

Alpha₁-Adrenoceptor Activation Mediates the Infarct Size-limiting Effect of Ischemic Preconditioning through Augmentation of 5'-Nucleotidase Activity

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Abstract

We have reported that ischemic preconditioning may limit infarct size by increasing 5'-nucleotidase activity. The present study tested whether alpha₁-adrenoceptor stimulation in ischemic preconditioning mediates the infarct size-limiting effect through augmentation of 5'-nucleotidase activity. The coronary artery was occluded four times for 5 min separated by 5 min of reperfusion (ischemic preconditioning) in 82 dogs. Then the coronary artery was occluded for 90 min followed by 6 h of reperfusion. Infarct size normalized by risk area was smaller after ischemic preconditioning than in the control group (40.6±2.3 vs 6.7±2.0%, $P < 0.001$), even though no difference existed in endomyocardial collateral flow during ischemia (8.7±1.0 vs 8.9±1.0 ml/100 g per min). Ectosomal and cytosolic 5'-nucleotidase activity was increased after ischemic preconditioning. However, prazosin blunted the infarct size-limiting effect of ischemic preconditioning (infarct size: 42.8±3.7%). Intermittent alpha₁-adrenoceptor stimulation by methoxamine mimicked the increase in 5'-nucleotidase activity and the infarct size-limiting effect, which were abolished by alpha, beta,-methyleadenosine 5'-diphosphate. Identical results were obtained in the conscious model ($n = 20$). Therefore, we conclude that increases in ectosomal 5'-nucleotidase activity due to alpha₁-adrenoceptor activation may contribute to the infarct size-limiting effect of ischemic preconditioning. (*J. Clin. Invest.* 1994. 93:2197–2205.) Key words: adenosine • ischemic preconditioning • 5'-nucleotidase • prazosin • methoxamine

Introduction

Brief periods of ischemia that precede sustained ischemia limit infarct size markedly, a phenomenon known as ischemic preconditioning (1–3). The mechanisms underlying this phenomenon have been studied extensively (4–8) with an eye to developing new treatments for acute myocardial infarction, and evidence now exists which suggests that ischemic preconditioning is clinically beneficial (9, 10). Recently, Liu et al. (11) have demonstrated experimentally that exposure to 8-sulfophe-

nyltheophylline blunts the infarct size-limiting effect of ischemic preconditioning, and Liu et al. (11) and Thornton et al. (12) have shown that adenosine A₁ receptor activation is responsible for limiting infarct size. Other investigators have reached the same conclusion (13–16). Furthermore, we have reported previously that ischemic preconditioning increases 5'-nucleotidase activity (17, 18). Additionally, alpha₁-adrenoceptor activation and/or protein kinase C activation have been shown to mimic the infarct size-limiting effect of ischemic preconditioning (17–19). Although these two mechanisms of ischemic preconditioning, activations of 5'-nucleotidase and protein kinase C, seem to be independent, these two factors appear tightly linked; we have reported that alpha₁-adrenoceptor activation increases 5'-nucleotidase activity through activation of protein kinase C (20–22). This suggests that catecholamine release (23) and/or translocation of protein kinase C (24) during ischemia may mediate ischemic preconditioning through increased 5'-nucleotidase activity.

In this study, we measured ectosomal and cytosolic 5'-nucleotidase activity and infarct size with and without administration of prazosin. We also tested whether intermittent exposure to methoxamine would mimic the infarct size-limiting effect of ischemic preconditioning.

Methods

Instrumentation

82 mongrel dogs (protocols I–III) weighing 14–20 kg were anesthetized with sodium pentobarbital (30 mg/kg, intravenous), intubated, and ventilated with room air mixed with oxygen (100% O₂ at 1.0–1.5 liter/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 U/kg), we cannulated and perfused the left anterior descending (LAD)¹ 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 introduced 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. The left atrium was catheterized for microsphere injection. Hydration was maintained by a slow normal saline infusion. Heart rate averaged 138±1 beats per minute during control conditions and did not change during the study. The pH, pO₂, and pCO₂ in the systemic arterial blood before instituting the protocols were 7.40±0.03, 104±4, and 37.5±1.9 mmHg, respectively.

1. Abbreviations used in this paper: ANCOVA, analysis of covariance; AOPCP, alpha, beta,-methyleadenosine 5'-diphosphate; CBF, coronary blood flow; CPP, coronary perfusion pressure; IP, ischemic preconditioning; LAD, left anterior descending; SAH, S-adenosylhomocysteine; TTC, 2,3,5-triphenyltetrazolium.

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20 other mongrel dogs (protocol IV) weighing 15–19 kg were anesthetized with sodium pentobarbital (30 mg/kg, intravenous), intubated, and ventilated with room air mixed with oxygen (100% O₂ at 1.0–1.5 liter/min). Surgery was performed under aseptic conditions, and animals received 1.5 × 10⁶ U of intramuscular penicillin as prophylaxis against infection (25). The chest was opened through the left fifth intercostal space. The LAD coronary artery was isolated, and a strip of moistened umbilical tape was passed around the vessels for the occlusion, which was accomplished by snaring it into a small plastic tube. The left atrium was catheterized for microsphere injection. After completion of the experimental protocol, the chest wound was closed after the removal of the catheter to the left atrium, and the animals were allowed to survive for 7 d to delineate the necrotic cardiac muscle. At the completion of the experiment, the animals were reanesthetized and intravenously given 500 U/kg of heparin to aid postmortem coronary perfusion. Hearts then were excised for analysis of area at risk, regional myocardial blood flow, and histological infarct size.

