Infarct Size–Limiting Effect of Ischemic Preconditioning Is Blunted by Inhibition of 5′-Nucleotidase Activity and Attenuation of Adenosine Release

Masafumi Kitakaze, MD, PhD; Masatsugu Hori, MD, PhD; Toshikazu Morioka, MD; Tetsuo Minamino, MD; Seiji Takashima, MD, PhD; Hiroshi Sato, MD, PhD; Yoshiro Shinozaki; Mitsuaki Chujo, MSc; Hidezo Mori, MD, PhD; Michitoshi Inoue, MD, PhD; Takenobu Kamada, MD, PhD

Background We have previously reported that ischemic preconditioning increases 5′-nucleotidase activity and adenosine release during ischemia and reperfusion. However, its direct cause-and-effect relation has not been proven. To test the idea that the infarct size–limiting effect of ischemic preconditioning is blunted by inhibition of ectosolic 5′-nucleotidase activity, we assessed 5′-nucleotidase activity, adenosine release, and infarct size caused by sustained ischemia with and without an exposure to α,β-methylene adenosine 5′-diphosphate (AOPCP) in the ischemia-preconditioned myocardium.

Methods and Results In 67 open-chest dogs, the left anterior descending coronary artery was cannulated and perfused with an extracorporeal bypass tube from the carotid artery. After hemodynamic stabilization, the coronary artery was occluded four times for 5 minutes separated by 5 minutes of reperfusion (ischemic preconditioning, n=10). After this procedure, the coronary artery was occluded for 90 minutes followed by 6 hours of reperfusion. Infarct size normalized by the risk area was smaller than the control group (n=8, 41.0±2.6% versus 6.8±1.9%), although there were no significant differences in the endomyocardial collateral flow measured at 80 minutes of ischemia (8.5±1.1 versus 9.4±1.0 mL/100 g per minute). Ectosolic and cytosolic 5′-nucleotidase activity and adenosine release were increased during reperfusion in the ischemic preconditioning group compared with the control group, and the activity of ectosolic 5′-nucleotidase was markedly reduced by AOPCP (n=10). AOPCP affected neither adenosine-induced coronary vasodilation nor increases in myocardial oxygen consumption during an intracoronary infusion of isoproterenol (n=10). To test whether the increase in 5′-nucleotidase activity decreases infarct size, we infused AOPCP 10 minutes before the ischemic preconditioning procedure and continued for 60 minutes after the onset of reperfusion (n=8), AOPCP blunted the infarct size–limiting effect (infarct size, 38.8±4.9%). AOPCP without ischemic preconditioning did not increase infarct size (n=9). Furthermore, when AOPCP was infused during the ischemic preconditioning procedure (n=6) or during 60 minutes of reperfusion (n=6), the infarct size–limiting effect was partially blunted (infarct size, 21.3±2.5% and 19.5±2.4%, respectively).

Conclusions Increases in ectosolic 5′-nucleotidase activity and adenosine release are primarily responsible for the infarct size–limiting effect of ischemic preconditioning. Exposures to adenosine during the ischemic preconditioning procedure and enhanced release of adenosine during reperfusion synergistically contribute to the infarct size–limiting effects. (Circulation. 1994;89:1237-1246.)

Key Words • myocardium • ischemia • reperfusion • purine • 5′-AMP

When brief periods of ischemia precede sustained ischemia, infarct size is markedly limited, a phenomenon known as ischemic preconditioning.1-3 The precise mechanisms underlying this phenomenon have been investigated extensively4-9 because identification of the primary mediator of ischemic preconditioning may contribute to the development of the potential treatment of acute myocardial infarction. Indeed, several lines of evidence suggest that beneficial effects of ischemic preconditioning are observed in clinical settings.10,11 Recently, Liu et al12 experimentally demonstrated that an exposure to 8-sulphophenyltheophylline blunts the infarct size–limiting effect of ischemic preconditioning and that brief periods of exposure to adenosine, instead of transient ischemia, mimic ischemic preconditioning. Liu et al12 and Thornton et al13 also showed that adenosine A1-receptor activation is responsible for the infarct size–limiting effect of ischemic preconditioning. Several lines of evidence support this observation.14-18 Furthermore, we have previously reported that ischemic preconditioning increases 5′-nucleotidase activity and enhances adenosine release during the ischemic preconditioning procedure and early reperfusion after sustained ischemia.19,20 Since an intracoronary infusion of adenosine during ischemia and reperfusion is known to limit infarct size,21,22 the augmentation of endogenous adenosine release caused by increased 5′-nucleotidase activity during
the ischemic preconditioning procedure and reperfusion after sustained ischemia may contribute to the infarct size–limiting effect of ischemic preconditioning. However, we have not proven the direct cause-and-effect relation between increased 5'-nucleotidase activity and attenuation of infarct size in ischemic preconditioning.

To test this cause-and-effect relation, we measured adenosine release and infarct size with and without administration of an inhibitor of 5'-nucleotidase, αβ-methylene adenosine 5'-diphosphate (AOPCP). Furthermore, we infused AOPCP during the ischemic preconditioning procedure or during the reperfusion period to discriminate the roles of increased 5'-nucleotidase activity and adenosine release during these two periods.

