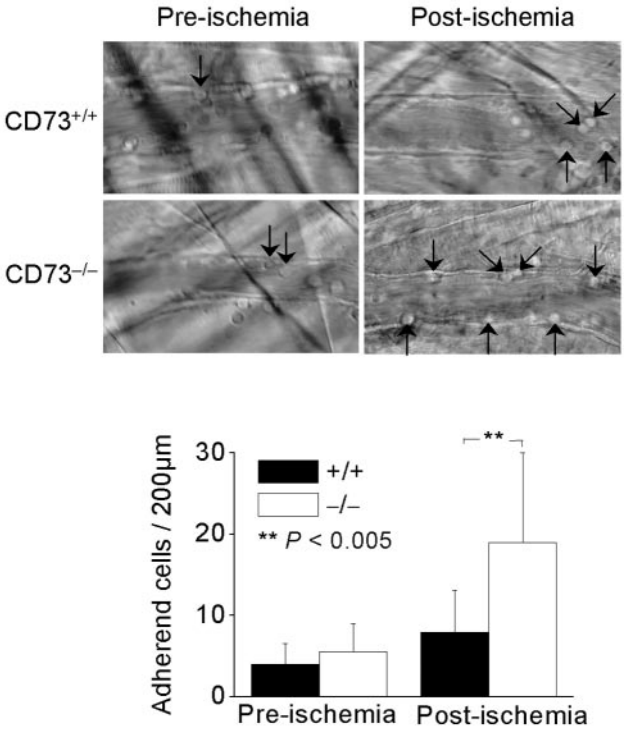

Figure 6. Leukocyte adhesion before and after ischemia. a, Arrows indicate adherent leukocytes under normal conditions and after ischemia–reperfusion within venules of the cremaster muscle in WT and *cd73* mutant mice. b, Under nonischemic conditions, a tendency of increased cell adhesion is observed in *cd73*$^{-/-}$ mice. In cases of ischemia–reperfusion, leukocyte adhesion is augmented 2 to 3× in mutants. Symbols show means±SD for at least 15 vessels per group.

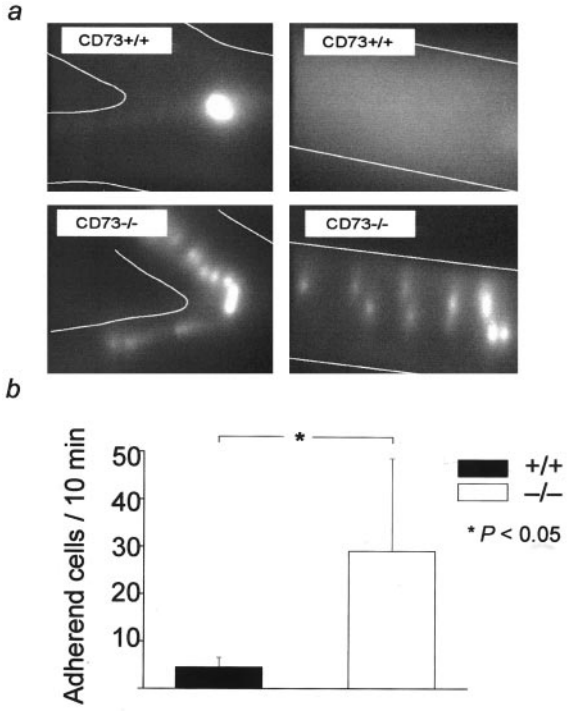

Figure 7. Monocyte arrest in carotid arteries perfused ex vivo. a, Representative images of fluorescently labeled monocytic Mono Mac 6 cells firmly adherent in carotid arteries of WT and *cd73*$^{-/-}$ mice perfused ex vivo. The bifurcation area (left) and a more proximal segment (right) of the common carotid artery are shown. b, Quantification after 10 minutes of perfusion confirms an increase in firm monocyte adhesion in carotid arteries of *cd73*$^{-/-}$ mice compared with WT mice. Data are given as mean±SD (n=4 arteries per group).

by 35% in *cd73*$^{-/-}$ mice but did not reach the level of significance because of the large SE associated with this method (Figure 5b). Platelet cAMP but not cGMP was significantly lower in *cd73*$^{-/-}$ mice (Figure 5c), suggesting that under in vivo conditions, the concentration of plasma adenosine was reduced in *cd73*$^{-/-}$ mice. We also were unable to detect CD73 on platelets by flow cytometry using mutant platelets as negative controls. Under in vitro conditions, platelet aggregation in response to ADP (dose range 0.05 to 10 μmol/L) was undistinguishable between platelets isolated from *cd73*$^{-/-}$ mice and WT littermates (data not shown). This is not surprising given the turnover of cellular cAMP and the short half life of plasma adenosine.[25]