Experimental protocols

Protocol I: the effect of prazosin administration on ischemic preconditioning (IP). 33 dogs were used in this protocol. Both CPP and CBF were measured continuously. Coronary arterial and venous blood were sampled for blood gas analysis and measurement of the plasma adenosine concentration. After hemodynamic stabilization, four cycles of 5 min of coronary occlusion and subsequent 5 min of reperfusion were performed to precondition the myocardium to sustained ischemia ($n = 9$, the IP group). In the IP group, coronary venous blood was sampled before and immediately after each period of coronary occlusion. Arterial blood was sampled at 60–90-min intervals to monitor the condition of the dogs. As a control, instead of IP, after 40 min of hemodynamic stabilization, the coronary artery was occluded for 90 min and reperfused for 360 min ($n = 9$, the control group). Coronary arterial and venous blood were sampled at 20-min intervals during the 40 min of hemodynamic stabilization.

In eight dogs, constant infusion of prazosin (4 µg/kg per min, 240 µg/ml with an infusion rate of 0.0167 ml/kg per min) into the LAD coronary artery was begun 5 min before IP and continued through the first 60 min of the reperfusion period, except during coronary occlusion (the prazosin with IP group). Prazosin (Sigma Chemical Co., St. Louis, MO) was diluted with distilled water. In seven dogs, prazosin was infused into the LAD coronary artery beginning 40 min before ischemia without IP and continued for 60 min of reperfusion, except during coronary occlusion (the prazosin group). Arterial and coronary venous blood sampling in both groups were performed as in the control and IP groups, respectively.

Protocol II: the effects of intermittent methoxamine exposure on the infarct size-limiting effect of IP. To test whether alpha₁-adrenoceptor stimulation mimics the infarct size-limiting effect of IP, we administered methoxamine dissolved in saline (40 µg/kg per min, intracoronary, 2.4 mg/ml with an infusion rate of 0.0167 ml/kg per min; Sigma Chemical Co.) for four cycles for 5 min at 5-min intervals (the methoxamine group, $n = 10$). After methoxamine exposure, 90 min of coronary occlusion and 6 h of reperfusion were imposed. Furthermore, to examine the role of increased ectosolic 5'-nucleotidase activity due to exposures of methoxamine (40 µg/kg per min intracoronary, 2.4 mg/ml with an infusion rate of 0.0167 ml/kg per min) we concomitantly infused alpha,beta,-methyleneadenosine 5'-diphosphate dissolved in saline (AOPCP, 80 µg/kg per min, 4.8 mg/kg per min with an infusion rate of 0.0167 ml/kg per min; Sigma Chemical Co.) 5 min before IP and continued for 60 min of reperfusion, except during 90 min of coronary occlusion to animals treated with methoxamine (the methoxamine with AOPCP group, $n = 7$). In another group, we determined the effect of AOPCP on infarct size (the AOPCP group, $n = 7$). In this group, AOPCP was administered before 40 min of coronary occlusion and during 1 h of reperfusion after 90 min of coronary occlusion. AOPCP is a specific and competitive inhibitor of ectosolic 5'-nucleotidase. In our preliminary study, this dose of AOPCP was sufficient to attenuate ectosolic 5'-nucleotidase activity of the canine myocardium.

At 5 min of exposure to AOPCP, ectosolic 5'-nucleotidase activity was reduced to 3.2±4.9 from 37.1±4.1 nmol/mg protein per min ($P < 0.001$, $n = 5$). Arterial and coronary venous blood sampling in both groups were performed according to the same schedule as in the IP group in protocol I.

Protocol III: the effect of prazosin and methoxamine administrations on myocardial ectosolic and cytosolic 5'-nucleotidase activity with and without IP. We used five dogs in five groups each. With and without IP (the preconditioning and control groups, respectively), we measured 5'-nucleotidase activity (26) in the endocardial and epicardial myocardium before sustained ischemia. We sampled myocardial tissue to measure ectosolic and cytosolic 5'-nucleotidase activity. Furthermore, we also examined 5'-nucleotidase activity in the endocardial and epicardial myocardium before the sustained ischemia with (the prazosin with IP group, $n = 5$) and without (the prazosin group, $n = 5$) IP with prazosin administration. Finally, we examined 5'-nucleotidase activity in the endocardial and epicardial myocardium after four cycles of 5-min exposures to methoxamine (the methoxamine group, $n = 5$). We quickly sampled myocardial tissue at the conclusion of the protocol and stored it at -80°C.

Protocol IV: the effect of prazosin administration on the infarct size-limiting effect of IP assessed in the conscious dog model. Since prazosin may have membrane-stabilizing effects, administration of prazosin may have attenuated the extent of the efflux of dehydrogenase from the cardiomyocytes without affecting necrosis size. If this were the case, we may erroneously estimate infarct size when we use prazosin. Therefore, in this protocol, we used the conscious dog model to validate the role of prazosin in the infarct size-limiting effect. 20 dogs were used in this protocol. Blood pressure was measured, and systemic blood was sampled for blood gas analysis to monitor the condition of the dogs. After hemodynamic stabilization, four cycles of 5 min of coronary occlusion and subsequent 5 min of reperfusion were performed to precondition the myocardium to sustained ischemia ($n = 5$, the IP group). As a control, instead of IP, after 40 min of hemodynamic stabilization, the coronary artery was occluded for 90 min and then reperfused ($n = 5$, the control group).