Methods

Instrumentation

Sixty-seven mongrel dogs weighing 12 to 21 kg were anesthetized with sodium pentobarbital (30 mg/kg IV), intubated, and ventilated with room air mixed with oxygen (100% O2 at 1 to 2 L/min). The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After intravenous administration of heparin (500 units/kg), we cannulated and perfused the left anterior descending (LAD) coronary artery with blood from the left carotid artery through an extracorporeal bypass tube. Coronary blood flow (CBF) in the perfused area was measured using an electromagnetic flow probe attached to the bypass tube, and coronary perfusion pressure (CPP) was monitored at the tip of the coronary artery cannula. A small-caliber (1 mm), short (7 cm) collecting tube was cannulated into a small coronary vein near the center of the perfused area to sample coronary venous blood. Drained venous blood was collected in a reservoir placed at the level of the left atrium and was returned to the jugular vein. Heart rate averaged 137±3 beats per minute during control conditions and did not change during the study with and without an intracoronary infusion of AOPCP. The pH, PO2, and PCO2 in the systemic arterial blood before the protocol were 7.41±0.02, 121±5, and 39.6±2.5 mm Hg, respectively.

Experimental Protocols

Protocol 1: Effects of AOPCP on the Infarct Size–Limiting Effect of Ischemic Preconditioning

Forty-seven dogs were used in this protocol. CPP and CBF were measured continuously. Coronary arterial and venous blood were sampled for blood gas analysis and measurement of plasma adenosine concentration. After hemodynamic stabilization, four cycles of coronary occlusion and subsequent reperfusion for 5 minutes each were performed to precondition the myocardium to sustained ischemia (n=10, ischemic preconditioning [IP] group). In this ischemic preconditioning group, coronary venous blood was sampled before and immediately after each 5 minutes of coronary occlusion. Arterial blood was also sampled at 60- to 90-minute intervals to check the condition of the dogs. After 90 minutes of coronary occlusion, coronary venous blood was sampled after 1, 5, 10, 30, 60, and 120 minutes of reperfusion. As a control, instead of the ischemic preconditioning procedure, after 40 minutes of hemodynamic stabilization, the coronary artery was occluded for 90 minutes and reperfused for 360 minutes (n=8, control group). Coronary arterial and venous blood was sampled at 20-minute intervals during 40 minutes of hemodynamic stabilization and was sampled after 1, 5, 10, 30, 60, and 120 minutes of reperfusion after 90 minutes of ischemia.

In 8 other dogs, 80 μg·kg⁻¹·min⁻¹ AOPCP (Sigma Chemical Co) was administered into the LAD coronary artery 5 minutes before the ischemic preconditioning procedures and was continued for 60 minutes of reperfusion except for the coronary occlusion period (the AOPCP treatment with IP group). In 9 dogs, AOPCP was administered into the LAD coronary artery 40 minutes before ischemia and was continued for 60 minutes of reperfusion except for the coronary occlusion period (the AOPCP treatment group). AOPCP was dissolved with saline and infused into the perfusion line at the rate of 0.33 mL/min.

To discriminate the role of increases in 5'-nucleotidase activity during the ischemic preconditioning procedure (the AOPCP pretreatment with IP group, n=6) or during reperfusion (the AOPCP during reperfusion with IP group, n=6) on the infarct size–limiting effect, we infused AOPCP (80 μg·kg⁻¹·min⁻¹ IC) only during the ischemic preconditioning procedure or only during reperfusion up to 60 minutes in the ischemia-preconditioned dogs.

Arterial and coronary venous blood samplings in these groups were performed at the same timing as in the control and IP groups, respectively.

Protocol 2: Effects of Ischemic Preconditioning on Ectosolic and Cytosolic 5'-Nucleotidase Activity in the Myocardium

Ten dogs were used in protocol 2. Using two groups of 5 dogs each with and without ischemic preconditioning, we measured 5'-nucleotidase activity in the endocardial and epicardial myocardium before sustained ischemia after separation of the myocardium into membrane and cytosolic fractions. Furthermore, we measured 5'-nucleotidase activity of each fraction obtained from myocardium of the control left circumflex (LCx) coronary artery at 5, 40, and 100 minutes of exposure to AOPCP (10⁻⁸ mol/L) and 10 minutes after washout of AOPCP. AOPCP 80 μg·kg⁻¹·min⁻¹ (IC) was approximately comparable to the concentration of 2 to 4·10⁻⁸ mol/L. Myocardial tissue was sampled and frozen with precooled stainless scissors and tongs in liquid nitrogen and immediately stored at −80°C.

