Background—In the heart with acute myocardial infarction, production of prostaglandin (PG) E₂ increases significantly. In addition, several subtypes of PGE₂ receptors (EPs) have been reported to be expressed in the heart. The role of PGE₂ in cardiac ischemia-reperfusion (I/R) injury, however, remains unknown. We intended to clarify the role of PGE₂ via EP₄, an EP subtype, in I/R injury using mice lacking EP₄ (EP₄⁻/⁻ mice). 

Methods and Results—In murine cardiac ventricle, competitive reverse transcription–polymerase chain reaction revealed the highest expression level of EP₂ mRNA among EP mRNA. EP₂⁻/⁻ mice had larger infarct size than wild-type mice in a model of I/R; the left anterior descending coronary artery was occluded for 1 hour, followed by 24 hours of reperfusion. In addition, isolated EP₂⁻/⁻ hearts perfused according to the Langendorff technique had greater functional and biochemical derangements in response to I/R than wild-type hearts. In vitro, AE1-329, an EP₄ agonist, raised cAMP concentration remarkably in noncardiomyocytes, whereas the action was weak in cardiomyocytes. When 4819-CD, another EP₄ agonist, was administered 1 hour before coronary occlusion, it reduced infarct size significantly in wild-type mice. Notably, a similar cardioprotective effect was observed even when it was administered 50 minutes after coronary occlusion.

Conclusions—Both endogenous PGE₂ and an exogenous EP₄ agonist protect the heart from I/R injury via EP₄. The potent cardioprotective effects of 4819-CD suggest that the compound would be useful for treatment of acute myocardial infarction. (Circulation. 2004;109:2462-2468.)

Key Words: prostaglandins ■ ischemia ■ reperfusion ■ myocardial infarction

Acute myocardial infarction (AMI) is still a leading cause of death in developed countries, and it is usually caused by acute thrombotic occlusion of the coronary artery that has become atherosclerotic. The underlying pathophysiology of AMI is ischemia-reperfusion (I/R) injury of the heart. During cardiac I/R, a variety of mediators, such as adenosine, endothelin, and nitric oxide, are produced in the heart and have all been suggested as modulating the course of cardiac I/R injury. Recently, cyclooxygenase (COX)-2, a rate-limiting enzyme for the synthesis of prostaglandins (PGs), has been reported to be induced in the heart during I/R. This result is consistent with the fact that production of PGE₂ in the heart increases significantly during ischemia, suggesting that it has an important role in cardiac I/R injury. The contribution of endogenous PGE₂ to cardiac I/R injury, however, remains unknown.

PGE₂ exerts its action through the specific receptor subtypes encoded by different genes called EPs: EP₁, EP₂, EP₃, and EP₄, all of which belong to the family of G-protein–coupled receptors but show different signaling. EP₁ couples to G₄ and raises intracellular Ca²⁺ concentration. EP₂ and EP₃ couple to Gₛ and raise intracellular cAMP concentration ([cAMP]ᵢ). In contrast, EP₄ couples to G₁ and inhibits the increase in [cAMP]ᵢ. When examined with Northern blot analysis, the heart shows expression of several kinds of EPs. Although the expression levels of the mRNAs for each EP vary among the species studied, a high expression level of the EP₄ mRNA has been reported in the hearts of several species, including humans. There have been no reports, however, on the role of the EP₄-mediated signaling in the heart.

To clarify the role of PGE₂ via EP₄ in cardiac I/R injury, we used mice lacking EP₄ (EP₄⁻/⁻ mice) and also used recently developed EP₄-specific agonists. We found that endogenous PGE₂ had a cardioprotective effect against I/R injury via EP₄ both in vivo and in vitro. Furthermore, we demonstrated that an exogenous EP₄ agonist protected the heart from I/R injury in vivo.
Methods

Mice
Generation and maintenance of EP4−/− mice has been reported.11 Most EP4−/− mice die postnatally as a result of patent ductus arteriosus and do not survive at all in the C57BL/6 background. Therefore, F2 progenies of surviving EP4−/− mice and their wild-type littermates were maintained independently in a mixed genetic background of 129/Ola and C57BL/6. All experiments, which were approved by the Asahikawa Medical College Committee on Animal Research, were performed in 10- to 16-week-old male mice.