To determine the role of endogenous adenosine produced through the CD73 pathway in the vascular inflammatory response, mouse cremaster muscles were subjected to ischemia–reperfusion injury as described.[26] As shown in Figure 6, ischemia (30 minutes) followed by reperfusion significantly increased the number of adherent leukocytes in *cd73*$^{-/-}$ mice. In WT littermates, ischemia–reperfusion increased the number of adherent leukocytes by 98%, whereas in *cd73* mutants, the respective increase was 245% ($P<0.005$). Even under basal unstressed conditions, the number of adherent leukocytes tended to be higher in *cd73*$^{-/-}$ mice compared with that in WT but did not reach the level of significance. Maximal increase in adherent leukocytes occurred between 10 and 15 minutes after the onset of reperfusion. There was no change

of blood flow in microvessels of *cd73*$^{-/-}$ versus control mice. Average centerline velocity was 2.1±0.8 mm per second in *cd73*$^{+/+}$ mice and 1.9±0.9 mm per second in *cd73*$^{-/-}$ mutants (mean±SD).

Because a strong activity of CD73 was found in carotid and coronary arteries, the ex vivo perfusion of carotid arteries was performed to further elaborate on a constitutive role of CD73 in anti-inflammatory vasoprotection. Whereas the background adhesion of monocytes perfused in carotid arteries of WT mice was negligible, perfusion of monocytes in carotid arteries of *cd73*$^{-/-}$ mice resulted in a remarkable (> 6-fold) and significant ($P<0.05$) increase in accumulation after 10 minutes of perfusion (Figure 7). In contrast to monocyte accumulation on early atherosclerotic endothelium in carotid arteries of *apoE*$^{-/-}$ mice, where arrest occurs preferentially at the lesion-prone predeliction site of the bifurcation, enhanced arrest in *cd73*$^{-/-}$ was also seen in other segments of the perfused artery.[21] Rolling interactions of monocytes analyzed over 10 minutes were not significantly altered in *cd73*$^{-/-}$ arteries (data not shown).

Discussion

In the present study, we obtained convincing evidence for successful gene targeting and generation of *cd73*-deficient mice by Southern blot analysis (Figure 1b), RT-PCR (Figure 2a), and Western blot analysis (Figure 2b) of selected tissues known to exhibit RNA transcripts and ecto-5′-nucleotidase activity (Figure 2c). We deleted exon 2 encoding the "Phosphoesterase Signature Motive," a sequence pattern involved in phosphoester bond cleavage conserved in a wide variety of phosphoesterases.[27] Use of the Cre-loxP system involving 3 loxP sites (Figure 1) enabled us to generate, in one experiment, animals containing constitutive and conditional knockout of the *cd73* gene, the latter being important in future studies on tissue-specific *cd73* deletion.[28] Our approach also enabled the effective removal of a selectable marker cassette, thereby eliminating the possibility of influencing the expression of the neighboring genes, which can lead to unexpected phenotypes in "knockout" mice.[29]

AMP-hydrolyzing activity of cell membranes comprises CD73 and AP activity, the former being a high-affinity enzyme and the latter being of low affinity.[30] Thus, ecto-5′-nucleotidase is expected to be more efficient than AP at producing adenosine at physiological AMP and pH. CD73 is a prominent enzyme in the heart and liver under V_{max} conditions (Figure 2c), whereas in the isolated perfused heart, AMP hydrolyzing activity attributable to CD73 amounts to ≈60% of total hydrolytic activity during single passage through the coronary vasculature (Figure 4b). Histochemical data revealed CD73 to be the predominant AMPase associated with the vascular endothelium of large conduit vessels such as the aorta, carotid, and coronary artery with no measurable AP. However, at the coronary microcirculation, there is substantial local heterogeneity (Figure 3c).