In five dogs, constant infusion of prazosin (8 µg/kg per min, 480 µg/ml with an infusion rate of 0.0167 ml/kg per min) into the systemic vein was begun 5 min before IP and continued through 60 min of reperfusion (the prazosin with IP group). In five dogs, prazosin was infused into the systemic vein beginning 40 min before ischemia without IP and continued for 60 min of reperfusion (the prazosin group). We assessed infarct size 7 d after this procedure.

Measurement of infarct size

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

On the other hand, in protocol IV, 7 d after reperfusion, while LAD was reoccluded and perfused with autologous blood with the catheter from the carotid artery, Evans blue dye was injected into a systemic vein to determine the anatomic risk area and the nonischemic area in the hearts. The heart was then removed immediately. The nonischemic area was identified by blue stain, the ischemic region was sectioned into eight transverse slices, and each slice was weighed. Remaining tissue was used for the microsphere measurement. These slices were then fixed by immersion in a large volume of phosphate-buffered 10% formalin, pH 7.4. After fixation for at least 2 d, histological blocks which

encompassed the entire area at risk were prepared, and two sections for each tissue sample were cut and stained with hematoxylin and eosin. The histological sections were observed and photographed for slides. The photographic slides of the entire risk area of the heart slice were projected ($\times 10$) and traced. Extents of the area at risk and area of necrosis in each slice were then quantified by planimetry, corrected for the weight of the tissue slice, and summed for each heart. Infarct size was expressed as a percentage of the area at risk.

For the randomization of the study, the area of necrosis and the risk area were measured for all dogs at the completion of the protocol, without knowledge of the treatment in each heart.

Measurement of regional coronary blood flow

Regional myocardial blood flow was determined by the microsphere technique, which uses nonradioactive microspheres (Sekisui Plastic Co., Ltd., Tokyo, Japan) made of inert plastic labeled with different stable heavy elements, as described in detail previously (27, 28). In the present study, microspheres labeled with Br or Zr were used. The mean diameter was $15 \mu\text{m}$, and the specific gravity was 1.34 for Br and 1.36 for Zr. Microspheres were suspended in isotonic saline with 0.01% Tween 80 to prevent aggregation. The microspheres were ultrasonicated for 5 min followed by 5 min of vortexing immediately before injection. 1 ml of the microsphere suspension ($2-4 \times 10^6$ spheres) was injected into the left atrium followed by several warm (37°C) saline flushes (5 ml). Microspheres were administered at 80 min 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 min.

The x-ray fluorescence of the stable heavy elements was measured by a wavelength dispersive spectrometer (PW 1480; Phillips Co., Ltd., Almelo, The Netherlands). The specifications of this x-ray fluorescence

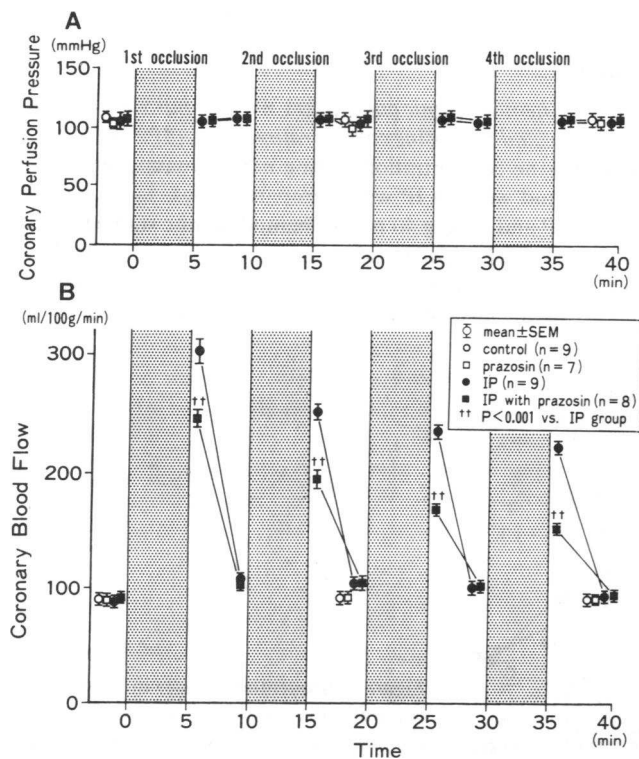


Figure 1. Sequential changes in coronary perfusion pressure (A) and coronary blood flow (B) during four cycles of IP with (closed square) and without (closed circle) prazosin administration and during a 40-min steady state observation period with (open square) and without (open circle) prazosin administration before the onset of sustained ischemia.