Reverse Transcription–Polymerase Chain Reaction
To determine the expression levels of EP mRNAs, we prepared total RNA from the cardiac ventricles of adult mice and from the cultured fetal noncardiomyocytes using Isogen (Nippon Gene) and performed reverse transcription–polymerase chain reaction (RT-PCR) and competitive RT-PCR as reported.14 We also prepared total RNA from the cardiomyocytes, which were isolated from the ventricles of adult mice according to the reported method.15

Murine Model of AMI
We used a murine model of AMI to examine the role of both endogenous PGE2 and an exogenous EP4 agonist in cardiac I/R injury in vivo, in which the left anterior descending coronary artery (LAD) was occluded for 1 hour, followed by 24 hours of reperfusion, as reported.16 When examining the effect of an exogenously applied agonist on cardiac I/R injury, we injected 4819-CD (300 μg/kg SC) subcutaneously 1 hour before or 50 minutes after occlusion of the LAD. We determined infarct size, size of area at risk (AAR), and left ventricular size (LVS) as reported.16

Heart Perfusion Model
We examined the role of endogenous PGE2 in cardiac I/R injury in vitro by use of a heart perfusion model as reported.16 In short, we perfused excised hearts according to the Langendorff technique. After a stabilization period, the hearts were subjected to 40 minutes of global ischemia followed by 40 minutes of reperfusion. The developed and diastolic tensions, creatine kinase (CK) release, and coronary flow rate were monitored during the I/R period.

Cell Cultures
We performed cultures of cardiomyocytes and noncardiomyocytes as reported,17 with minor modifications. In short, ventricles from 18- to 20-day fetal mice were treated with a solution containing collagenase. The released cells were harvested and filtered through a nylon mesh, then the cells were preplated into a dish to separate the cardiomyocytes from the noncardiomyocytes. Unattached cardiomyocytes were harvested, resuspended in a culture medium, and plated for the examinations of cAMP accumulation. Attached noncardiomyocytes were grown to near confluence and used for a similar examination.

Measurements of cAMP Accumulation
The cardiomyocytes were stimulated with AE1-329 or vehicle for 30 minutes at 37°C in DME/F-12 medium containing ITS-X (Invitrogen), 10 μmol/L indomethacin, and 1 mmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich). The noncardiomyocytes were stimulated similarly in DME/F-12 medium containing 10 μmol/L indomethacin and 1 mmol/L 3-isobutyl-1-methylxanthine. The intracellular cAMP contents were measured as reported.14 We determined the protein contents of the cells by use of a BCA protein assay kit (Pierce Chemical).

Measurement of Blood Pressure and Heart Rate
Blood pressure and heart rate of conscious mice were measured by a tail-cuff method (BP-98A, Softron).

Results
Expression of mRNAs for the EPs in the Cardiac Ventricle
We first examined which subtypes of the EPs were expressed in the cardiac ventricle of adult wild-type mice by RT-PCR, because the cardiac ventricle is the main site of cardiac I/R injury. We found expression of the mRNAs for EP1, EP2, and EP3, but not for EP4 (Figure 1A). The expression levels of the mRNAs determined by competitive RT-PCR were 54.3 ± 7.4, 13.2 ± 2.3, and 1.3 ± 0.5 copies/ng total RNA for EP1, EP2, and EP3, respectively, suggesting that EP1 is most abundantly expressed among the EPs (Figure 1B). The expression levels of EP4 mRNA in cardiomyocytes and noncardiomyocytes were found to be 19.1 ± 3.9 and 62.6 ± 18.5 copies/ng total RNA, respectively, indicating high expression levels of the EP4 mRNA in both cardiomyocytes and noncardiomyocytes. On the basis of these results, we performed the following experiments to determine whether PGE2 protects the heart from I/R injury via EP4.