Protocol 3: Effects of AOPCP on Adenosine-Induced Coronary Vasodilation and Isoproterenol-Induced Increases in Myocardial Oxygen Consumption

AOPCP inhibits ectosolic 5'-nucleotidase activity; however, AOPCP may affect adenosine receptors because the structure of AOPCP is similar to adenosine: The activation of adenosine A1-receptors inhibits increases in myocardial oxygen consumption caused by β-adrenoceptor stimulation, and the activation of adenosine A2-receptors induces coronary vasodilation. To exclude this possibility, we infused three doses of adenosine (0.5, 1.0, and 2.0 μg·kg⁻¹·min⁻¹ IC) with and without an infusion of AOPCP (80 μg·kg⁻¹·min⁻¹ IC) in 5 dogs. Furthermore, we measured increases in myocardial oxygen consumption caused by isoproterenol exposure (50 and 150 μg·kg⁻¹·min⁻¹ IC) with and without intracoronary infusion of AOPCP in 5 dogs.

Measurements of Infarct Size

After 6 hours of reperfusion in protocol 1, while the LAD was reoccluded and perfused with autologous blood, Evans blue dye was injected into the systemic vein to determine the anatomic risk area and the nonischemic area in the hearts. The heart was removed immediately and sliced into serial transverse sections 6 to 7 mm in width. The nonischemic area was separated as the blue-staining normal area, and the ischemic region was incubated at 37°C for 20 to 30 minutes in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Chemical Co) in 0.1 mol/L phosphate buffer adjusted to pH 7.4. TTC stained the noninfarcted myocardium a brick-red color, indicating the presence of a formazin precipitate that results from the reduction of TTC by dehydrogenase enzymes present in
viable tissues. Infarct size was expressed as a percent of the area at risk.

**Measurements of Regional Coronary Blood Flow**

Regional myocardial blood flow was determined by the microsphere technique as previously reported. In the present study, microspheres labeled with bromine or zirconium were used. Mean diameter was 15 μm, and specific gravity was 1.34 for bromine and 1.36 for zirconium. Microspheres were suspended in isotonic saline with 0.01% Tween 80 to prevent aggregation. The microspheres were ultrasonicated for 5 minutes followed by 5 minutes of vortexing immediately before injection. Approximately 1 mL of the microsphere suspension (2 to 4×10⁶ microspheres) was injected into the left atrium followed by several warm (37°C) saline flushes (5 mL). Microspheres were administered at 80 minutes after the onset of coronary occlusion. Just before microsphere administration, a reference blood flow sample was withdrawn from the femoral artery at a constant rate of 8 mL/min for 2 minutes.

The x-ray fluorescence activity of stable heavy elements was measured by a wavelength dispersive spectrometer (PW 1480, Phillips Co, Ltd). Specification of this x-ray fluorescence spectrometer has been described previously. In brief, when the microspheres are irradiated by the primary x-ray beam, the electrons fall back to a lower orbit and emit measurable energy. This energy level of the x-ray fluorescence is characteristic for each element. Therefore, it is possible to quantify the x-ray fluorescence of several differently labeled microspheres in a mixture. Myocardial blood flow was calculated according to the formula time flow equals tissue counts times reference flow divided by reference counts and was expressed in milliliters per minute per gram of wet weight. We measured endomyocardial blood flow of the inner half of the left ventricular wall.

**Myocardial Metabolism**

The coronary arterial and venous blood oxygen difference (AVO₂D) was determined as the difference between coronary arterial and venous oxygen content. MVO₂ (mL/100 g per minute) was calculated as CBF (mL/100 g per minute) times AVO₂D (mL/dL).

**Adenosine Measurement**

The technique for measuring the plasma adenosine concentration (pmol/mL) has been reported previously. Briefly, 1 mL of blood was drawn into a syringe containing 0.5 mL of dipyridamole (0.02%) and 100 μL of 2'-deoxycoformycin (0.1 mg/mL) with EDTA (500 mmol/L) to block both the uptake of adenosine by red blood cells and degradation of adenosine. After centrifugation, the supernatant was collected and its adenosine content was measured by radioimmunoassay. The plasma adenosine (100 μL) was succinylated with 100 μL of dioxane containing succinic acid anhydride and triethylamine. After a 20-minute incubation, the mixture was diluted with 100 μL of dioxane 2',3'-O-disuccinyl-3-['²²²°]-iodotyrosine methyl ester (0.5 pmol/L) and 100 μL of diluted antidigoxigenin serum. The mixture was kept in a cold water bath (4°C) for 18 hours, and the second antibody solution (goat anti-rabbit IgG antisem, 500 μL) was added. After incubation at 4°C for 1 hour, the unreacted material was removed by centrifugation at 3000 rpm at 4°C for 20 minutes. The radioactivity remaining in the tube was counted using a gamma counter. The amount of adenosine degraded during this blood sampling procedure has been reported to be negligible.