Adenosine is a primordial signaling molecule that has evolved to modulate a multitude of physiological responses in all mammalian tissues.[5] Pharmacological activation of A_{2A} receptors through Gs activation causes vasodilatation of coronary arteries and produces a constellation of responses that can be classified as anti-inflammatory.[31] The present study is the first to demonstrate that adenosine generated by the extracellular hydrolysis of adenine nucleotides by action of CD73 is an important endogenous pathway to modulate the inflammatory vascular response. After a brief period of ischemia–reperfusion, we observed significantly increased leukocyte adherence to the vascular endothelium only in the *cd73* mutant (Figure 6). Thus, under physiological conditions with unperturbed extracellular hydrolysis of AMP, adenosine generated by this pathway appears to be quantitatively sufficient to keep circulating leukocytes in a nonactivated state.

Modulation of leukocyte adhesion by CD73 generating adenosine under physiological conditions was corroborated by findings that monocyte adhesion was constitutively increased in carotid arteries of *cd73*$^{-/-}$ mice, whereas arteries of WT mice displaying strong CD73 activity supported only little arrest. The absence of paracrine effects by adenosine on both endothelial cells and monocytes may thus account for the mechanism of enhanced monocyte accumulation in *cd73*$^{-/-}$ mice. The adhesion of monocytes on endothelium in carotid arteries of *apoE*$^{-/-}$ mice has been shown to depend on interactions of $\alpha 4\beta 1$ integrin (very-late antigen-4 [VLA-4]) with vascular cell adhesion molecule (VCAM)-1 and is triggered by the murine interleukin-8 (IL-8) ortholog keratinocyte-derived chemokine.[21,22] Conversely, adenosine has been shown to dose-dependently reduce the expression of VCAM-1 and the arrest chemokine IL-8 by activated endothelial cells,[32] providing one possible explanation for a suppressive interference with this crucial axis by endogenous adenosine. Furthermore, neutrophil–endothelial cell adhesion can be attenuated by elevating intracellular cAMP. This effect can be attributed to inhibition of $\alpha M\beta 2$ integrin upregulation by formyl-methionyl-leucyl-phenylalanine. Prevention by adenosine deaminase implicates endogenous adenosine as a comodulator of inhibition.[33] In neutrophils, adenosine binding to A_{2A} receptors has been shown to counteract the stimulation of VLA-4 expression and binding to VCAM-1 by chemoattractants via a cAMP-mediated pathway.[34] This may not only explain the increase in leukocyte attachment observed after ischemia—reperfusion; because VLA-4 is the primary adhesive receptor in monocytes, it is conceivable that chemokine-triggered VLA-4 adhesiveness mediating monocyte arrest in arteries may also be affected by endothelial-derived adenosine. The generation of adenosine by CD73 may thus provide a general vasoprotection against leukocyte adhesion.

Blood platelets maintain vascular integrity and promote primary and secondary hemostasis after interruption of vessel continuity. They undergo profound shape changes in the presence of ADP mediated by cell surface P2Y receptors. The proaggregatory action of ADP is sensitively inhibited by adenosine and mediated by antiaggregatory A_{2A} receptors.[5] It has been postulated for quite some time that adenosine formed at the endothelial platelet interface may limit thrombus formation.[35,36] Our study provides experimental evidence that this hypothesis is valid. We found that the time to carotid occlusion after $FeCl_3$ application was reduced by 20% in *cd73* mutants (Figure 5). However, we found no differences in the aggregatory response of platelets to ADP in vitro, which is

consistent with no measurable CD73 activity on mouse platelets and the extremely short plasma half life of adenosine.[25] The decreased platelet cAMP combined with unchanged cGMP in *cd73* mutants is most likely a consequence of a lower in vivo adenosine level in the immediate milieu of the vascular endothelium.

For the extracellular cascade from ATP to adenosine to be functionally relevant during thromboregulation and inflammation, an intact endothelium capable of releasing adenine nucleotides on external stimuli is required. Cellular ATP release may involve transport through ATP-binding cassette proteins, vesicular release,[37] and connexin hemichannels in endothelial cells.[38] Ischemia–reperfusion but not hypoxia was shown by us to release ATP from the coronary endothelium,[39] and more recently, an increased flow was reported to release ATP from endothelial cells in pulmonary circulation.[40] Thus, there appears to be a basal flux of purines through this extracellular nucleotide cascade that can be further augmented by ATP/ADP release from activated platelets and neutrophils under pathophysiological conditions.

