Adora2b-elicited Per2 stabilization promotes a HIF-dependent metabolic switch crucial for myocardial adaptation to ischemia

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Adenosine signaling has been implicated in cardiac adaptation to limited oxygen availability. In a wide search for adenosine receptor A2b (Adora2b)-elicited cardioadaptive responses, we identified the circadian rhythm protein period 2 (Per2) as an Adora2b target. Adora2b signaling led to Per2 stabilization during myocardial ischemia, and in this setting, Per2−/− mice had larger infarct sizes compared to wild-type mice and loss of the cardioprotection conferred by ischemic preconditioning. Metabolic studies uncovered a limited ability of ischemic hearts in Per2−/− mice to use carbohydrates for oxygen-efficient glycolysis. This impairment was caused by a failure to stabilize hypoxia-inducible factor-1α (Hif-1α). Moreover, stabilization of Per2 in the heart by exposing mice to intense light resulted in the transcriptional induction of glycolytic enzymes and Per2-dependent cardioprotection from ischemia. Together, these studies identify adenosine-elicited stabilization of Per2 in the control of HIF-dependent cardiac metabolism and ischemia tolerance and implicate Per2 stabilization as a potential new strategy for treating myocardial ischemia.

Metabolic adaptation during environmental stress is currently an area of intense investigation because of the potential implications of metabolic alterations in human disease. For example, myocardial ischemia leads to the activation of pathways directed toward enhancing myocardial oxygen efficiency. A metabolic switch from a more ‘energy-efficient’ utilization of fatty acids to a more ‘oxygen-efficient’ utilization of glucose as the main source for energy generation is pivotal for allowing the myocardium to function under ischemic conditions.

Signal by extracellular adenosine has been implicated in cellular adaptation to hypoxia. In the extracellular compartment, adenosine is produced from the phosphohydrolysis of AMP by ecto-5’-nucleotidase (NT5E) and signals through four adenosine receptors (adenosine receptor A1 (ADORA1), ADORA2A, ADORA2B and ADORA3). During conditions of hypoxia, adenosine generation is markedly enhanced, and the activation of adenosine receptors has a key role in counterbalancing the deleterious effects of hypoxia. Particularly during conditions of myocardial ischemia, adenosine signaling events have been implicated in cardioprotection from ischemia. Similarly, cardioprotective responses elicited by ischemic preconditioning are abolished after pharmacological inhibition or genetic ablation of extracellular adenosine production or signaling.

Here we identify the circadian rhythm protein Per2 as a key mediator of Adora2b-elicited cardioprotection by enhancing the glycolytic capacity of the ischemic heart.

RESULTS

The circadian rhythm gene Per2 as an Adora2b target gene

Previous studies have implicated adenosine receptor signaling in myocardial adaptation to ischemia or hypoxia in mice. Here we studied these pathways in cardiac tissues obtained from patients with ischemic heart disease (average ejection fraction (EF%) of the left ventricle, 18.75; Supplementary Tables 1 and 2). Compared to cardiac tissues derived from healthy hearts, we found a selective induction of ADORA2B expression in diseased hearts (Fig. 1a). Together with previous studies in gene-targeted mice, these findings in human patients implicate extracellular adenosine signaling through ADORA2B in cardioprotection from ischemia.

Given the prominent role of adenosine receptor signaling in ischemic preconditioning, we performed microarray studies to compare the transcriptional responses elicited by ischemic preconditioning (Supplementary Fig. 1) in wild-type and Adora2b−/− mice (Fig. 1b, Supplementary Figs. 2–4 and Supplementary Table 3). The gene with highest differential readout was Per2, encoding a circadian rhythm protein, which was exclusively upregulated in wild-type hearts. Per2, a member of the Period family of genes, is expressed in a circadian rhythm pattern in the suprachiasmatic nucleus. In addition to Per2, the microarray studies also showed a similar regulatory pattern for Per1, whereas other circadian rhythm genes were not affected (Supplementary Figs. 3–5 and Supplementary Table 3).

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We found that ischemic conditioning did not induce mRNA expression of the circadian control proteins Clock, Cryptochrome 1 (Cry1) or Timeless, and protein stabilization occurred in an Adora2b-independent manner (Supplementary Fig. 5). As studies in Per1−/− mice did not identify a functional role for Per1 in myocardial ischemia (Supplementary Figs. 6 and 7), we instead focused on Per2. We found that cardiac Per2 mRNA was expressed in a circadian pattern, as measured over a 24-h zeitgeber period (measured according to zeitgeber time, ZT, where ZT0 denotes the time of lights on and ZT12 indicates the time of lights off), and that this pattern was abolished in Adora2b−/− mice. Moreover, the induction of Per2 mRNA and protein expression after ischemic preconditioning was abolished in the hearts from Adora2b−/− mice (Fig. 1c–f, Supplementary Fig. 1 and Supplementary Table 4). Studies in isolated cardiac myocytes exposed to in vitro hypoxic preconditioning (HPC; Supplementary Fig. 8) showed enhanced Adora2b and Per2 mRNA expression in wild-type but not Adora2b−/− myocytes (Fig. 1g). Moreover, we found elevated PER2 mRNA and protein expression in the cardiac