**Myocardial 5'-Nucleotidase Activity**

A biopsy specimen of subendocardial myocardium supplied by the LAD and LCx coronary arteries was obtained before sustained coronary occlusion with and without ischemic preconditioning in protocol 2. The myocardial tissue samples were frozen and stored under liquid nitrogen, and the ectosolic and cytosolic 5'-nucleotidase activity were measured at membrane and cytosolic fractions. The myocardium was separated into its membrane and cytosolic fractions as follows: Myocardial tissue was homogenized using a Potter-Elvehjem homogenizer (30 strokes) for 5 minutes in 10 vol of ice-cold 10 mmol/L N,2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid–potassium hydroxide (HEPES-KOH) buffer (pH 7.4) containing 0.25 mol/L sucrose, 1 mmol/L MgCl₂, and 1 mmol/L mercaptoethanol at 0°C. The crude homogenate was strained through a double-layered nylon sieve and homogenized again for 1 minute. To prepare a crude

**TABLE 1. Sequential Changes in Coronary Hemodynamics During Four Cycles of Ischemic Preconditioning**

<table>
<thead>
<tr>
<th>IP Group (n=10)</th>
<th>AOPCP Treatment With IP Group (n=8)</th>
<th>AOPCP Pretreatment With IP Group (n=6)</th>
<th>AOPCP During Reperfusion With IP Group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, min</td>
<td>Cycle of ischemia</td>
<td>CPP, mm Hg</td>
<td>CBF, mL/100 g per min</td>
</tr>
<tr>
<td>Before 0</td>
<td>1</td>
<td>105±5</td>
<td>90±2</td>
</tr>
<tr>
<td>Reperfusion 5</td>
<td></td>
<td>105±5</td>
<td>307±10</td>
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<tr>
<td>Before 10</td>
<td>2</td>
<td>107±5</td>
<td>102±3</td>
</tr>
<tr>
<td>Reperfusion 15</td>
<td></td>
<td>106±5</td>
<td>252±5</td>
</tr>
<tr>
<td>Before 20</td>
<td>3</td>
<td>105±4</td>
<td>102±3</td>
</tr>
<tr>
<td>Reperfusion 25</td>
<td></td>
<td>103±6</td>
<td>234±6</td>
</tr>
<tr>
<td>Before 30</td>
<td>4</td>
<td>103±6</td>
<td>100±2</td>
</tr>
<tr>
<td>Reperfusion 35</td>
<td></td>
<td>104±4</td>
<td>225±8</td>
</tr>
<tr>
<td>5 Min after</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IP indicates ischemic preconditioning; AOPCP, α,β-methylene adenosine 5'-diphosphate; CPP, coronary perfusion pressure; CBF, coronary blood flow; Before, before the onset of 5 minutes of coronary occlusion; Reperfusion, immediately after the onset of 5 minutes of reperfusion; and 5 Min after, 5 minutes after the end of the fourth occlusion.

*P<.005 and †P<.001 compared with the IP group, using a modified Bonferroni's test. Values are expressed as mean±SEM.
TABLE 2. Sequential Changes in Coronary Hemodynamics During a 40-Minute Steady State Before Ischemia in Control and AOPCP Treatment Groups

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Control Group (n=8)</th>
<th>AOPCP Treatment Group (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPP, mm Hg</td>
<td>CBF, ml/100 g per min</td>
</tr>
<tr>
<td>Baseline</td>
<td>109±6</td>
<td>91±2</td>
</tr>
<tr>
<td>20</td>
<td>108±4</td>
<td>91±2</td>
</tr>
<tr>
<td>40</td>
<td>106±4</td>
<td>91±2</td>
</tr>
</tbody>
</table>

AOPCP indicates α,β-methylene adenosine 5'-diphosphate; CPP, coronary perfusion pressure; and CBF, coronary blood flow.

Statistical significances between the control and AOPCP treatment groups were tested by unpaired t test with an adjusted level of significance according to the modified Bonferroni's method. Values are expressed as mean±SEM.

membrane fraction, part of the homogenate was centrifuged at 1000g for 10 minutes. The resulting pellet was washed three times and resuspended in the HEPES-KOH buffer. To prepare the cytosolic fraction, the remaining part of the homogenate was first centrifuged at 3000g for 10 minutes, and the supernatant was centrifuged at 200 000g for 1 hour. The membrane and cytosolic fractions were dia lyzed at 4°C for 4 hours against 10 mmol/L HEPES-KOH (pH 7.4) containing 1 mmol/L MgCl₂, 1 mmol/L mercaptoethanol, and 0.01% activated charcoal and were divided into aliquots that were frozen immediately and stored at −80°C. In the preliminary study, we examined the recovery of the 5'-nucleotidase activity in the membrane fraction in this procedure and observed that the recovery of cytosolic 5'-nucleotidase is 97±2% (n=5). This recovery is highly reproducible.²⁰

5'-Nucleotidase activity was assessed by the enzymatic assay technique²⁰,²⁸ and is reported as units of nmol/mg protein per minute. Protein concentration was measured by the method of Lowry et al.,²⁹ using bovine serum albumin as standard.

Statistical Analysis

Statistical analyses were performed using paired and unpaired t tests,²⁰ and the significance level was adjusted according to a modified Bonferroni's method. When the data were compared among groups, a modified Bonferroni's test was used to determine significance at the P<.05 level for group pairs that exhibited statistically significant differences.²¹ ANCOVA, by regional collateral flow in the inner-half left ventricular wall as the covariate, was used to account for the effect of collateral blood flow on infarct size. Each value was expressed as the mean±SEM, with P<.05 considered significant.

