Roles of Prostaglandin I₂ and Thromboxane A₂ in Cardiac Ischemia-Reperfusion Injury

A Study Using Mice Lacking Their Respective Receptors

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Background—Prostaglandin (PG) I₂ and thromboxane (TX) A₂, the most common prostanoids in the cardiovascular system, are produced abundantly during cardiac ischemia/reperfusion (I/R); their roles in I/R injury, however, remain undetermined. We intended to clarify these roles of PGI₂ and TXA₂ using mice lacking the PGI₂ receptor, IP⁻/⁻ mice, or the TXA₂ receptor, TP⁻/⁻ mice.

Methods and Results—The left anterior descending coronary artery was occluded for 1 hour and then reperfused for 24 hours. The size of myocardial infarct in IP⁻/⁻ mice was significantly larger than that in wild-type mice, although the size of the area at risk was similar between the 2 groups of mice. In contrast, there was no such difference between TP⁻/⁻ and wild-type mice. To further determine whether PGI₂ and TXA₂ act directly on the cardiac tissue or indirectly through their action on blood constituents, we perfused excised heart according to the Langendorff technique. The isolated heart was then subjected to global ischemia followed by reperfusion. In IP⁻/⁻ mice, developed tension and coronary flow rate during reperfusion were significantly lower and release of creatine kinase was significantly higher than those in wild-type mice. There were no such differences, however, between TP⁻/⁻ and wild-type mice.

Conclusions—PGI₂, which was produced endogenously during cardiac I/R, exerts a protective effect on cardiomyocytes independent of its effects on platelets and neutrophils. In contrast, TXA₂ has little role in the cardiac I/R injury. (Circulation. 2001;104:2210-2215.)

Key Words: prostaglandins • thromboxane • ischemia • reperfusion • myocardium

Prostaglandin (PG) I₂ and thromboxane (TX) A₂ are major prostanoids in the cardiovascular system.¹ Their opposite actions on vasculature and platelets are well known, and their balance is critical in various vascular occlusive diseases, including coronary heart disease.² Their roles in cardiac ischemia/reperfusion (I/R) injury, however, have been largely unknown. In the vascular system, PGI₂ and TXA₂ are produced mainly by the vascular endothelial cells and platelets, respectively.¹ They have also been found to be produced by cardiomyocytes, suggesting some role in the heart. Moreover, their synthesis is significantly increased during cardiac I/R,³⁻⁴ and their respective rhodopsin-type receptors, IP and TP, were reported to be expressed on cardiomyocytes.⁵⁻⁶

PGI₂ and its analogues have been reported to attenuate cardiac I/R injury when used exogenously in vivo.⁷⁻⁸ Their mechanisms of protection were proposed to be a result of their inhibitory effects on platelets⁹ and neutrophils² and ill-defined membrane-stabilizing effects. Several investigators have also examined their effects on isolated and perfused hearts, with somewhat conflicting results.¹⁰⁻¹³ Moreover, to date, there has been no antagonist for IP, which has hindered evaluation of the roles of endogenous PGI₂.

TX synthase inhibitors and/or TP antagonists have been reported to reduce myocardial infarct size in animal studies in vivo.¹⁴⁻¹⁶ The beneficial effects of TX synthase inhibitors on cardiac I/R injury were attributed to enhanced generation of PGI₂ derived from increased availability of its precursor, resulting in inhibition of neutrophil adhesion to endothelial cells.¹¹⁻¹³ The cardioprotective effects of TP antagonists, however, are variable, according to the reports,¹⁶⁻¹⁷ and therefore, the role of TXA₂ in I/R injury has not been established.

To define the precise roles of endogenously produced PGI₂ and TXA₂ in cardiac I/R injury, the present study was designed to determine their roles in both in vivo and ex vivo models using IP⁻/⁻ and TP⁻/⁻ mice.

Basic Science Reports

Received December 31, 2000; revision received August 6, 2001; accepted August 15, 2001.