**Figure 1** Consequences of adenosine signaling on Per2 induction. (a) Transcript concentrations of individual adenosine receptors (ADORA1, ADORA2A, ADORA2B and ADORA3) in cardiac tissue from patients with severe ischemic heart disease (IHD) or controls (C). n = 10 patients per condition. Statistical significance was determined by Student’s t test. Data are mean ± s.d. (b) Canonical pathway analysis. A canonical pathway analysis was used to identify the pathways from the IPA (Ingenuity Pathway Analysis) library of canonical (typical) pathways that were most significant to the dataset. (c–f) Hearts from Adora2b−/− and WT littermate controls were analyzed. (c) Per2 mRNA levels in hearts over a period of 24 hours (with ZT0 set as 6 am). n = 6 mice per group. *P < 0.05 by one-way analysis of variance (ANOVA) with Dunnett’s post-hoc test. Data are mean ± s.d. NS, not significant. (d) Per2 mRNA concentrations in hearts subjected to in situ ischemic preconditioning (IP) followed by reperfusion for the indicated time periods. n = 6 mice per group. Statistical significance was calculated by one-way ANOVA followed by Dunnett’s post-hoc test compared to controls (C). Data are mean ± s.d. (e) Per2 protein expression determined by western blot following ischemic preconditioning without reperfusion. WT, wild-type; KO, Adora2b−/−. One representative blot of three is shown. Actb, β-actin. (f) Comparison of the immunoreactivity for Per2 in preconditioned cardiac tissue (IP-WT and IP-Adora2b−/−) or sham-operated control tissue (C-WT and C-Adora2b−/−). One representative image of three for each condition is shown. Scale bar, 100 μm. (g) Adora2b and Per2 mRNA concentrations in isolated adult mouse cardiomyocytes from wild-type or Adora2b−/− mice after in vitro exposure to HPC. n = 4 hearts per group. Statistical significance was calculated by Student’s t test. Data are mean ± s.d. (h) PER2 mRNA (left) or protein (right) concentrations in cardiac tissues from patients with severe ischemic heart disease (IHD) or controls (C). n = 10 patients per condition. Statistical significance was determined by Student’s t test. Data are mean ± s.d. (i) Chromatin immunoprecipitation analysis to detect CREB protein binding to the PER2 promoter using HMEC-1 cells treated with BAY 60-6583 for 20 min. Real-time RT-PCR for human PER2 promoter or satellite DNA (negative control) (top). Products obtained by PCR for the PER2 promoter analyzed on a 1% agarose gel (bottom). Data are mean ± s.d. n = 3. *P < 0.05 by Student’s t test. Ab, antibody; IgG, immunoglobulin G. (j) Luciferase activity measured in transfected HMEC-1 cells with a full-length PER2 promoter construct (FLPER2) or the indicated truncated versions, subcloned into the pGL4 luciferase reporter vector. The pGL4 vector used alone served as a negative control. As a positive control, a pMetLuc reporter vector containing a specific promoter for CREB was used (CREB). The cells were cotransfected with vehicle or BAY 60-6583 or were cotransfected with a dominant negative CREB (Dom neg CREB) construct, as indicated. Schematic diagrams of the truncated versions of the PER2 promoter, as well as the location of putative CREB binding sites, are shown in Supplementary Figure 11. *P < 0.05 by one-way ANOVA with Dunnett’s post-hoc test for luciferase activity over baseline. Data are mean ± s.d. n = 6.
Figure 2 Influence of transcriptional, translational and post-translational mechanisms on PER2 protein expression. (a) Immunoblot analysis of PER2 protein using synchronized HMEC-1 cells treated with vehicle or BAY 60-6583 for the indicated time periods. One of three representative experiments is shown and is quantified below; boxed protein signals indicate the largest difference in PER2 signal strength between controls and cells treated with BAY 60-6583. *P < 0.05 by Student’s t-test. Data are mean ± s.d. n = 3. (b, c) Immunoblot analysis of PER2 protein in synchronized HMEC-1 cells treated with vehicle, BAY 60-6583, or forskolin with or without actinomycin (ACT) (b) or cycloheximide (CMX) (c). Protein signals were quantified using densitometry, and the fold change over control is given below the blots. The effectiveness of ACT and CMX is shown in Supplementary Figure 12a, b. (d) Proposed model of adenosine-dependent alteration on post-translational PER2 protein stability (left). Immunoblot analysis of PER2 protein in synchronized HMEC-1 cells with or without addition of the proteasome inhibitor AM114 (right). One of three representative blot is shown. (e) Protein lysates were obtained from synchronized HMEC-1 cells treated with vehicle or BAY 60-6583 and were immunoprecipitated with PER2 antibody. The presence of ubiquitin (UBC) in the immunoprecipitate was determined by immunoblot analysis (left). Co-IP, coimmunoprecipitation. One representative blot of three is shown. Changes in protein concentration of PER2 (middle) and coimmunoprecipitated UBC (right) after BAY 60-6583 treatment, as determined by densitometry. *P < 0.05 by Student’s t-test. n = 3. (f) Immunoblot analysis for total CUL1 or neddylated CUL1 (CUL1(NEDD8)) in synchronized HMEC-1 cells treated with vehicle or BAY 60-6583. One of three representative blots is shown. (g) Immunoblot analysis for CUL1(NEDD8) in synchronized HMEC-1 cells treated with BAY 60-6583 alone or after pretreatment with the ADORA2B antagonist PSB1115. One of three representative experiments is shown. (h, i) Immunoblot analysis for CUL1(NEDD8) (h) and PER2 (i) in synchronized HMEC-1 cells after siRNA knockdown of CSN5 (siCSN5) or treatment with nonspecific control siRNA (csiR). The cells were treated with vehicle or BAY 60-6583 as indicated. One of three representative experiments is shown. (j) Immunoblot analysis for CUL1(NEDD8) and PER2 in cardiac myocytes isolated from wild-type or Adora2b−/− mice exposed to HPC or control conditions. One representative blot of three independent experiments is shown, n = 1 mouse per experiment.