Results

CPP, CBF, and adenosine concentration during the period of hemodynamic stabilization with and without the ischemic preconditioning procedure were compared (Tables 1 through 4). During 40 minutes of hemodynamic stabilization (Tables 2 and 4), CPP, CBF, nor adenosine concentrations in the coronary arterial and venous blood changed significantly among the groups. During the ischemic preconditioning procedure with and without AOPCP administration (Tables 1 and 3), although CPP was unchanged, CBF was increased during reperfusion after brief periods of ischemia. AOPCP attenuated the extent of the reactive coronary hyperemic flow during reperfusion after brief periods of ischemia in the ischemic preconditioning procedure (Table 1). The adenosine concentration in coronary venous blood collected during reperfusion was significantly increased (P<.001), and it gradually augmented with repeated cycles of reperfusion after coronary occlusion in the ischemic preconditioning group (Table 3). AOPCP treatment during the ischemic preconditioning procedure in the AOPCP treatment with IP group and the AOPCP pretreatment with IP group attenuated adenosine release during reperfusion after brief periods of ischemia compared with the IP group (Table 3). In all of the groups in which we performed ischemic preconditioning (Tables 1 and 3), 5 minutes after the fourth cycle of coronary occlusion, CPP, CBF, and adenosine concentration in the coronary venous blood returned to the control levels (Tables 2 and 4). As observed in the previous study,²⁰ Table 5 shows that ischemic precondi-

TABLE 3. Sequential Changes in Adenosine Concentration (pmol/mL) in Coronary Arterial and Venous Blood During Four Cycles of Ischemic Preconditioning

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Cycle of Ischemia</th>
<th>IP Group (n=10)</th>
<th>AOPCP Treatment With IP Group (n=8)</th>
<th>AOPCP Pretreatment With IP Group (n=6)</th>
<th>AOPCP During Reperfusion With IP Group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ado(a)</td>
<td>Ado(v)</td>
<td>Ado(a)</td>
<td>Ado(v)</td>
</tr>
<tr>
<td>Before</td>
<td>0</td>
<td>43±2</td>
<td>54±3</td>
<td>39±3</td>
<td>57±2</td>
</tr>
<tr>
<td></td>
<td>Reperfusion</td>
<td>5</td>
<td>133±6</td>
<td>96±4</td>
<td>89±8*</td>
</tr>
<tr>
<td>Before</td>
<td>10</td>
<td>2</td>
<td>42±2</td>
<td>61±3</td>
<td>41±2</td>
</tr>
<tr>
<td></td>
<td>Reperfusion</td>
<td>15</td>
<td>160±5</td>
<td>103±5†</td>
<td>107±5†</td>
</tr>
<tr>
<td>Before</td>
<td>20</td>
<td>3</td>
<td>45±3</td>
<td>60±3</td>
<td>38±6</td>
</tr>
<tr>
<td></td>
<td>Reperfusion</td>
<td>25</td>
<td>175±6</td>
<td>104±4†</td>
<td>105±5†</td>
</tr>
<tr>
<td>Before</td>
<td>30</td>
<td>4</td>
<td>39±2</td>
<td>58±3</td>
<td>39±4</td>
</tr>
<tr>
<td></td>
<td>Reperfusion</td>
<td>35</td>
<td>209±9</td>
<td>104±3†</td>
<td>109±4†</td>
</tr>
<tr>
<td>5 Min after</td>
<td>40</td>
<td>44±2</td>
<td>59±2</td>
<td>38±3</td>
<td>55±2</td>
</tr>
</tbody>
</table>

IP indicates ischemic preconditioning; AOPCP, α,β-methylene adenosine 5'-diphosphate; Ado, adenosine; a, arterial; v, venous; Before, before 5 minutes of coronary occlusion; Reperfusion, immediately after 5 minutes of reperfusion; and 5 Min after, 5 minutes after the end of the fourth occlusion.

*P<.005 and †P<.001 compared with the IP group by using a modified Bonferroni's test. Values are expressed as mean±SEM.
tioning significantly increases both ectosolic and cytosolic 5'-nucleotidase activity of the myocardium compared with the control group. Table 6 shows the time course of decreases in 5'-nucleotidase activity in membrane and cytosolic fraction after exposure to 10^{-4} mol/L AOPCP. Five minutes after exposure to AOPCP, ectosolic 5'-nucleotidase activity was markedly reduced, which is the same extent of decreases in ectosolic 5'-nucleotidase activity at 40 and 100 minutes of exposure to AOPCP. The inhibitory effect of AOPCP was reversible in 10 minutes of washout of AOPCP. These results indicate that a 5-minute exposure to AOPCP drastically reduces ectosolic 5'-nucleotidase activity.