It is generally assumed that during hypoxia, the intracellular formation from parenchymal cells dominates and may be responsible for the phenomenon of metabolic coronary vasodilation. Here we show that in the mouse, adenosine formed by the extracellular nucleotide cascade is involved in setting basal coronary vascular tone postulated previously by pharmacological evidence to be adenosine independent.[41] Thus, CD73-derived adenosine, most likely attributable to short diffusion distances, can directly influence vascular smooth muscle tone. How this finding translates to the blood perfused heart in vivo is difficult to ascertain at present. We found blood pressure and cardiac output to be normal in *cd73* mutants, suggesting that in vivo at the given high oxygen extraction rate of the heart, the loss of CD73-derived adenosine may have been compensated by other factors.

Whereas ATP acts on extracellular purinergic P2 receptors, its degradation to adenosine activates P1 receptors. Interruption of the cascade from ATP to adenosine thus converts a P1 to a P2 environment at the cell surface irrespective of the site of blockade. The recently published disruption of the *cd39*/ATP diphosphohydrolase[3] and the *cd73* mutant reported here interrupt the extracellular nucleotide cascade, yet at different stages. However, the formation of adenosine should be equally reduced in both mutants, and given our present findings, changes in adenosine are likely to have importantly contributed to the reported phenotype in the former model. It also should be noted that lack of elimination of nucleotides in the *cd73* mutant may have altered local levels of upstream ADP and ATP, and thereby could have contributed to inflammation.

In summary, the present study provides the first evidence that the terminal step of the extracellular adenine nucleotide cascade on endothelial cells, which converts AMP to adenosine, modulates coronary vascular tone and inhibits platelet function and leukocyte adhesion. This implicates the CD73-mediated pathway as a key innate mechanism to attenuate tissue inflammation.

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References

1. Zimmermann H. 5′-Nucleotidase: molecular structure and functional aspects. *Biochem J*. 1992;285:345–365.
2. Deussen A, Bading B, Kelm M, Schrader J. Formation and salvage of adenosine by macrovascular endothelial cells. *Am J Physiol*. 1993;264: H692–H700.
3. Enjyoji K, Sevigny J, Lin Y, Frenette PS, Christie PD, Esch JS, Imai M, Edelberg JM, Rayburn H, Lech M, Beeler DL, Csizmadia E, Wagner DD, Robson SC, Rosenberg RD. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat Med*. 1999;5:1010–1017.
4. Shryock JC, Belardinelli L. Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am J Cardiol*. 1997;79:2–10.
5. Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol*. 2001;41: 775–787.
6. Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via adenosine A2 receptors. *J Immunol*. 1992;148:2201–2206.
7. Bouma MG, Jeunhomme TM, Boyle DL, Dentener MA, Voitenok NN, van den Wildenberg FA, Buurman WA. Adenosine inhibits neutrophil degranulation in activated human whole blood: involvement of adenosine A2 and A3 receptors. *J Immunol*. 1997;158:5400–5408.
8. Knight D, Zheng X, Rocchini C, Jacobson M, Bai T, Walker B. Adenosine A3 receptor stimulation inhibits migration of human eosinophils. *J Leukoc Biol*. 1997;62:465–468.
9. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature*. 2001;414:916–920.
10. Kawashima Y, Nagasawa T, Ninomiya H. Contribution of ecto-5′-nucleotidase to the inhibition of platelet aggregation by human endothelial cells. *Blood*. 2000;96:2157–2162.
11. Deussen A. Metabolic flux rates of adenosine in the heart. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362:351–363.
12. Decking UK, Schlieper G, Kroll K, Schrader J. Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. *Circ Res*. 1997;81:154–164.
13. Marcus AJ, Safier LB, Broekman MJ, Islam N, Fliessbach JH, Hajjar KA, Kaminski WE, Jendraschak E, Silverstein RL, von Schacky C. Thrombosis and inflammation as multicellular processes: significance of cell-cell interactions. *Thromb Haemost*. 1995;74:213–217.
14. Braun N, Zhu Y, Krieglstein J, Culmsee C, Zimmermann H. Upregulation of the enzyme chain hydrolyzing extracellular ATP after transient forebrain ischemia in the rat. *J Neurosci*. 1998;18:4891–4900.
15. Vogel M, Kowalewski H, Zimmermann H, Hooper NM, Turner AJ. Soluble low-Km 5′-nucleotidase from electric-ray (Torpedo marmorata) electric organ and bovine cerebral cortex is derived from the glycosylphosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. *Biochem J*. 1992;284:621–624.
16. Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem*. 1979;100: 95–97.
17. Gödecke A, Molojavyi A, Heger J, Flögel U, Ding Z, Jacoby C, Schrader J. Myoglobin protects the heart from inducible nitric-oxide synthase (iNOS)-mediated nitrosative stress. *J Biol Chem*. 2003;278:21761–21766.
18. Haink G, Deussen A. Liquid chromatography method for the analysis of adenosine compounds. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2003;784:189–193.
19. Kurz KD, Main BW, Sandusky GE. Rat model of arterial thrombosis induced by ferric chloride. *Thromb Res*. 1990;60:269–280.
20. Baez S. An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc Res*. 1973;5:384–394.