ADORA2B attenuates proteasomal degradation of PER2

The rapid kinetics of PER2 accumulation after adenosine receptor activation together with previous reports demonstrating post-translational regulation of PER2 expression12 prompted us to investigate post-translational mechanisms of ADORA2B-dependent regulation of PER2. As the difference in PER2 expression between non-treated control and ADORA2B-agonist–treated HMEC-1 cells was at a maximum at 6 h after treatment (Fig. 2a), we examined the influence of the inhibition of transcription by actinomycin or the inhibition of translation with cycloheximide at this time point (Fig. 2b, c and Supplementary Figure 12a). These experiments provided evidence for a combination of transcriptional and post-translational mechanisms in ADORA2B-dependent regulation of PER2 expression: whereas actinomycin or cycloheximide effectively inhibited transcription or translation (Supplementary Figure 12a), respectively, ADORA2B-agonist treatment was still able to enhance PER2 protein stabilization with either treatment. However, the highest level of PER2 protein accumulation was seen with ADORA2B-agonist treatment alone.

We next investigated whether ADORA2B signaling could interfere with proteasomal degradation of PER2 (Fig. 2d). We pretreated HMEC-1 cells with the proteasome inhibitor AM114, which resulted in prominent PER2 stabilization (Fig. 2d). Previous studies have indicated that post-translational degradation of Per2 involves the SCF E3 ubiquitin ligase complex, resulting in polyubiquitination of Per2 and subsequent degradation by the 26S proteasome13. The SCF complex is active only when cullin 1 (CUL1) is covalently modified by the ubiquitin-like protein NEDD8 (ref. 14). Indeed, immunoprecipitation of PER2 and immunoblotting for ubiquitin showed attenuated PER2 ubiquitination after treatment with the ADORA2B agonist BAY 60-6583 (Fig. 2e). Given that ADORA2B signaling has been shown to deneddylate CUL1 (ref. 15), we investigated ADORA2B-dependent alterations of the neddylation status of CUL1. We confirmed that deneddylation of CUL1 was enhanced after treatment with the ADORA2B agonist (Fig. 2f and Supplementary Figure 12c, d). Furthermore, pretreatment with an ADORA2B antagonist (PSB1115) blocked ADORA2B-agonist–dependent cullin deneddylation (Fig. 2g).

Cullin deneddylation is accomplished through interaction with subunits of the COP9 signalosome (for example, CSN5, also known as JAB1)16. We therefore examined whether decreasing CSN5 expression (and, thereby, inhibition of cullin deneddylation) might influence...
ADORA2B-dependent stabilization of PER2. Cullin deneddylated induced by BAY 60-6583 was attenuated in HMEC-1 cells that had been pretreated with CSN5-targeted siRNA (Fig. 2h, Supplementary Fig. 12e,f and Supplementary Table 5). Moreover, cells treated with CSN5-targeted siRNA lost their ability to stabilize PER2 in response to BAY 60-6583 (Fig. 2i). We then tested isolated adult cardiac myocytes from wild-type and Adora2b−/− mice. Exposure to HPC was associated with PER2 stabilization in wild-type but not Adora2b−/− myocytes. Similarly, HPC-induced cullin deneddylated was seen in wild-type but not Adora2b−/− myocytes (Fig. 2j). Taken together, these results indicate that ADORA2B signaling increases PER2 expression by effects on both PER2 transcription and PER2 protein stability.