After 90 minutes of coronary occlusion, CPP was not different in all of the groups (control group, 105±3 mm Hg; IP group, 103±4 mm Hg; AOPCP treatment with IP group, 102±3 mm Hg; AOPCP treatment group, 107±4 mm Hg; AOPCP pretreatment with IP group, 106±4 mm Hg; and AOPCP during reperfusion with IP group, 103±4 mm Hg). Adenosine concentration in coronary venous blood during reperfusion was elevated during early reperfusion periods (Table 7) compared with that before 90 minutes of ischemia (Table 3). In the IP group, adenosine release was significantly higher after the onset of coronary reperfusion compared with the control group (Table 7). In the AOPCP treatment with IP group and AOPCP during reperfusion with IP group, adenosine release was not augmented to the levels of the control group but was attenuated to the levels of the AOPCP treatment group (Table 7). This dose of AOPCP affected neither adenosine-induced coronary vasodilation (coronary blood flow [mL/100 g per minute] with and without AOPCP administration, 91±1 versus 91±2 at baseline, 144±4 versus 143±3 at 0.5 μg·kg^{-1}·min^{-1} of adenosine infusion, 161±3 versus 169±3 at 1.0 μg·kg^{-1}·min^{-1} of adenosine infusion, and 205±3 versus 207±3 at 2.0 μg·kg^{-1}·min^{-1} of adenosine infusion) nor isoproteanol-induced increases in myocardial oxygen consumption (myocardial oxygen consumption [mL/100 g per minute] with and without AOPCP administration, 6.9±0.1 versus 7.0±0.1 at baseline, 8.9±0.1 versus 8.7±0.2 at 50 ng·kg^{-1}·min^{-1} of isoproterenol infusion, 12.6±0.3 versus 12.4±0.4 at 150 ng·kg^{-1}·min^{-1} of isoproterenol infusion).

Fig 1 depicts risk area and collateral flow during ischemia in the control group, the IP group, the AOPCP treatment with IP group, the AOPCP treatment group, the AOPCP pretreatment with IP group, and the AOPCP during reperfusion with IP group. There are no significant differences in risk area and collateral flow during ischemia between these six groups. Fig 2 depicts infarct size in the six groups. Ischemic preconditioning markedly attenuated infarct size (40.1±2.6% versus 6.8±1.9%, *P<.001). AOPCP completely abolished the infarct size-limiting effect of ischemic preconditioning (infarct size in the AOPCP group and the AOPCP treatment with IP group, 45.7±4.2% versus 38.8±4.9%, NS), arguing the cause-and-effect relation between the increased ectosolic 5'-nucleotidase activity and the infarct size-limiting effect in ischemic preconditioning. In the AOPCP pretreatment with IP and the AOPCP during reperfusion with IP groups, infarct size was partially attenuated compared with the AOPCP treatment with IP group and the AOPCP treatment group. Infarct sizes of these two groups were smaller (*P<.001) than the control and AOPCP groups and larger (P<.001) than that in the IP group. Fig 3 depicts the relation between infarct size normalized by risk area and collateral blood flow to inner half of the LAD-

### TABLE 4. Sequential Changes in Adenosine Concentrations (pmol/mL) of Coronary Arterial and Venous Blood During a 40-Minute Steady State Before Ischemia in Control and AOPCP Treatment Groups

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Control Group (n=8)</th>
<th>AOPCP Treatment Group (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>37±4 57±3</td>
<td>38±3 57±2</td>
</tr>
<tr>
<td>20</td>
<td>42±4 56±3</td>
<td>39±2 57±2</td>
</tr>
<tr>
<td>40</td>
<td>39±4 56±3</td>
<td>37±3 59±2</td>
</tr>
</tbody>
</table>

AOPCP indicates α,β,-methylene adenosine 5'-diphosphate; Ado(a) and Ado(v), adenosine concentrations in coronary arterial and venous blood, respectively. Statistical significances between the control and AOPCP treatment groups were tested by unpaired t test with an adjusted level of significance according to a modified Bonferroni's method. Values are expressed as mean±SEM.

### TABLE 5. Myocardial Ectosolic and Cytosolic 5'-Nucleotidase Activity With and Without Ischemic Preconditioning

<table>
<thead>
<tr>
<th></th>
<th>Ectosolic 5'-Nucleotidase Activity, nmol/mg protein per min</th>
<th>Cytosolic 5'-Nucleotidase Activity, nmol/mg protein per min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LAD Area</td>
<td>LCx Area</td>
</tr>
<tr>
<td>Epicardial myocardium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>34.8±3.0</td>
<td>35.1±1.2</td>
</tr>
<tr>
<td>IP group</td>
<td>62.2±3.7†</td>
<td>44.4±1.8*</td>
</tr>
<tr>
<td>Endocardial myocardium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>37.5±2.9</td>
<td>41.8±1.2</td>
</tr>
<tr>
<td>IP group</td>
<td>70.4±1.9†</td>
<td>47.8±2.1</td>
</tr>
</tbody>
</table>

LAD, left anterior descending coronary artery; LCx, left circumflex artery; and IP, ischemic preconditioning. *P<.05, †P<.005, ‡P<.001 compared with the control group, tested by unpaired t test. Numbers of the data in the control and IP groups are 5 each. Values are expressed as mean±SEM.
dependent myocardium during a sustained ischemic period. Fig 3 also clearly indicates that (1) ischemic preconditioning attenuates \( P<.001 \) infarct size, (2) AOPCP treatment blunts the infarct size-limiting effect of ischemic preconditioning \( P<.001 \) between the IP group and AOPCP treatment with IP group, and (3) administration of AOPCP during ischemic preconditioning procedures and during the reperfusion period partially blunts the infarct size-limiting effect. These results indicate that both increased 5'-nucleotidase activity and adenosine release during ischemic preconditioning procedures and during early reperfusion synergistically contribute to the infarct size-limiting effect of ischemic preconditioning.