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### Methods

#### Mice

The generation and maintenance of IP⁻/⁻ mice have been reported, and those of TP⁻/⁻ mice will be reported elsewhere. These mice and wild-type control mice have a genetic background similar to that of C57BL/6 mice. All experiments, which were approved by the Asahikawa Medical College Committee on Animal Research, were performed in 10- to 12-week-old male mice.

#### In Vivo Experiment

**Measurement of Blood Pressure and Heart Rate**

Blood pressures and heart rates of conscious mice at a steady state were measured by the tail-cuff method (BP-98A, Softron).

**Animal Model**

The coronary artery was occluded and reperfused according to a reported method. Mice anesthetized with ketamine (100 mg/kg IP) and xylazine (5 mg/kg IP) were maintained under a respirator (model 480-T, Shinano Industry). After a left thoracotomy, the left anterior descending coronary artery (LAD) was occluded along with PE-10 tubing (1 mm in length). Myocardial ischemia was verified by blanching of the left ventricle (LV) and by ischemic change in the ECG. After occlusion for 1 hour, blood flow was restored by removal of the ligature and PE tubing.

**Assessment of Area at Risk and Infarct Size**

After 24 hours of reperfusion, the LAD was reoccluded, and 5% Evans blue dye (0.2 mL) was injected into the LV cavity with a needle, which was kept in place for 2 to 3 minutes. Then the heart was excised and rinsed in saline, and the LV was frozen at −80°C for 30 minutes. The frozen LV was cut transversely into 5 slices. These samples were further stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC). The areas of the ischemic region, infarcted tissue, and LV were measured by digital planimetry using computer software, NIH Image (ver 1.61). The sizes of the ischemic region (area at risk), infarcted tissue, and LV, defined as AAR, infarct size, and LVS, respectively, were determined according to the reported method. The variability of infarct size measurement among the wild-type, IP⁻/⁻, and TP⁻/⁻ mice was small, and SEM values were within 6% of respective mean values from independent experiments (n=10).

#### Ex Vivo Experiment

**Heart Perfusion Model**

Mice were anesthetized with sodium pentobarbital (50 mg/kg IP) after heparin injection (1000 U/kg IP). The excised hearts were perfused according to the Langendorff technique at a constant pressure of 80 cm H₂O with Krebs-Henseleit bicarbonate buffer equilibrated with gas (95% O₂, 5% CO₂). After a stabilization period, the hearts were subjected to 40 or 60 minutes of global ischemia followed by 40 minutes of reperfusion. The tension was monitored through a hook attached to the LV apex under 1 g of preload. The coronary effluent was collected every minute to determine the amount of creatine kinase (CK) and the coronary flow rate. To measure the tissue levels of ATP and creatine phosphate (CrP), the hearts were immediately frozen at the end of reperfusion. In nonischemic control groups, hearts were perfused without global ischemia for the same period as in other groups. During the reperfusion, the heart rate was kept constant at a rate of 500 bpm with an electronic stimulator (3F46, San-Ei Instruments).

**Biochemical Analysis**

The frozen heart, pulverized in a mortar, was used for determination of the tissue contents of ATP and CrP according to a standard enzymatic procedure. The CK activity in the coronary effluent was measured spectrophotometrically according to an enzymatic method with a CK assay kit (No. 4720, Sigma Chemical Co).

### Roles of PGI₂ and TXA₂ in Ischemic Hearts

#### TABLE 1. Basal Values of Heart Rate and Blood Pressure in Wild-Type, IP⁻/⁻, and TP⁻/⁻ Mice

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Heart Rate, bpm</th>
<th>SBP, mm Hg</th>
<th>MBP, mm Hg</th>
<th>DBP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>14</td>
<td>610±16</td>
<td>104±2</td>
<td>83±2</td>
<td>72±3</td>
</tr>
<tr>
<td>IP⁻/⁻</td>
<td>13</td>
<td>571±15</td>
<td>103±2</td>
<td>82±2</td>
<td>71±2</td>
</tr>
<tr>
<td>TP⁻/⁻</td>
<td>13</td>
<td>607±15</td>
<td>101±3</td>
<td>82±2</td>
<td>73±3</td>
</tr>
</tbody>
</table>

Heart rate, systolic blood pressure (SBP), mean blood pressure (MBP), and diastolic blood pressure (DBP) of conscious mice were measured by the tail-cuff method. Values are mean±SEM.