**Impaired myocardial adaptation to ischemia in Per2−/− mice**

We next studied Per2 gene-targeted mice that have a deletion of the entire PAS-B domain (Supplementary Fig. 13a,b). Per2−/− mice had increased tissue injury after myocardial ischemia and an abolishment of the cardioprotection conferred by ischemic preconditioning (Fig. 3a,b). Moreover, treatment with BAY 60-6583 led to a significant reduction of the size of infarcted tissue and the concentration of troponin I in plasma samples from wild-type but not Per2−/− mice, indicating that Adora2b-dependent cardioprotection is abolished in Per2−/− mice (Fig. 3b). Baseline electron microscopic imaging of the cardiac ultrastructure in Per2−/− mice showed isolated mitochondrial swelling and glycogen accumulation, with no major structural alterations of the myofibrillar apparatus (Fig. 3d). Baseline cardiac glycogen concentrations were elevated (Supplementary Fig. 13b), whereas long-chain fatty acid concentrations were lower, in Per2−/− compared to wild-type mice (Supplementary Fig. 13c). Consistent with these findings, Per2−/− mice had elevated protein concentrations of glycogen synthase 1 (Gys1) (Supplementary Fig. 13d) and carnitine palmitoyltransferase 1 (Cpt) (Supplementary Fig. 13e) compared to wild-type mice. However, baseline cardiac function assessed by echocardiography was unaltered in Per2−/− mice (Supplementary Fig. 13f). Consistent with recent studies of the role of Per2 in fatty acid metabolism, we found that either at baseline or after ischemic preconditioning, Per2−/− mice had decreased amounts of long-chain fatty acids and increased amounts of Cpt1 protein compared to wild-type mice (Fig. 3e). Because these studies indicate that Per2−/− hearts have enhanced fatty acid metabolism at baseline as well as after myocardial ischemia, we used nuclear magnetic resonance (NMR) studies to characterize the metabolic role of Per2. We exposed Per2−/− or wild-type mice to ischemia alone (60 min) or to a protocol in which ischemia was preceded by ischemic preconditioning. Although baseline creatine phosphate concentrations in cardiac tissue were similar in all groups, ischemia-associated creatine phosphate depletion was significantly enhanced in Per2−/− mice compared to wild-type mice, and the conservation of creatine phosphate concentrations by ischemic preconditioning was abolished in Per2−/− mice (Fig. 3f). Parallel measurements of lactate concentrations showed that ischemia-induced increases in the concentration of cardiac lactate were attenuated in Per2−/− mice (Fig. 3g,h), indicating a role for Per2 in the glycolytic utilization of carbohydrates during myocardial ischemia.

**Impaired cardiac glycolysis during ischemia in Per2−/− mice**

Ischemic-preconditioning elicited an induction of glycolytic enzyme mRNA expression in the hearts of wild-type mice that was abolished in Per2−/− mice (Supplementary Fig. 14 and Supplementary Table 6). We used liquid chromatography–tandem mass spectrometry studies to quantify lactate and lactate production during ischemia. Figure 3
after the infusion of 13C-glucose into wild-type and Per2−/− mice to assess glucose metabolism during ischemia or reperfusion. Although we observed no differences between these mice in 13C-glucose uptake at baseline, during ischemia or at reperfusion (Fig. 4a and Supplementary Fig. 15a), a detailed analysis of the glycolytic flux revealed that ischemia elicited an increase of 13C-fructose-1,6-bisphosphate concentrations in wild-type but not Per2−/− mice, indicating that hypoxia-elicted enhancement of glycolysis during ischemia involves Per2 (Fig. 4b and Supplementary Fig. 15b). Similarly, ischemia induced elevations of 13C-pyruvate and 13C-lactate concentrations in wild-type but not Per2−/− mice (Fig. 4c,d and Supplementary Fig. 15c). In addition, whereas ischemia in wild-type mice attenuated the tricarboxylic acid (TCA) cycle flux of 13C-glucose, the TCA cycle flux of 13C-glucose in Per2−/− mice was increased during ischemia (Fig. 4e). Finally, ischemic preconditioning of wild-type mice was associated with an additional reduction in TCA cycle flux, a reduction that was abolished in Per2−/− mice (Fig. 4f and Supplementary Fig. 15d).

Although glycolytic utilization of carbohydrates is a key adaptive mechanism during ischemia18, increased glycolysis during reperfusion is considered detrimental, as it frequently indicates mitochondrial dysfunction19. Indeed, tissue-reperfusion–attenuated glycolysis was associated with a reduced production of 13C-fructose-1,6-bisphosphate, 13C-pyruvate and 13C-lactate in wild-type mice (Fig. 4b–d). Although ischemia alone did not enhance glycolysis in Per2−/− mice, the glycolytic flux in these mice was increased during reperfusion (Fig. 4e,f). In wild-type mice, ischemic preconditioning led to a further reduction of glycolysis after reperfusion compared to ischemia that was not preceded by ischemic preconditioning, consistent with previous results20, and to a restoration of glucose metabolism to a level comparable to that seen under baseline conditions (Fig. 4f and Supplementary Fig. 15b–d). In contrast, Per2−/− mice maintained lactate production after ischemia preceded by ischemic preconditioning, indicating an uncoupling of glycolysis from glucose oxidation, which is a sign of mitochondrial dysfunction21 (Supplementary Fig. 15c). As we did not observe differences in glucose uptake between wild-type and Per2−/− mice during ischemia or reperfusion (Fig. 4a), we next analyzed the effect of metabolic changes on cardiac glycogen concentrations. Ischemia significantly reduced glycogen storage in both wild-type and Per2−/− mice, despite the fact that Per2−/− mice had a higher glycogen concentration at baseline (Fig. 4g and Supplementary Fig. 15e). Whereas reperfusion led to the restoration of glycogen concentrations in wild-type mice, this effect was abolished in Per2−/− mice. Together, these data indicate that Per2−/− mice are severely compromised in effectively using carbohydrates during ischemia or reperfusion (Fig. 4h and Supplementary Table 7).