### Discussion

**Linkage Between the Infarct Size–Limiting Effect and Adenosine in Ischemic Preconditioning**

Ischemic preconditioning has been the focus of studies by basic and clinical investigators in cardiology. Several laboratories have reported that ischemic preconditioning potently attenuates infarct size.\(^{1-3,12-20}\) However, the subcellular mechanisms must be elucidated to apply the results of ischemic preconditioning in the treatment of acute myocardial infarction because it is impractical to initiate brief periods of ischemia in clinical settings. Interestingly and timely, we have demonstrated that ischemic preconditioning increases both adenosine release and 5'-nucleotidase activity during ischemia and reperfusion.\(^{19,20}\) Moreover, the present study revealed that inhibition of ectosomal 5'-nucleotidase activity and attenuation of enhanced release of adenosine blunts the infarct size-limiting effect of ischemic preconditioning. Since adenosine is known to limit infarct size\(^{21,22}\) and to attenuate contractile dysfunction\(^{32-34}\) during ischemia and reperfusion, this augmentation of adenosine release during reperfusion after sustained ischemia can attenuate the infarct size in ischemic preconditioning.

Several lines of evidence support the idea that a transient exposure to adenosine modulates myocardial cellular function and attenuates infarct size.\(^{12-18}\) This observation is consistent with our previous\(^{19,20}\) and present results. We have reported that both ectosomal 5'-nucleotidase activity and adenosine release are increased during the ischemic preconditioning procedure as well as during reperfusion after sustained ischemia. Furthermore, in the present study, when adenosine release during the ischemic preconditioning procedure was attenuated by pretreatment with AOPCP, attenuation of infarct size was partially blunted, although AOPCP pretreatment did not attenuate the enhanced release of adenosine during reperfusion after sustained ischemia compared with the control group. We have

### Table 6. Sequential Changes in Differences of Adenosine Concentration (pmol/mL) in Coronary Arterial and Venous Blood After the Onset of Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Time After Onset of Reperfusion, Min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control group (n=8)</td>
<td>358±11</td>
</tr>
<tr>
<td>IP group (n=10)</td>
<td>618±19§</td>
</tr>
<tr>
<td>AOPCP treatment group (n=9)</td>
<td>196±21§</td>
</tr>
<tr>
<td>AOPCP treatment with IP group (n=8)</td>
<td>230±18§</td>
</tr>
<tr>
<td>AOPCP pretreatment with IP group (n=6)</td>
<td>620±23§</td>
</tr>
<tr>
<td>AOPCP during reperfusion with IP group (n=6)</td>
<td>229±19§</td>
</tr>
</tbody>
</table>

IP indicates ischemic preconditioning; AOPCP, \( \alpha,\beta \)-methylene adenosine 5'-diphosphate.

\( ^*P<.05, ^{†}P<.01, ^{‡}P<.005, ^{§}P<.001 \) compared with the control group, using a modified Bonferroni’s test. Values are expressed as mean±SEM.
further revealed in the present study that increases in 5'-nucleotidase activity and subsequent enhancements of adenosine release during reperfusion also contribute to the attenuation of infarct size, because AOPCP administration only during an early reperfusion period also partially blunted the infarct size–limiting effect of ischemic preconditioning. Deleterious events such as Ca overload,35-37 activation and adhesion of leukocytes, and norepinephrine release,38,39 all of which can cause irreversible cellular injury, are most prominent during reperfusion.35-37 Therefore, we argue that not only the enhanced release of adenosine during the ischemic preconditioning procedure but also the increased adenosine release during reperfusion synergistically contribute to the infarct size–limiting effect of ischemic preconditioning.

Although we showed that ectosolic and cytosolic 5'-nucleotidase activity is increased before and at the end of the sustained ischemia and that adenosine release during the ischemic preconditioning procedures and during reperfusion are increased, this observation does not necessarily indicate that adenosine release is augmented during ischemia. Indeed, we did not measure adenosine release during the sustained period of ischemia. Moreover, there is a report showing that increases in adenosine concentration in the interstitial space is not augmented during the sustained ischemia in ischemic preconditioning.40 One possibility to explain the differences between this and our observations is that the activities of the other enzymes, which also regulate adenosine metabolism, eg, adenosine kinase and adenosine deaminase,41-43 may be augmented by the ischemic events, eg, acidosis or energy levels. If this is the case, adenosine production caused by increased 5'-nucleotidase activity during sustained ischemia may be attenuated, and an increase in interstitial adenosine concentration during ischemia may not be augmented. Another possibility is that adenosine uptake into the myocytes is also enhanced during ischemia in the IP group. Adenosine taken into the cardiomyocytes may be converted to ATP by 5'-AMP, which may contribute to the preservation of high-energy phosphates in ischemic preconditioning.