Endogenous PGE2 Protects the Heart From I/R Injury In Vivo
We examined the role of endogenous PGE2 in cardiac I/R injury using a murine model of AMI. There were no signif-
PGE2 protects the heart from I/R injury via EP4 in vivo. There are significant differences in basal heart weight and morphology between wild-type and EP\textsubscript{4}\textsuperscript{-/-} mice (data not shown). Representative photographs showing the transverse sections of wild-type and EP\textsubscript{4}\textsuperscript{-/-} hearts after I/R are shown in Figure 2A.

In an EP\textsubscript{4}\textsuperscript{-/-} heart, the white necrotic area was markedly enlarged compared with that in a wild-type heart. Summary data for the ratios of infarct size versus size of AAR and AAR as a percentage of LVS, respectively, showing that relative increase in infarct size in EP\textsubscript{4}\textsuperscript{-/-} hearts was 37% greater than that of wild-type mice. In wild-type mice, diastolic tension then declined quickly during reperfusion and reached a level of 125% of the preischemic value. In EP\textsubscript{4}\textsuperscript{-/-} hearts, however, recovery of diastolic tension was significantly delayed compared with that of wild-type hearts (Figure 3B). These results indicate that there was a greater degree of functional damage to the myocardium in EP\textsubscript{4}\textsuperscript{-/-} hearts compared with wild-type hearts. In accordance with this result, release of CK from the heart increased significantly during the reperfusion period in EP\textsubscript{4}\textsuperscript{-/-} hearts compared with wild-type hearts (Figure 3C), suggesting that endogenous PGE\textsubscript{2} had a protective effect on the cell damage induced by I/R. Within 5 minutes from the start of reperfusion, coronary flow rate was significantly higher than the baseline rate in both groups, showing that there was reactive vasodilation during the early period of reperfusion. There was no significant difference, however, in the coronary flow rate between wild-type and EP\textsubscript{4}\textsuperscript{-/-} hearts during the reperfusion period (Figure 3D), suggesting that the cardioprotective effects of PGE\textsubscript{2} via EP\textsubscript{4} are independent of its vasodilatory action.

**AE1-329 Increases [cAMP]\textsubscript{i} in Both Cardiomyocytes and Noncardiomyocytes**

To clarify whether PGE\textsubscript{2} acts on cardiomyocytes and/or noncardiomyocytes via EP\textsubscript{4}, we examined the effect of AE1-329, a recently developed agonist specific for EP\textsubscript{4}, on [cAMP]\textsubscript{i} in these cells. In noncardiomyocytes, AE1-329 at 10 \(\mu\)mol/L induced a slight but significant
increase in [cAMP], although it did not increase [cAMP] in cardiomyocytes from EP4/h11002 hearts (Figure 4B). These results show the possibility that both cardiomyocytes and noncardiomyocytes are the target cells of PGE2 via EP4.

An EP4 Agonist Shows Cardioprotective Action in I/R Injury In Vivo

Finally, we investigated whether the activation of EP4 by a specific agonist could attenuate cardiac I/R injury in vivo. To activate EP4, we used 4819-CD, a novel agonist for EP4, which has been developed for in vivo use as a kind of prodrug and liberates an active metabolite in the circulation. We injected 4819-CD (300 μg/kg SC) 1 hour before occlusion of the LAD. In wild-type mice, the EP4 agonist reduced the infarct size significantly (42% reduction compared with those in the absence of the agonist), whereas it did not reduce the infarct size in EP4/h11002 mice, indicating that the cardioprotective effect of 4819-CD was mediated by EP4 (Figure 5).

Moreover, when injected 50 minutes after occlusion of the LAD, 4819-CD showed a cardioprotective effect almost identical to that seen when injected before the occlusion of the LAD, indicating the therapeutic potency of the drug. At the dose used, 4819-CD did not affect blood pressure or heart rate in wild-type mice (Table), indicating that the cardioprotective effect of 4819-CD was independent of its effect on hemodynamics.