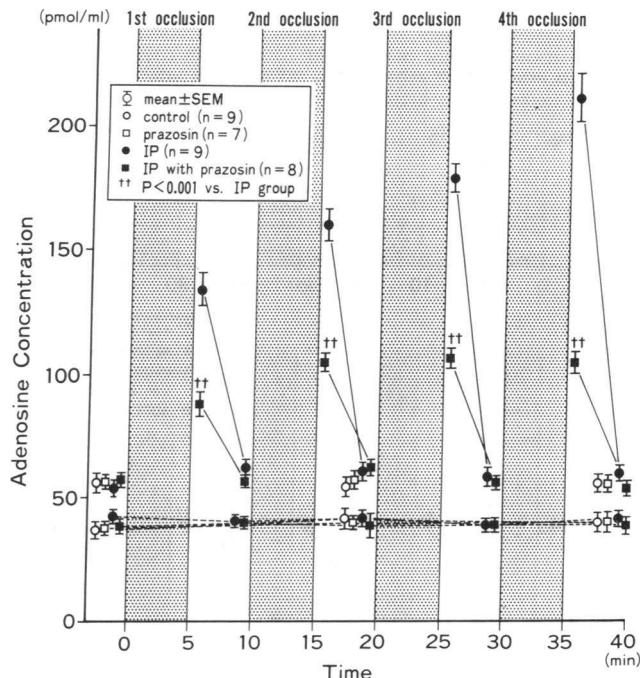


Figure 2. Sequential changes in adenosine concentration in coronary arterial (dotted line) and venous blood (solid line) during four cycles of IP with (closed square) and without (closed circle) prazosin administration and during a 40-min steady state observation period with (open square) and without (open circle) prazosin administration before the onset of sustained ischemia.

spectrometer have been described in detail (27, 28). Briefly, when the microspheres are irradiated by the primary x-ray beam, the electrons fall back to a lower orbit and emit measurable energy with a characteristic x-ray fluorescence energy level for each element. Therefore, it is possible to qualify the x-ray fluorescence of several species of labeled microspheres in a single mixture. Myocardial blood flow was calculated according to the formula: time flow = (tissue counts) \times (reference flow)/(reference counts) and was expressed in milliliters per minute per gram wet weight.

Myocardial metabolites

Adenosine measurement. The technique for measuring the plasma adenosine concentration has been reported previously (18, 20, 29, 30). 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 mM) to block both the uptake of adenosine by red blood cells and the 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-min incubation, the mixture was diluted with 100 μl of adenosine 2',3'-O-disuccinyl-3-[^{125}I]iodotyrosine methyl ester (0.5 pmol) and 100 μl of diluted antiadenosine serum. The mixture was kept in a cold water bath (4°C) for 18 h, and the second antibody solution (goat anti-rabbit IgG antiserum, 500 μl) was added. After incubation at 4°C for 1 h, unreacted material was removed by centrifugation at 3,000 rpm at 4°C for 20 min. Radioactivity remaining in the tube was counted using a gamma counter. Adenosine degradation during this blood sampling procedure has been reported to be negligible (29, 30). This method with the specific antibody was sensitive enough to detect as low as 5 pmol/ml of adenosine. The coefficient of variance of intraassay and interassay was observed 1.3–3.1 and 4.1–9.4%, respectively. This sensitive radioimmunoassay method for adenosine measurement does not need to remove protein, which is usually performed in the HPLC measurements for adenosine.

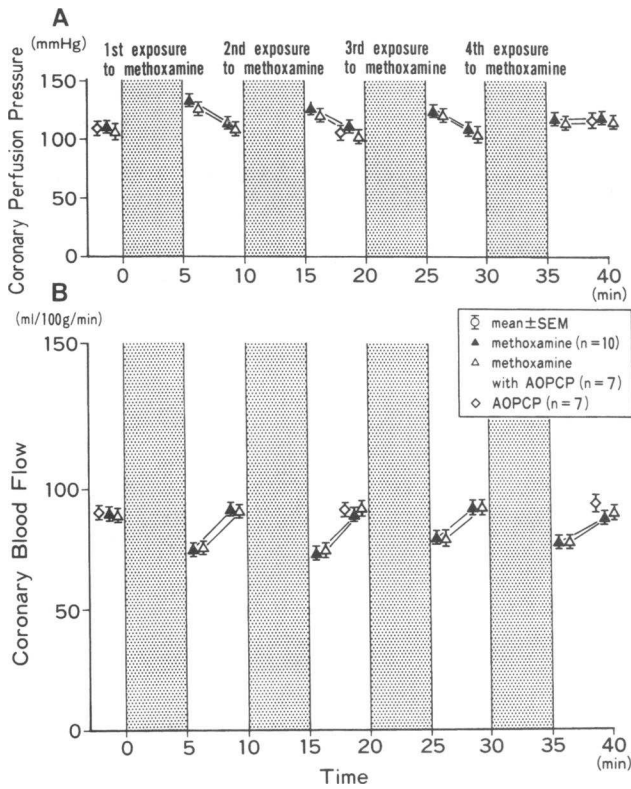


Figure 3. Sequential changes in coronary perfusion pressure (A) and coronary blood flow (B) during four cycles of intermittent methoxamine administration with (open triangle) and without (closed triangle) AOPCP administration and during a 40-min steady state observation period with AOPCP administration (open diamond) before the onset of sustained ischemia.

Measurement of 5'-nucleotidase activity. A biopsy specimen of myocardium supplied by the LAD was obtained before sustained coronary occlusion with and without IP in protocol III. The myocardial tissue samples were frozen and stored under liquid nitrogen, and the ectosolic and cytosolic 5'-nucleotidase activities were measured (31).