**Hif-1α as a link to Per2 regulated cardiac metabolism**

Ischemic preconditioning of wild-type mice was associated with a robust induction of glycolytic enzymes that was completely abolished in Per2−/− mice (Supplementary Table 6), prompting us to study the transcriptional mechanisms involved in this response (Fig. 5). Based on the notion that Hif-1α has a key role in the transcriptional control of the glycolytic pathway22, we used a Hif-1α reporter mouse23 to investigate the functional status of Hif-1α. Notably, over the course of a 24-h time period, we found circadian patterns for the concentrations of cardiac Hif-1α protein (Fig. 5a), Hif-1.1 and Hif-1.2 isoform mRNAs, and the glycolytic enzymes pyruvate dehydrogenase kinase, isozyme 1 (Pdk1) and lactate dehydrogenase (Ldh) in these mice (Supplementary Fig. 16). This was similar to the circadian pattern of cardiac Per2 protein observed in Per2 reporter mice (Fig. 5a) and of Per2 transcripts in hearts from wild-type mice (Fig. 1c). In Hif-1α reporter mice in which the Per2 knockout allele had been introduced, Hif-1α cycling was abolished (Fig. 5a). In addition, the stabilization of Hif-1α by ischemic preconditioning seen in wild-type mice was lost in Per2−/− mice (Fig. 5b). Similarly, hypoxic Hif-1α stabilization was abolished in isolated myocytes from Per2−/− mice (Fig. 5d). Ischemic preconditioning of the Hif-1α reporter mouse was associated
with increased reporter activity, which was abolished by genetic deletion of Per2 (Fig. 5e). These data indicate that Hif-1α stabilization by hypoxia or ischemia depends on Per2 function. We next tested whether HIF-1α was reciprocally required for normal regulation of Per2 expression. Mice in which the gene encoding HIF-1α was inductively deleted in their cardiac myocytes (Fig. 5c and Supplementary Fig. 17) retained their ability to stabilize Per2 by ischemic preconditioning (Fig. 5c). Taken together, these data indicate that although Per2 is needed for the hypoxic regulation of Hif-1α, Per2 stabilization is not dependent on Hif-1α in cardiac tissue.

We next assessed the transcriptional regulation of glycolytic enzymes in oxygen-stable HIF-1α-overexpressing HMEC-1 cells with or without siRNA-mediated PER2 knockdown. Oxygen-stable HIF-1α expression or treatment with the ADORA2B agonist BAY 60-6583 led to elevated glycolytic enzyme mRNA expression. Treatment with BAY 60-6583 further enhanced this elevation in oxygen-stable HIF-1α-overexpressing cells, an effect which was abolished by PER2 knockdown (Fig. 5f). Additional studies using coimmunoprecipitation indicated a direct protein–protein interaction between Hif-1α and Per2 in cardiac tissues from wild-type mice after exposure to ischemic preconditioning (Fig. 5g).

As our results show that Adora2b signaling has a crucial role in the transcriptional induction and protein stability of Per2, we next examined Hif-1α concentrations in the hearts of Adora2b−/− mice. Similarly to our findings in Per2−/− mice, we observed lower expression of Hif-1.1 and Hif-1.2 isoform mRNA in Adora2b−/− mice compared to wild-type mice, as well as abolished circadian expression over a 24-h period (Supplementary Figs. 16 and 18). Moreover, ischemia or hypoxia-induced stabilization of Hif-1α was abolished in Adora2b−/− mice (Fig. 5h,i). We also observed a defect in the transcriptional induction of glycolytic enzymes by cardiac ischemic preconditioning in Adora2b−/− mice, similar to that seen in Per2−/− mice (Fig. 5j). Together, these data indicate that Adora2b-dependent control of Per2 has a key role in the hypoxia-elicited induction of the glycolytic machinery during myocardial ischemia.