**Linkage Between Ectosolic 5'-Nucleotidase Activity and Adenosine Release in Ischemic Preconditioning**

The question arises as to what mechanisms are involved in enhanced production of adenosine by ischemic preconditioning. Adenosine concentration is mainly determined by (1) synthesis enzymes for adenosine, ie,
5’-nucleotidase and S-adenosylhomocysteine (SAH)-hydrolase, (2) degradation or salvage enzymes for adenosine, ie, adenosine deaminase and adenosine kinase, and (3) substrates, 5’-AMP, and S-adenosylhomocysteine. In the nonischemic myocardium, adenosine production is derived from SAH-hydrolase, and adenosine production in the ischemic myocardium is reported to be mainly attributable to 5’-nucleotidase. However, this does not necessarily indicate that all of the increased adenosine release is attributable to the increased 5’-nucleotidase activity. Indeed, AOPCP treatment did not completely abolish adenosine release, indicating that other factors aside from ectosolic 5’-nucleotidase may be involved in ischemic preconditioning. First of all, we did not measure the activities of adenosine kinase and adenosine deaminase with and without ischemic preconditioning. Second, if 5’-AMP accumulates during ischemic preconditioning, adenosine release may be enhanced: 5’-AMP is reported to increase after five 3-minute coronary occlusions, though 5’-AMP decreases after 20 and 40 3-minute coronary occlusions. Third, the ability of membrane transport of adenosine also may affect the amount of adenosine release. Fourth, cytosolic 5’-nucleotidase also may be involved in adenosine release during reperfusion since cytosolic 5’-nucleotidase has a higher $K_m$ (Michaelis constant) for 5’-AMP than does ectosolic 5’-nucleotidase but may easily access 5’-AMP degraded from ATP and ADP in cytoplasm. However, ectosolic 5’-nucleotidase activity, which is thought to link with ischemic preconditioning, has lower $K_m$ and easily produces adenosine. We showed in the present study that ischemic preconditioning increases 5’-nucleotidase activity in the myocardium, and in turn, attenuation of ectosolic 5’-nucleotidase activity blunts adenosine release and the infarct size-limiting effect in ischemic preconditioning. Thus, we suggest the cause-and-effect relation between increases in adenosine production and activity of ectosolic 5’-nucleotidase. Interestingly, since ectosolic 5’-nucleotidase produces adenosine outside the cardiomyocytes and endothelial cells, this topical advantage to exert the action of adenosine on its $A_1$-receptors may primarily contribute to attenuation of infarct size.

In the present study, we did not clarify the mechanisms of increased 5’-nucleotidase activity. We have recently observed that protein kinase C increases ectosolic and cytosolic 5’-nucleotidase activity in the in vivo measurements. Since protein kinase C is activated by ischemia per se and by norepinephrine released during brief periods of ischemia, the ischemic preconditioning procedure may increase ectosolic and cytosolic 5’-nucleotidase activity through protein kinase C activation and therefore precondition the myocardium. If protein kinase C is responsible for increased 5’-nucleotidase activity and thereby the infarct size-limiting effects in ischemic preconditioning, $\alpha_1$-adrenoceptor stimulation may mimic ischemic preconditioning. Indeed, several groups of investigators, including those of our laboratory, preliminarily observed that $\alpha_1$-adrenoceptor activation is tightly involved in ischemic preconditioning

**Clinical Relevance**

The present study revealed that adenosine release through ectosolic 5’-nucleotidase activity potently limits infarct size in ischemic preconditioning, which may lead to two clinical applications for the treatment of acute myocardial infarction. One strategy to limit infarct size is to find the method to increase 5’-nucleotidase activity. As we observed, protein kinase C activation by administration of $\alpha_1$-adrenoceptor stimulators or phorbol esters may increase both 5’-nucleotidase activity and adenosine production and may merit attenuation of infarct size. Furthermore, exposures to cytokines are reported to increase 5’-nucleotidase activities. Oxygen-derived free radicals are also reported to attenuate the degradation of ectosolic 5’-nucleotidase activity. Therefore, administration of superoxide dismutase during ischemia and reperfusion may lessen the tendency for the decreased activity of ectosolic 5’-nucleotidase activity.

Another possibility arises from increased 5’-nucleotidase activity is to enhance adenosine release. Administration of a potentiator of adenosine production, eg, acadesine, dilazep, and dipryridamole, may be effective for the attenuation of infarct size. These hypoth-
es are worth testing in clinical settings to find and develop a new strategy for the treatment of acute myocardial infarction, although further basic understanding of ischemic preconditioning is necessary.

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M Kitakaze, M Hori, T Morioka, T Minamino, S Takashima, H Sato, Y Shinozaki, M Chujo, H Mori and M Inoue

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