The myocardium was separated into its membrane and cytosolic fractions using the following technique. Myocardial tissue was homoge-

nized using a Potter-Elvehjem homogenizer (30 strokes) for 5 min in 10 vol of ice-cold 10 mM Hepes-KOH buffer (pH 7.4) containing 0.25 M sucrose, 1 mM MgCl₂, and 1 mM mercaptoethanol at 0°C. The crude homogenate was strained through a double-layer nylon sieve and homogenized again for 1 min. To prepare a crude membrane fraction, part of the homogenate was centrifuged at 1,000 g for 10 min. 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 3,000 g for 10 min, and the supernatant was centrifuged at 200,000 g for 1 h. The membrane and cytosolic fractions were dialyzed at 4°C for 4 h against 10 mM Hepes-KOH (pH 7.4) containing 1 mM MgCl₂, 1 mM mercaptoethanol, and 0.01% activated charcoal and divided into aliquots which were frozen immediately and stored at -80°C. In a preliminary study, we examined the recovery of the 5'-nucleotidase activity in the membrane fraction using this procedure and found that the recovery of ectosolic 5'-nucleotidase is 97±2% (n = 5). This recovery rate is highly reproducible (19).

5'-Nucleotidase activity was assessed by the enzymatic assay technique (18, 31) and is reported as units of nanomoles/milligram protein per minute. The protein concentration was measured by the method of Lowry et al. (32) using bovine serum albumin as a standard.

Statistical analysis

Statistical analyses were performed using paired and unpaired *t* tests (33, 34), and the significance level was adjusted according to a modified Bonferroni's method. When the data were compared among groups, a modified Bonferroni test was used to determine significance at the *P* < 0.05 level for group pairs that exhibited statistically significant differences (33, 34). Analysis of covariance (ANCOVA), by regional collateral flow in the inner half left ventricle 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* < 0.05 considered significant.

Results

Changes in hemodynamic parameters, myocardial 5'-nucleotidase activity, and adenosine concentration during IP. CPP, CBF, and adenosine concentration during hemodynamic stabilization with and without IP were compared (Figs. 1-4). Before and after IP and during 40 min of hemodynamic stabilization, neither CPP, CBF (Figs. 1 and 3), nor adenosine concentration (Figs. 2 and 4) in the coronary arterial or venous blood changed significantly in any group. In the IP group, the coro-

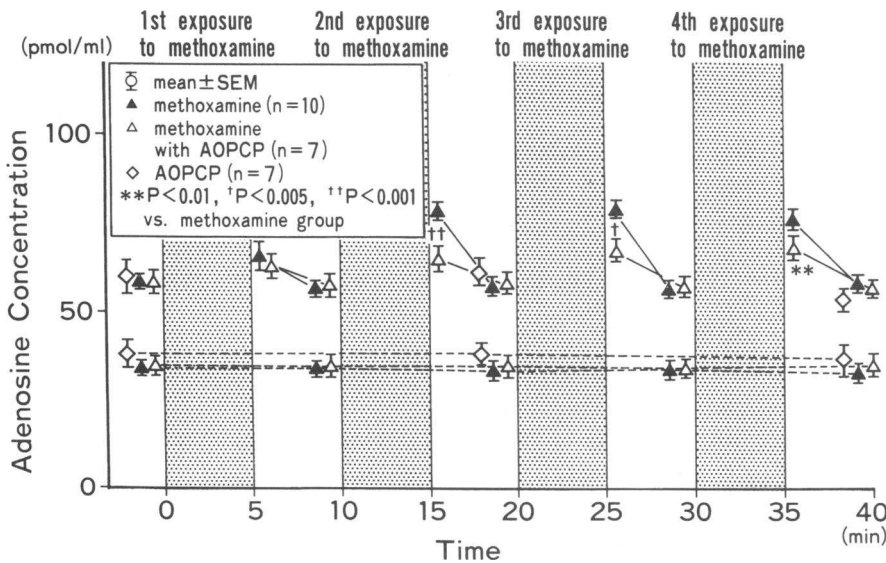


Figure 4. Sequential changes in adenosine concentration in coronary arterial (dotted line) and venous blood (solid line) during four cycles of intermittent methoxamine administration with (open triangle) and without (closed triangle) AOPCP administration and during a 40-min steady state observation period with AOPCP administration (open diamond) before the onset of sustained ischemia.

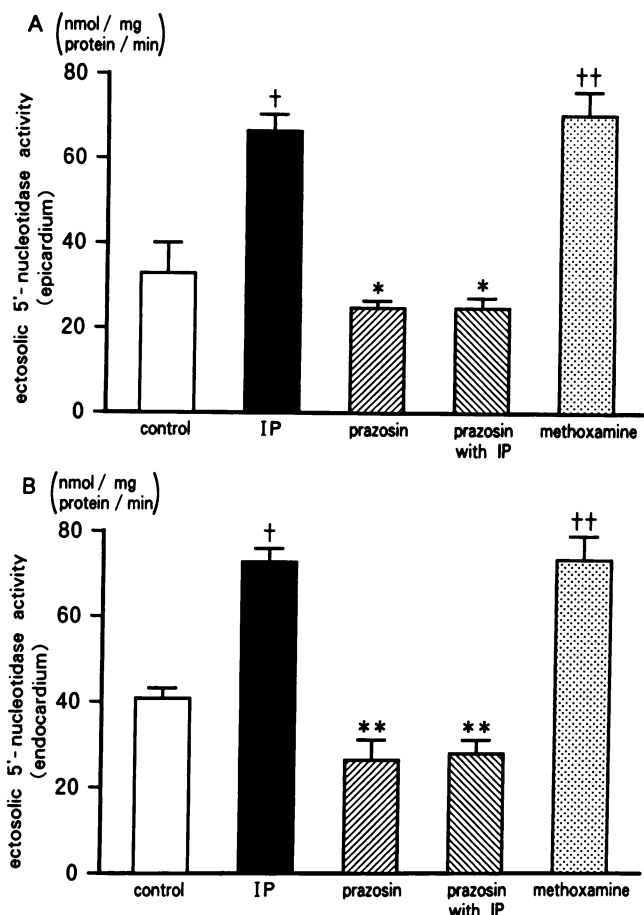


Figure 5. Comparison of canine ectosolic 5'-nucleotidase activity in the epicardium (A) and endocardium (B) after 40 min of steady state observation (control, $n = 5$), 40 min of prazosin administration ($n = 5$), IP with ($n = 5$) and without ($n = 5$) prazosin administration, and intermittent exposure to methoxamine ($n = 5$). IP increased ectosolic 5'-nucleotidase activity, which was blunted by prazosin and mimicked by methoxamine. All values are mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; † $P < 0.005$; and †† $P < 0.001$ vs control.