### Statistical Analysis

All values are expressed as mean±SEM. When changes in mechanical function, coronary flow rate, and CK release were to be compared, statistical analysis was performed with a 2-way repeated-measures ANOVA followed by Dunnett's test for multiple comparisons. If a significant difference was obtained, further comparisons at each time point were performed with Dunnett's test. When other data were to be compared, statistical analysis was performed with a 1-way ANOVA followed by Dunnett's test for multiple comparisons. A difference was considered significant at a value of P<0.05.

### Results

#### In Vivo Experiments

Blood pressures and heart rates of mice are summarized in Table 1. There were no significant differences in these parameters among wild-type, IP⁻/⁻, and TP⁻/⁻ mice. This result suggests that PGI₂ and TXA₂ have a minor role in maintaining heart rate and blood pressure under steady-state conditions, although their potent and direct actions on the blood vessels are well known.

Representative photomicrographs showing AAR and infarct area in wild-type, IP⁻/⁻, and TP⁻/⁻ mice after I/R are presented in Figure 1A. In an IP⁻/⁻ mouse, the white necrotic area within the AAR was markedly enlarged compared with that in a wild-type mouse, in which areas stained red and representing living cells were abundant. No difference was apparent between wild-type and TP⁻/⁻ mice. Summary data for infarct size, size of AAR, and LVS are shown in Figure 1B. AAR as a percentage of LVS was similar in the 3 groups, showing no difference in the size of the areas perfused by the LAD. In IP⁻/⁻ mice, the infarct size as a percentage of either AAR or LVS (50.3% and 24.1%, respectively) was significantly increased compared with that in wild-type mice (39.5% and 17.9%, respectively). These values indicate that the infarct size in IP⁻/⁻ mice as a percentage of either AAR or LVS increased 27.3% or 34.6%, respectively, compared with those for wild-type mice. There were no significant differences in infarct size, however, between TP⁻/⁻ and wild-type mice. Mortalities of mice during the I/R period were 26.7% (4/15), 37.5% (6/16), and 23.8% (5/21) in wild-type, IP⁻/⁻, and TP⁻/⁻ mice, respectively.

#### Ex Vivo Experiments

Before ischemia, there were no significant differences in diastolic tension or developed tension among the 3 groups (data not shown). Global ischemia markedly decreased developed tension and caused a gradual rise in diastolic tension in 3 groups of mice to a similar extent. In wild-type mice,
developed tension recovered up to 90% of the preischemic value within 5 minutes from the start of reperfusion after 40 minutes of ischemia and then gradually declined to 60% of the preischemic value and remained fairly constant. In IP\(_{2}/2\) mice, however, recovery of developed tension was significantly suppressed compared with that of the wild-type mice and was 40% of the preischemic value at a steady state, indicating that there was a greater degree of damage to the myocardium (Figure 2A). In contrast, there was no significant difference in developed tension between wild-type and TP\(_{2}/2\) mice (Figure 2A).

Diastolic tension rose during 40 minutes of ischemia and, at the end of ischemia, reached the maximum values of 291±64%, 303±19%, and 265±46% of the respective preischemic values in wild-type, IP\(_{2}/2\), and TP\(_{2}/2\) mice. Diastolic tension declined quickly during reperfusion and reached a level of 115% to 125% of the preischemic value. No significant difference was found in diastolic tension during reperfusion among the 3 groups. Although the reason why there was no difference in diastolic tension between wild-type and TP\(_{2}/2\) mice is unclear, it may indicate that the decrease in developed tension is a more sensitive marker of myocardial damage than the elevation of diastolic tension in I/R injury, at least in this model. In relation to this point, iloprost, an IP agonist, was reported to show a beneficial effect on postischemic function of the stunned myocardium, in which the developed tension is more susceptible to injury than the diastolic tension.20