Discussion

Recent studies have shown upregulation of COX-2 and increased PGE2 synthesis in the heart during I/R, suggesting that PGE2 plays some role in cardiac I/R injury. To elucidate this role, we first examined which subtypes of the PGE2 receptors are expressed. We detected mRNAs for EP2, EP3, and EP4 in adult murine ventricles. Among the EPs, the EP4 mRNA showed by far the highest expression level (Figure 1). This result is in good agreement with previous reports showing the high expression level of EP4 mRNA in the heart from various species, suggesting that EP4 mediates the effect of PGE2 in the heart. The role of PGE2 in I/R injury, however, remains unknown, primarily because of lack of antagonists specific for EP4. Until recently, there were no specific agonists for EP4, but now agonists showing high specificities to EP4 are available. In the present study, to clarify the role of PGE2 in cardiac I/R injury, we used EP4/h11002 mice and these EP4 agonists.

The present study provided direct evidence that EP4 deficiency significantly aggravates cardiac I/R injury in vivo (Figure 2). To the best of our knowledge, this is the first report showing that endogenous PGE2 is able to attenuate cardiac I/R injury and that the effect of PGE2 is mediated by EP4. Because there is significant production of PGE2 during I/R and abundant expression of the EP4 mRNA in the heart, PGE2 should exert its cardioprotective action by acting directly on the cardiac tissue. EP4, however, has a potent vasodilatory action and also an inhibitory effect on the neutrophil function via EP4, both of which might contribute to the EP4-mediated cardioprotective effect of PGE2. Therefore, to examine further whether the EP4-mediated cardioprotective effect of PGE2 depends on the effects on the neutrophils and/or vasculatures, we used Langendorff hearts. In this model, EP4/−/− hearts developed a greater degree of myocar-
Heart rate, bpm

There was no difference in coronary flow rate during I/R between EP₄⁻/⁻ and wild-type hearts (Figure 3D), precluding the EP₄-mediated vasodilatory action of PGE₂ from its cardioprotective action. This result clearly indicates that the cardioprotective action of PGE₂ is independent of its actions on blood constituents and vasculatures, although the possibility of some contribution by these actions to cardioprotection in vivo could not be ruled out by the present study.

The present study also revealed the target cells of PGE₂, AE1-329, an EP₄ agonist, increased [cAMP], in both cardiomyocytes and noncardiomyocytes from wild-type mice but not in those from EP₄⁻/⁻ mice, indicating that PGE₂ acts on these cells via EP₄ (Figure 4). Although it is not known how the rise in [cAMP], in these cells causes the cardioprotection in vivo, several reports suggest possible mechanisms. First, the increase in [cAMP], inhibits secretion of tumor necrosis factor (TNF)-α by inhibiting the activation of nuclear factor-κB and stimulates secretion of interleukin (IL)-10 by stimulating the cAMP-responsive nuclear factors in several types of cells. In agreement with these findings, PGE₂ inhibits the production of TNF-α and stimulates the production of IL-10 via EP₄ in macrophages. TNF-α is known to be produced during cardiac I/R by both cardiomyocytes and noncardiomyocytes and to aggravate cardiac injury. In contrast, endogenous IL-10 has been reported to protect the heart from I/R injury. These results suggest that PGE₂ may protect the heart by regulating the production of these cytokines. Second, when stimulated by PGE₂, the mesenchymal fibroblastic cell, a major cell type among the noncardiomyocytes, releases hepatocyte growth factor (HGF), a factor showing a growth-promoting effect on various types of cells. In addition, endogenous HGF exerts a cardioprotective action against I/R injury. These results suggest that PGE₂ could stimulate the production of HGF in noncardiomyocytes during cardiac I/R and that this action may explain the cardioprotective effect of PGE₂ at least in part. The precise mechanism of the EP₄-mediated signaling leading to cardioprotection, however, remains to be clarified.