**Light-induced cardiac Per2 mediates cardioprotection**

We next attempted to achieve enhanced cardiac Per2 stabilization using light exposure22,26. We exposed mice to light with the same intensity as daylight (13,000 lx), which was achieved by using a light box with an ultraviolet filter, over a period of 4 h and assessed their cardiac Per2 protein concentrations (Fig. 6a). We found time-dependent increases in cardiac Per2 concentrations compared to mice maintained at room light (200 lx) over the same time period. The same accumulation of cardiac Per2 as seen with 13,000 lx was normally achieved after a 12-h period at room light (Fig. 6b and Supplementary Fig. 19a). Exposure to illumination with an equivalent intensity to that of
Figure 6 Light-induced stabilization of cardiac Per2 expression provides potent protection from myocardial ischemia. (a) Experimental model for studying light-induced stabilization of cardiac Per2. (b) Immunoblotting for Per2 in the hearts of wild-type mice after exposure to 12 h of darkness and then to indicated time periods of intense light (13,000 lx; daylight) compared to control mice maintained at room light (top). Quantitation of the results by densitometry (below). n = 4 mice per group. *P < 0.05 by Student’s t-test. Data are mean ± s.d. (c) Transcript concentrations of Pfkm, Pgk1, Pk and Pdk1 in the hearts of Per2−/− or WT littermate control mice after 4 h of daylight exposure. n = 4 mice per condition. Statistical significance was calculated by one-way ANOVA with Dunnett’s post-hoc test. Data are mean ± s.d. (d,e) Myocardial injury, as assessed by infarct size (d) or measurement of troponin I plasma concentrations (e), in Per2−/− or WT littermate control mice exposed to light over the indicated time periods followed by exposure to in situ myocardial ischemia-reperfusion, n = 6 mice per group. Statistical significance was calculated by two-factor ANOVA with Bonferroni’s post-hoc test.

DISCUSSION

Myocardial adaptation to conditions of limited oxygen availability involves a metabolic switch toward a more oxygen-efficient utilization of carbohydrates. Our results show that ADORA2B-dependent stabilization of PER2 has a key role in this cardioadaptive response. We found that Per2−/− mice have diminished amounts of cellular energy stores during ischemia and did not generate lactate. Per2−/− mice lacked the capacity to enhance oxygen-efficient glycolysis, ultimately resulting in the depletion of energy-rich phosphates and leading to increased myocardial cell death during ischemia. Together, these studies indicate a previously unrecognized role for Per2 as a master metabolic switch during cardioadaptation to ischemia, driving the utilization of oxygen-efficient carbohydrate-dependent metabolic pathways.

We found that Adora2b signaling increased Per2 expression and protein stability. A previous study showed that Adora2b signaling activates mitogen-activated protein kinases (MAPK) through the cAMP-CREB pathway, which have been implicated in Per2 regulation. Consistent with the notion that Adora2b signaling alters intracellular cAMP responses, recent studies have identified CLOCK protein-independent regulation of Per2 by cAMP-dependent signaling. Other studies have shown that Adora2b signaling regulates the stability of target proteins. For example, study of hypoxic preconditioning of the lungs showed that Adora2b...
signaling has protective and anti-inflammatory effects by regulating the post-translational stability of NF-κB. Our studies show that cardiac Per2 stabilization can be achieved by daylight exposure. Indeed, stabilization of Per2 within the suprachiasmatic nuclei of the hypothalamus involves light-induced Creb phosphorylation. Moreover, previous studies found that the timing of light exposure markedly affects the circadian profiles of Per1 or Per2 protein concentrations in the lungs and hearts of rats. The mechanisms linking central circadian rhythm regulation and peripheral Per2 stabilization are currently under investigation and may involve hormonal pathways or cyclic alterations in 5′-AMP or adenosine concentrations. Indeed, a recent study identified 5′-AMP in the initiation of hypometabolism, similar to a torpor-like state found in hibernating mammals.

It is intriguing to consider the findings of light-dependent stabilization of Per2 and cardioprotection from ischemia in the context of well-documented variations in the frequency of the onset of acute myocardial infarction. Two recent studies found that patients have larger infarct sizes in the early morning hours compared to other times of the day; moreover, myocardial infarct size and left ventricular function after acute myocardial infarction have a circadian dependence on the time of day of the onset of ischemia. However, another study reported a 12-h delay in the oscillation of cardiac Per2 concentrations in humans as compared to mice. In this respect, observations in patients indicating larger infarct sizes in the morning hours are in contrast to our present findings. Notably, the study characterizing the oscillation of cardiac Per2 using human tissue obtained during orthotopic heart transplantation included a high proportion of patients with coronary heart disease and cardiomyopathy compared to healthy controls (patients without cardiac disease who died from head injury). Therefore, it is not clear whether the PER2 expression pattern observed is characteristic of hearts in normal, healthy individuals, and this may help explain the apparent difference in the circadian pattern of PER2 expression between humans and mice. Moreover, epidemiologic studies in humans have indicated that sunlight is the dominant zeitgeber (a signal from the environment that synchronizes the human circadian system) compared to other zeitgebers such as social cues. This would indicate that the oscillation of cardiac Per2 could be very similar in humans as in mice, as both mice and humans are exposed to the same day-night rhythm. However, the present findings in mice cannot simply be extrapolated to the circadian rhythmicity of myocardial ischemia in humans. Additional studies will be necessary to define the expression and circadian rhythmicity of PER2 in the human heart, as well as its functional role in human heart disease.