21. Huo Y, Hafezi-Moghadam A, Ley K. Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions. *Circ Res*. 2000;87:153–159.
22. Huo Y, Weber C, Forlow SB, Sperandio M, Thatte J, Mack M, Jung S, Littman DR, Ley K. The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J Clin Invest*. 2001;108:1307–1314.
23. Van Belle H. Alkaline phosphatase. I. Kinetics and inhibition by levamisole of purified isoenzymes from humans. *Clin Chem*. 1976;22: 972–976.
24. Mlodzik K, Loffing J, Le Hir M, Kaissling B. Ecto-5′-nucleotidase is expressed by pericytes and fibroblasts in the rat heart. *Histochem Cell Biol*. 1995;103:227–236.
25. Moser GH, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. *Am J Physiol*. 1989;256:C799–C806.
26. Prorock AJ, Hafezi-Moghadam A, Laubach VE, Liao JK, Ley K. Vascular protection by estrogen in ischemia-reperfusion injury requires endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol*. 2003;284:H133–H140.
27. Koonin EV. Conserved sequence pattern in a wide variety of phosphoesterases. *Protein Sci*. 1994;3:356–358.
28. Meyers EN, Lewandoski M, Martin GR. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat Genet*. 1998;18: 136–141.
29. Pham CT, MacIvor DM, Hug BA, Heusel JW, Ley TJ. Long-range disruption of gene expression by a selectable marker cassette. *Proc Natl Acad Sci U S A*. 1996;93:13090–13095.
30. Picher M, Burch LH, Hirsh AJ, Spychala J, Boucher RC. Ecto-5′-nucleotidase and nonspecific alkaline phosphatase. Two AMP-hydrolyzing ectoenzymes with distinct roles in human airways. *J Biol Chem*. 2003;278:13468–13479.
31. Sullivan GW. Adenosine A2A receptor agonists as anti-inflammatory agents. *Curr Opin Investig Drugs*. 2003;4:1313–1319.
32. Bouma MG, van den Wildenberg FA, Buurman WA. Adenosine inhibits cytokine release and expression of adhesion molecules by activated human endothelial cells. *Am J Physiol*. 1996;270:C522–C529.
33. Derian CK, Santulli RJ, Rao PE, Solomon HF, Barrett JA. Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators. *J Immunol*. 1995;154:308–317.
34. Sullivan GW, Lee DD, Ross WG, DiVietro JA, Lappas CM, Lawrence MB, Linden J. Activation of A2A adenosine receptors inhibits expression of alpha 4/beta 1 integrin (very late antigen-4) on stimulated human neutrophils. *J Leukoc Biol*. 2004;75:127–134.
35. Gerlach E, Nees S, Becker BF. The vascular endothelium: a survey of some newly evolving biochemical and physiological features. *Basic Res Cardiol*. 1985;80:459–474.
36. Gordon EL, Pearson JD, Slakey LL. The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta. Feed-forward inhibition of adenosine production at the cell surface. *J Biol Chem*. 1986;261:15496–15507.
37. Novak I. ATP as a signaling molecule: the exocrine focus. *News Physiol Sci*. 2003;18:12–17.
38. Leybaert L, Braet K, Vandamme W, Cabooter L, Martin PE, Evans WH. Connexin channels, connexin mimetic peptides and ATP release. *Cell Commun Adhes*. 2003;10:251–257.
39. Borst MM, Schrader J. Adenine nucleotide release from isolated perfused guinea pig hearts and extracellular formation of adenosine. *Circ Res*. 1991;68:797–806.
40. Konduri GG, Mattei J. Role of oxidative phosphorylation and ATP release in mediating birth-related pulmonary vasodilation in fetal lambs. *Am J Physiol Heart Circ Physiol*. 2002;283:H1600–H1608.
41. Kroll K, Feigl EO. Adenosine is unimportant in controlling coronary blood flow in unstressed dog hearts. *Am J Physiol*. 1985;249:H1176–H1187.