nary venous adenosine concentration rose during the first cycle of reperfusion ($P < 0.001$) and continued to increase gradually with subsequent cycles of reperfusion, remaining higher than the control group (Fig. 2). Prazosin administration during IP procedure attenuated the extent of reactive hyperemic flow (Fig. 1) and adenosine release during reperfusion (Fig. 2). CBF decreased after administration of methoxamine in the methoxamine group but returned to the control level 5 min after the fourth exposure to methoxamine (Fig. 3). CPP increased during methoxamine administration but returned to the control level 5 min after the fourth exposure to methoxamine. Adenosine concentration in coronary venous blood release increased during intracoronary methoxamine infusion but also returned to the control level 5 min after the fourth exposure to methoxamine (Fig. 4). AOPCP slightly attenuated adenosine release during methoxamine administration (Fig. 4). After the fourth exposure to methoxamine with AOPCP, coronary venous adenosine concentration was not significantly different compared with the group which received AOPCP without methoxamine (Fig. 4). From the dose of each chemical and coronary blood flow at the baseline condition, we calculated the concentration

of each chemical in the coronary arterial blood. Concentrations of prazosin in coronary arterial blood were 2.29 ± 0.04 and 2.42 ± 0.04 $\mu\text{g/ml}$ in the prazosin and the prazosin with IP groups, respectively. Concentrations of methoxamine in coronary arterial blood were 25.1 ± 1.4 and 26.9 ± 1.6 $\mu\text{g/ml}$ in the methoxamine and the methoxamine with AOPCP groups, respectively. Concentrations of AOPCP in coronary arterial blood were 58.5 ± 4.9 and 53.8 ± 3.2 $\mu\text{g/ml}$ in the AOPCP and the methoxamine with AOPCP groups, respectively.

IP significantly increased both ectosolic (Fig. 5) and cytosolic (Fig. 6) 5'-nucleotidase activity in the myocardium. Prazosin administration without IP decreased ectosolic and cytosolic 5'-nucleotidase activity and blunted the increases in ectosolic and cytosolic 5'-nucleotidase activity due to IP. Methoxamine increased both ectosolic and cytosolic 5'-nucleotidase activity to the levels obtained with IP.

The infarct size-limiting effect of IP and its relation to α_1 -adrenoceptor activity. 90 min of coronary occlusion did not change CPP in any group (106 ± 2 mmHg). Fig. 7 shows that IP markedly attenuates infarct size compared with controls, although collateral flow was not different in the two groups (the control group and the IP groups: 8.7 ± 1.0 vs 8.9 ± 1.0 ml/100 g per min, NS; risk area in the left ventricle in the control and the IP groups: 36.1 ± 2.8 vs $37.6 \pm 2.9\%$, NS).

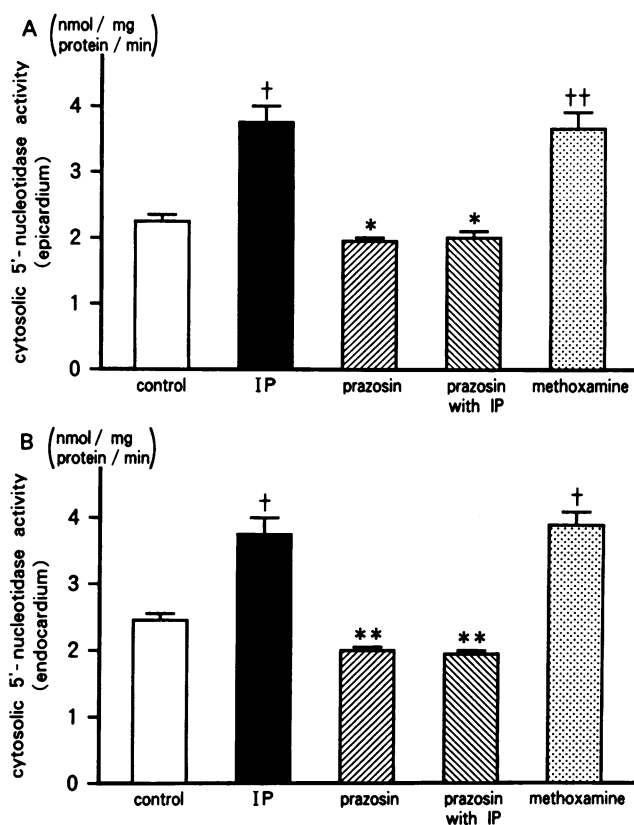


Figure 6. Comparison of canine cytosolic 5'-nucleotidase activity in the epicardium (A) and endocardium (B) after 40 min of steady state observation (control, $n = 5$), 40 min of prazosin administration ($n = 5$), IP with ($n = 5$) and without ($n = 5$) prazosin administration, and intermittent exposure to methoxamine ($n = 5$). IP increased cytosolic 5'-nucleotidase activity, which was blunted by prazosin and mimicked by methoxamine. All values are mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; † $P < 0.005$; and †† $P < 0.001$ vs control.