The key role of anaerobic glycolysis in providing ATP in severe ischemia has been well documented in mice. After low-flow myocardial ischemia, mice deficient in the glucose transporter Glut4 (ref. 41) or in AMP kinase have reduced lactate production, associated with increased tissue injury, and upon reperfusion have diminished regeneration of high-energy phosphate compounds. These findings are in line with our studies showing larger infarct sizes in Per2−/− mice, which did not utilize glycolysis during ischemia or restore glycogen concentrations during reperfusion. Similarly to Glut4-deficient mice, Per2−/− mice showed higher glycogen concentrations at baseline but were unable to sufficiently utilize carbohydrates. Although diminished uptake of exogenous glucose contributes to the phenotype of Glut4-deficient mice, we did not observe any differences in 31C-6-glucose uptake between wild-type and Per2−/− mice. Our results indicate that Hif-1α regulates the Per2-dependent enhancement of glycolytic capacity during ischemia. These findings are consistent with recent studies of myocardial ischemia in mice that are hypomorphic for HIF-1α.

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AUTHOR CONTRIBUTIONS
T.E. designed and supervised the study, wrote the manuscript and did mouse surgery. K.H. did western blots, RT-PCRs and siRNA knockdown studies. S.B. did western blots, coimmunoprecipitation, promoter studies, ELISAs and animal experiments. S.R. did western blots, RT-PCRs, ELISAs and mouse experiments. M.M. did immunohistochemistry and electron microscopy. L.A.W. isolated mouse myocytes and supervised the study. B.D.L. provided human heart samples. J.H., M.M. did immunohistochemistry and electron microscopy. P.M.R., S.P.C. and H.K.E. supervised the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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**ONLINE METHODS**

**Human cardiac tissue.** Patient heart samples were obtained from patients undergoing orthotopic cardiac transplantation. The clinical samples that were screened are listed in **Supplementary Table 1**. The collection and use of the patient samples was approved by the appropriate institutional review boards of each participating institution; in addition, the study received approval from the Colorado Multiple Institutional Review Board (COMIRB). Informed consent from the patients was obtained by the collecting institution (Division of Cardiology, Department of Medicine, University of Colorado Denver, Aurora, Colorado, USA).

**Mice.** For each group of mice, male mice were used only (12–16 weeks of age). Gene-targeted mice or littermate control mice were matched in age, gender and weight. Experimental protocols were approved by the institutional review board at the University of Colorado Denver and were in accordance with the Protection of Animals and the National Institutes of Health guidelines for the use of live animals. Adora2btm1Dgen (Adora2b−/−) mice were generated by Deltagen, and C57BL/6J (wild type), B6.Cg-Per2tm1Brd Tyrc-Brd/J (ref. 11; Per2−/−), B6.129-Hif1atm3Rjo/J (ref. 54; Hif1a−/−) mice possess loxP sites on either side of exon 2 of Hif1α, B6.FVB(129)-Tg[Myh6-cre/Esr1+]1Jmk/J (ref. 55; MerCreMer, tamoxifen-inducible Cre recombinase expressed specifically in cardiomyocytes), B6.12986-Per2tm1H/+ (used as Per2 reporter mice) and FVB.129S6-Per2tm1Jt/J (used as Per2 reporter mice) were obtained from Jackson Laboratories. To obtain mice with cardiac-specific knockout of Hif1α, Hif1α(−/−) mice were crossed with MerCreMer mice. Tissue-specific knockout was achieved using a 5 d treatment of tamoxifen (1 mg per day intraperitoneally).

**Mouse model for cardiac ischemia.** The mouse model for in situ ischemia and ischemic preconditioning of the heart was performed using a hanging weight system.57

**Microarray analysis.** Array data have been deposited at http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE19875). Data were analyzed through the use of IPA (Ingenuity Systems, www.ingenuity.com). Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the dataset. Molecules from the dataset that met the fold change cutoff of 2 and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the dataset and the canonical pathway was measured in two ways: (i) the ratio of the number of molecules from the dataset that map to the pathway divided by the total number of molecules that map to the canonical pathway. (ii) Fisher's exact test was used to calculate a P value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

**Transcriptional analysis.** Total RNA was isolated from human heart tissue or human microvascular endothelial cells (HMEC-1; cells were a gift of Sean P. Colgan, Mucosal Inflammation Program, Denver, CO), and transcript concentrations were determined by real-time RT-PCR (iCycler; Bio-Rad Laboratories Inc.).

**Immunoblotting experiments.** All antibodies used were rabbit polyclonal antibodies if not stated otherwise, and were used to detect the following proteins: Perl (Abcam, ab3443; 1:1,000), Clock (Abcam, ab43106; mouse polyclonal, 1:1,000), Timeless (Abcam, ab84502; 1:500), Cry1 (Abcam, ab104736; 1:1,000), Prkaa1 (Abcam, ab32047; rabbit monoclonal, 1:1,000; Y365), Per2 (Abcam, ab467 and ab46446; 1:2,000), ubiquitin (Cell Signaling, ab3933; 1:1,000), Neddf8 (Abcam, ab38634; 1:1,000), JAB1 (CSN5) (Abcam, ab12323; 1:5,000), Cull1 (Abcam, ab2964; 1:1,000), Gly1 (Abcam, ab2479; 1:1,000), Cpt1 (Alpha Diagnostic, CPT1M11-A; 1:1,000) and Hif-1α (Abcam, ab1; mouse monoclonal; 1:2,000; H1067).