Figure 2. Developed and diastolic tensions during reperfusion period in wild-type, IP\(_{2}/2\), and TP\(_{2}/2\) mice. Isolated hearts were perfused according to Langendorff technique and subjected to global ischemia for 40 minutes followed by 40 minutes of reperfusion. Values of developed (A) and diastolic (B) tensions are percentage of preischemic values. Points show mean values; error bars show SEM (n=6 to 18). \(*P<0.05\) vs wild-type group.
between wild-type and IP\(^{-/-}\) mice, it was significantly higher thereafter during reperfusion in IP\(^{+/+}\) mice than in wild-type mice, suggesting that endogenous PGI\(_2\) had a protective effect on the damage induced by reperfusion. In contrast, there was no such difference between TP\(^{-/-}\) and wild-type mice.

The tissue level of ATP declined significantly after I/R in wild-type mice (Table 2), showing an alteration in the energy metabolism of the myocardium. In contrast, the tissue level of ATP declined markedly to a level of 35% of the preischemic value, and the diastolic tension rose to a peak level of 439% of the preischemic value, indicating that 65 minutes of ischemia induced a greater injury to the myocardium than that induced by 40 minutes of ischemia. There were no significant differences, however, in coronary flow rates among the 3 groups (Figure 3B). Coronary flow rate was suppressed in IP\(^{-/-}\) mice and augmented in TP\(^{-/-}\) mice, suggesting that severe myocardial damage induced by prolonged ischemia obscured the protective effect of PGI\(_2\). Conversely, significant differences were noted in coronary flow rates among the 3 groups (Figure 3B). Coronary flow rate was suppressed in IP\(^{-/-}\) mice and augmented in TP\(^{-/-}\) mice compared with that in wild-type mice, suggesting significant, although opposite, vascular actions of PGI\(_2\) and TXA\(_2\) after prolonged ischemia.

**Discussion**

The present study provided direct evidence that IP deficiency significantly aggravates cardiac I/R injury in vivo. This result clearly shows that endogenously produced PGI\(_2\) is able to attenuate cardiac I/R injury. PGI\(_2\) and its analogues have been demonstrated to have potent inhibitory effects on platelet activation\(^1\) and on leukocyte adhesion to endothelial cells.\(^{24}\)
Therefore, to further examine whether the augmented I/R injury in IP<sup>−/−</sup> mice is dependent on the loss of effects of PGI<sub>2</sub> on blood cells, excised hearts were perfused according to the Langendorff technique, which is free of influences of hemodynamic factors as well as blood constituents, including blood cells, hormones, coagulation factors, and proinflammatory cytokines. In this ex vivo model, IP<sup>−/−</sup> hearts had a greater I/R injury when assessed functionally and biochemically, suggesting that endogenous PGI<sub>1</sub> could attenuate I/R injury by acting directly on the cardiac tissue and that the action of PGI<sub>2</sub> is independent of its inhibitory effects on blood constituents.

Several mechanisms by which PGI<sub>2</sub> could provide cardioprotection may be considered. Endogenous PGI<sub>2</sub> contributes only to the reactive vasodilatation during reperfusion after 40 minutes of ischemia (Figure 3A), suggesting that the vasodilatory action of PGI<sub>2</sub> may participate little, if any, in the protective action of PGI<sub>2</sub>. Furthermore, the inability of PGI<sub>2</sub> to protect against cardiac I/R injury was observed in experiments with 65 minutes of ischemia, in which the protective effect of PGI<sub>2</sub> was no longer observed despite its apparent vasodilatory action (Figure 3B). Conversely, iloprost, a PGI<sub>2</sub> analogue, has been shown to activate calcium-activated potassium channels (K<sub>Ca</sub> channels), and activation of cardiac K<sub>Ca</sub> channels by 17β-estradiol has been reported to reduce infarct size. These observations suggest that the activation of K<sub>Ca</sub> channels might mediate the protective effects of PGI<sub>2</sub>. Another ion channel present on the sarcolemma, the K<sub>ATP</sub> channel, was reported to be positively regulated by protein kinase A, which is one of the target molecules in IP signaling, suggesting the participation of the activation of the K<sub>ATP</sub> channel as a mechanism in the protective effect of PGI<sub>2</sub>. Furthermore, several interactive protective mechanisms may work in cardiac I/R injury. For example, the cardioprotective effect of inhibitors of nitric oxide synthase was reported to be dependent on the increased production of PGI<sub>2</sub>. The precise mechanisms of the protective effects of PGI<sub>2</sub> on myocardial I/R injury, however, remain to be elucidated.