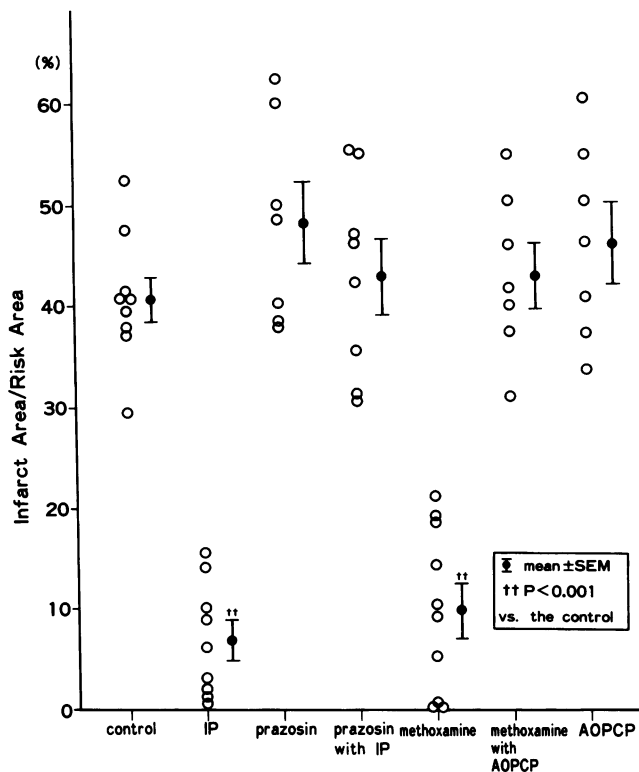


Figure 7. Infarct size in the control group ($n = 9$), the IP group ($n = 9$), the prazosin with IP group ($n = 8$), the prazosin group ($n = 7$), the methoxamine group ($n = 10$), the methoxamine with AOPCP group ($n = 7$), and the AOPCP group ($n = 7$). Infarct size was markedly decreased in the IP group. The infarct size-limiting effect of IP was completely abolished by administration of prazosin. On the other hand, methoxamine mimicked the infarct size-limiting effect of IP, which was blunted by AOPCP.

Fig. 7 also showed that prazosin completely abolished the infarct size-limiting effect of IP (collateral flow in the prazosin group and the prazosin with IP group: 8.5 ± 1.1 vs 8.4 ± 1.2 ml/100 g per min, NS; risk area in the left ventricle in the prazosin group and the prazosin with IP group: 40.8 ± 4.4 vs $41.9 \pm 3.1\%$, NS). With methoxamine administration, infarct size was attenuated to the level seen with IP (Fig. 7). However, the infarct size-limiting effect due to exposure of methoxamine was blunted by AOPCP administration, and no difference in infarct size existed between the methoxamine with AOPCP and the AOPCP groups (collateral flow in the methoxamine, the methoxamine with AOPCP, and the AOPCP groups: 7.9 ± 1.2 , 8.1 ± 1.2 , and 7.8 ± 1.4 ml/100 g per min, NS; risk area in the left ventricle in the methoxamine, the methoxamine with AOPCP, and the AOPCP groups: 41.7 ± 1.9 , 41.0 ± 2.4 , and $39.8 \pm 1.4\%$, NS). Fig. 8 illustrates the regression plots of infarct size, as a percentage of the area at risk against collateral flow in the preconditioned and control groups with and without prazosin administration and the methoxamine and control groups with and without AOPCP administration. IP significantly and markedly reduced infarct size, and prazosin blunted the infarct size-limiting effect at any level of collateral flow. Furthermore, transient methoxamine administration mimicked the infarct size-limiting effect of IP, which was blunted by AOPCP administration.