**Isolation of adult cardiomyocytes.** Myocytes from C57BL/6N, Adora2btm1Dgen, B6.Cg-Per2tm1Brd Tyrc-Brd/J or B6.129-Hif1atm3Rjo/J;B6.FVB(129)-Tg[Myh6-cre/Esr1+]1Jmk/J mice were isolated as previously described.59

**In vitro preconditioning.** Cellular preconditioning was performed on adult cardiomyocytes plated on either 6- or 24-well plates following a modified in vivo protocol optimized for cells.15

**Cell culture and treatments.** Human microvascular endothelial cells (HMEC-1) or HMEC-1 cells expressing oxygen-stable HIF-1α26 (a gift of S.P.C.) were cultured as described previously.58

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assays were performed using the ChIP-IT Express Enzymatic Kit from Active Motif (Carlsbad, CA, USA). We used a rabbit monoclonal antibody (Cell Signaling Technology, 9198, 2 μg) to detect phosphorylated CREB (pCREB (Ser133)) and normal rabbit IgG as a control (Cell Signaling Technology, 2729, 2 μg).

**PER2 promoter studies.** Full-length PER2 promoter constructs and truncated versions were subcloned into a pGL4 luciferase reporter vector (Promega). HMEC-1 cells were cotransfected with the pGL4 construct expressing firefly luciferase and with a pRL-TK plasmid expressing Renilla luciferase (Promega). To measure promoter activity, the activity of firefly luciferase was normalized to Renilla luciferase activity. To control for CREB activity from the time of transcription until the start of treatment, the cells were cotransfected with a CREB dominant-negative vector (pCMV-KCREB Vector; 631925) from Clontech. KCREB acts as a dominant repressor by forming an inactive dimer with CREB, blocking its ability to bind cAMP-regulated enhancer elements (CRE). As positive control, the Luciferase Reporter Assay Kit Ready-To-Glow Secreted Luciferase System from Clontech, which includes a pMetLuc Reporter Vector containing a specific promoter for CRE, was used.

**COP9 or PER2 suppression using RNA interference.** HMEC-1 cells were either grown on inserts or in 60-mm petri dishes. SMARTpool siRNAs targeting CSN5 or PER2 were synthesized by Dharmacon (Lafayette, CO, USA). The siRNA sequences used are listed in **Supplementary Table 5** (refs. 9,58).

**Electron microscopy.** The samples were imaged with an FEI Tecnai G2 Spirit BioTwin TEM (Hillsboro, OR) at an operating voltage of 120 kV.

**Glycogen and long-chain fatty acid measurements.** Glycogen and long-chain fatty acid concentrations were determined using the Glycogen Assay Kit and the Free Fatty Acid Quantification Kit from Biovision.

**Echocardiography.** Mice were anesthetized with 2% isoflurane and cardiac function was assessed by two-dimensional transthoracic echocardiography using a Visual Sonics Vevo 770 high-resolution ultrasound imager equipped with a 35-MHz transducer. Heart rates were maintained above 300 beats per min throughout.60

**Pyruvate kinase and LDH activity.** Tissues were homogenized and enzyme activity was determined using a Pyruvate Kinase Assay Kit and an LDH Assay kit from Biovision.

**NMR analysis on cell and tissue extracts.** All 1H-NMR spectra were obtained with a Bruker 500-MHz DRX NMR spectrometer using an inverse Bruker 5-mm TXI probe.

**Determination of 13C-glucose and 13C metabolites using liquid chromatography–tandem mass spectrometry (UPLC-MS).** Isotopically labeled 13C-glucose (U-13C6-glucose) was purchased from Cambridge Isotope Labs. All UPLC-MS data were acquired with a Waters Acquity UPLC system coupled to a Water Synapt HDMS quadrupole time-of-flight mass spectrometer. Experimental details are given in the Supplementary Methods.

**Coimmunoprecipitation studies.** Coimmunoprecipitation studies were performed using the Thermo Scientific Pierce Coimmunoprecipitation (Co-IP) Kit.

**Data analyses.** Data were compared by two-factor ANOVA with Bonferroni’s post-test or by Student's t test, where appropriate. Values are expressed as
mean ± s.d. from 3–8 mice per condition. For the analysis of changes in the transcripts, a one-way ANOVA was carried out, and multiple comparisons between the control and treatment groups were made using the Dunnett post test. Data are expressed as mean ± s.d. \( P < 0.05 \) was considered statistically significant. All numerical data are presented as mean ± s.d. from the replicate experiments. An unpaired \( t \) test and/or one-way ANOVA test were used to determine the differences between groups. The significance level was set at \( P < 0.05 \) for all tests. For all statistical analyses, GraphPad Prism 5.0 software for Windows XP was used.

Additional methods. Detailed methodology is described in the Supplementary Methods.

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