Another important finding in the present study is that lack of the TP did not significantly alter either the myocardial infarct size in vivo or the degree of I/R injury ex vivo. These results indicate that the inhibition of TP alone does not attenuate I/R injury. TXA<sub>2</sub> is known to be a potent vasoconstrictor. During reperfusion after 40 minutes of ischemia, we could not observe any significant difference in coronary flow rate between wild-type and TP<sup>−/−</sup> mice. However, 65 minutes of ischemia induced a significant increase in coronary flow rate in TP<sup>−/−</sup> mice (Figure 3B). This result is consistent with a previous report indicating that TXA<sub>2</sub> may not be produced during reperfusion if the ischemic period is <60 minutes and that endogenously produced TXA<sub>2</sub> decreases coronary flow after prolonged ischemia. The increase in coronary flow rate found in TP<sup>−/−</sup> mice, however, could not significantly attenuate mechanical or metabolic derangements induced by I/R, suggesting that the increase in coronary flow rate by itself could not protect myocardium from I/R injury. Moreover, the result of an in vivo study, in which platelet activation by TXA<sub>2</sub> would be expected to occur, suggests that platelet aggregation may not affect cardiac I/R injury. This result is consistent with a report suggesting that platelet aggregation plays a rather passive role in the process of infarction. Conversely, there are controversial reports regarding the cardioprotective effect of TP antagonists. Although beneficial effects of TP antagonists have been reported, an absence of their protective effects on cardiac I/R injury has also been reported. This discrepancy may result from the fact that some TP antagonists cross-act on other prostanoid receptors, such as EP<sub>2</sub>, through which PGE<sub>2</sub> was reported to exert protective effects on cardiac I/R injury. Another mechanism, that compensatory cardioprotective actions of PGI<sub>1</sub> may participate in the lack of effects of the TP deficiency, may be considered. This possibility is unlikely, however, because if PGI<sub>2</sub> were to show compensatory cardioprotective effects, the aggregating action of TXA<sub>2</sub> would be clearly detected in TP<sup>−/−</sup> mice as a decrease in the size of the infarct, which, however, was not detected in the present work. The present study clearly demonstrated that thromboxane A<sub>2</sub> has a small role, if any, in cardiac I/R injury. Nevertheless, the actions of TXA<sub>2</sub> on platelets and vasculature should contribute to the pathogenesis of atherosclerosis and/or vasospasm, which are prerequisites for coronary heart disease.

In conclusion, we have demonstrated significant aggravation in cardiac I/R injury in IP<sup>−/−</sup> mice in vivo and ex vivo. These results provide strong evidence that endogenously produced PGI<sub>2</sub> is able to protect the myocardium from I/R injury by acting directly on cardiac tissue.

Acknowledgments
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan and by a Research Grant for Cardiovascular Diseases (11C-1) from the Ministry of Health and Welfare. This work was also supported by grants from the Ono Medical Research Foundation, the Takeda Science Foundation, the Smoking Research Foundation, the Suhara Memorial Foundation, the Mochida Memorial Foundation, and the Sasaki Foundation Research Institute.

References


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_Circulation_. 2001;104:2210-2215
doi: 10.1161/hc4301.098058

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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