The increase in [cAMP], during cardiac I/R, such as caused by stimulation of β₁-adrenergic receptor, is generally thought...
to be deleterious to cardiomyocytes and leads to increases in cardiac work and energy consumption, resulting in severe cardiac injury. Therefore, the present results of the EP4-mediated stimulatory effect of PGE2 on adenylate cyclase in cardiomyocytes apparently disagree with the EP4-mediated cardioprotective action of PGE2. As to the effect of the increase in [cAMP], however, there are several interesting reports suggesting the subcellular compartmentalization of cAMP, leading to its different actions within cardiomyocytes.27 The β1-adrenergic receptor localizes mainly in the noncaveolin domain, and its stimulation increases [cAMP], in a particular fraction of cardiomyocytes, leading to a positive inotropic action.28 In contrast, the β2-adrenergic receptor localizes mainly in the caveolin-rich domain, and its stimulation increases [cAMP], in a soluble fraction of cardiomyocytes.28 The β-agonists, however, do not produce the positive inotropic action.29 These results suggest that the EP4-mediated increase in [cAMP] takes place in the soluble fraction of cardiomyocytes. In support of this suggestion, a previous study has shown that PGE2 does not produce the positive inotropic action, whereas it increased [cAMP], in the soluble fraction of cardiomyocytes.30 Therefore, the result of the present study, which demonstrated the stimulatory effect of PGE2 on adenylate cyclase in cardiomyocytes, may not necessary conflict with its cardioprotective action, although there is no report showing that an increase in [cAMP] in the noncaveolin fraction of cardiomyocytes. Thus, it is noteworthy that 4819-CD showed a potent cardioprotective action against I/R injury when applied exogenously.35,36 These results suggest that EP4 agonists will be useful for treatment of the ischemic heart disease.

Another important finding in the present study was that an exogenously applied agonist for EP4, significantly reduced the infarct size and that this effect was mediated by EP4. Furthermore, it is noteworthy that 4819-CD showed a potent cardioprotective effect not only when administered before occlusion of the LAD but also when administered after prolonged (50 minutes) occlusion of the LAD. According to previous findings, various anti-ischemic drugs, such as β-adrenoceptor antagonists and Ca2+ channel blockers, are effective when given before ischemia but not after.33,34 Therefore, EP4 agonists may be a novel type of anti-ischemic agent retaining effectiveness when given after an ischemic event had already occurred. This result strongly indicates that EP4 agonists are promising candidates as therapeutics for ischemic heart diseases such as AMI.

To the best of our knowledge, this is the first report verifying that the selective activation of an EP subtype can protect the heart from I/R injury. There have been several reports suggesting a cardioprotective effect of EP3 agonists against I/R injury when applied exogenously.35,36 These effects, however, have not been verified as being mediated by the EP4, because of lack of an EP4 antagonist. Taking into consideration that there is abundant expression of mRNA for EP4 compared with that for EP3 in the heart and that there is an apparent cardioprotective role of EP3 against I/R injury as shown in this study, the contribution of EP3 toward cardioprotection in I/R injury should be clarified in future studies.

In conclusion, we have demonstrated that there is significant aggravation of cardiac I/R injury in EP3−/− mice both in vivo and in vitro, providing strong evidence that endogenous PGE2 protects the heart from I/R injury via EP4. Therefore, inhibitors of cyclooxygenase, such as aspirin and indomethacin, may exacerbate ischemic heart disease, even though they have been widely used for the prevention and treatment of inflammation, thrombosis, and colon cancer. Furthermore, an exogenous EP4 agonist at a dose producing no hemodynamic effect showed a potent cardioprotective action against I/R injury in vivo, suggesting that EP4 agonists will be useful for treatment of the ischemic heart disease.

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References

Prostaglandin \( E_2 \) Protects the Heart From Ischemia-Reperfusion Injury via Its Receptor Subtype EP\textsubscript{4}


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