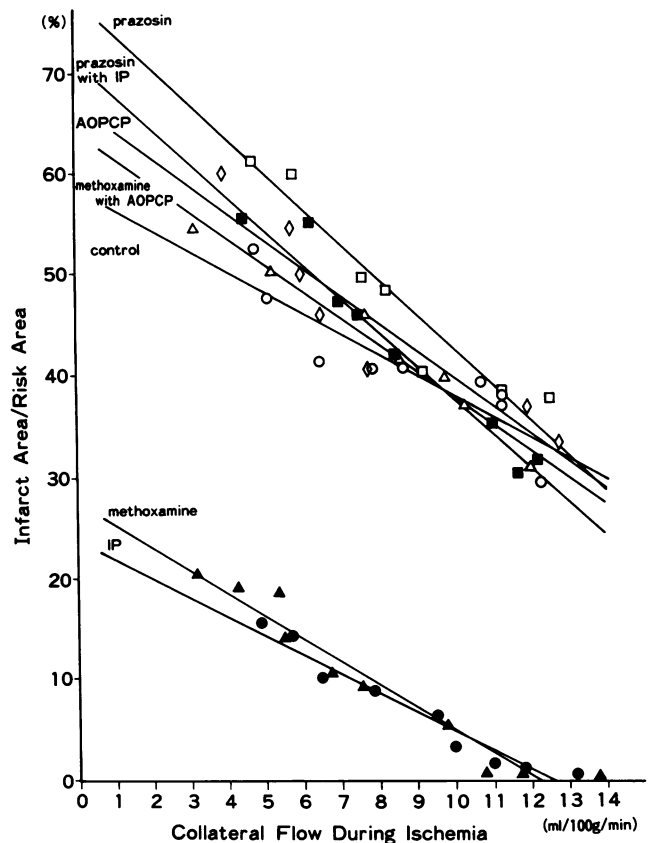


Figure 8. Plot of infarct size expressed as a percentage of the risk area and regional collateral flow during ischemia. In the control group (open circles, $n = 9$), the IP group (closed circles, $n = 9$), the prazosin with IP group (closed squares, $n = 8$), the prazosin group (open squares, $n = 7$), the methoxamine group (closed triangles, $n = 10$), the methoxamine with AOPCP group (open triangles, $n = 7$), and the AOPCP group (open diamonds, $n = 7$) there are inverse relations between normalized infarct area and collateral flow. Infarct size in the preconditioned hearts was smaller ($P < 0.001$) with respect to any given value for collateral blood flow than that in the control hearts. Infarct size was markedly increased ($P < 0.001$) in the prazosin with IP group compared with the IP group. Infarct size in the prazosin with IP group and the prazosin group were not different. On the other hand, transient methoxamine administration mimicked the infarct size-limiting effect of IP, which was blunted by AOPCP. Statistical significance was tested by ANCOVA.

In the conscious dog models, we obtained comparable results. Fig. 9 shows infarct size in the control and the IP groups with and without administration of prazosin. IP reduced infarct size compared with the control group, which was blunted by administration of prazosin (collateral flow in the control, IP, prazosin, and the prazosin with IP groups: 8.3 ± 1.6 , 8.4 ± 1.3 , 8.4 ± 1.6 , and 8.5 ± 1.3 ml/100 g per min, NS; risk area in the left ventricle in the control, IP, prazosin, and IP with prazosin groups: 38.9 ± 3.1 , 40.5 ± 2.3 , 41.5 ± 2.5 , and $39.9 \pm 1.9\%$, NS). Fig. 10 illustrates the regression plots of infarct size against the collateral flow preconditioned and control groups with and without prazosin administration. At any level of collateral flow, IP reduced infarct size, which was blunted by prazosin administration. These results indicate that increased cytosolic 5'-nucleotidase activity due to α_1 -adrenoceptor stimulation contributes to the infarct size-limiting effect in IP.

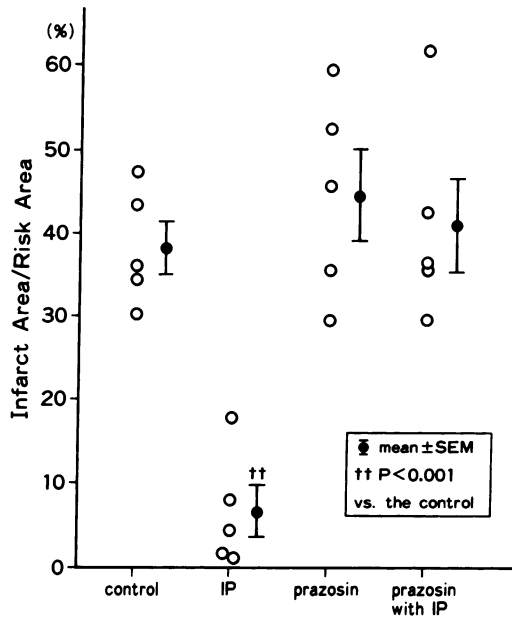


Figure 9. Infarct size in the control group ($n = 5$), the IP group ($n = 5$), the prazosin with IP group ($n = 5$), and the prazosin group ($n = 5$) in the conscious dog model. Infarct size was markedly decreased in the IP group. The infarct size-limiting effect of IP was completely abolished by administration of prazosin.

Discussion

The linkage between the infarct size-limiting effect and activation of ectosolic 5'-nucleotidase through α_1 -adrenoceptor activation in IP. IP has been the focus of intense study by basic and clinical investigators alike, because a number of laboratories have now confirmed that IP limits infarct size (1-3, 12-20). However, the subcellular mechanisms must be elucidated if IP is to be applied to the treatment of acute myocardial infarction.

Liu et al. (11) and Thornton et al. (12) have reported that adenosine A_1 receptor activation is involved in the infarct size-limiting effect of IP, and we have demonstrated that IP increases 5'-nucleotidase activity (17, 18). The present study shows that increases in ectosolic and cytosolic 5'-nucleotidase activity during IP are attributable to α_1 -adrenoceptor activation. Furthermore, when the increased ectosolic 5'-nucleotidase activity was inhibited by prazosin and AOPCP, the infarct size-limiting effect was abolished, indicating that increased ectosolic 5'-nucleotidase activity during IP contributes to the infarct size-limiting effect. This observation is consistent with previous studies. It has been reported that α_1 -adrenoceptor activation is intimately involved in attenuation of the severity of ischemia and reperfusion (35) and IP (17-22). The present results strongly suggest that α_1 -adrenoceptor stimulation mediates cardioprotection seen in IP, which is attributable to activation of ectosolic 5'-nucleotidase.

Nevertheless, before reaching any conclusion, we need to consider the other effects of augmentation of α_1 -adrenoceptor activity. Nathan and Feigl (36) have reported that α_1 -adrenoceptor stimulation during coronary hypoperfusion favorably affects intramyocardial flow distribution. Alterna-

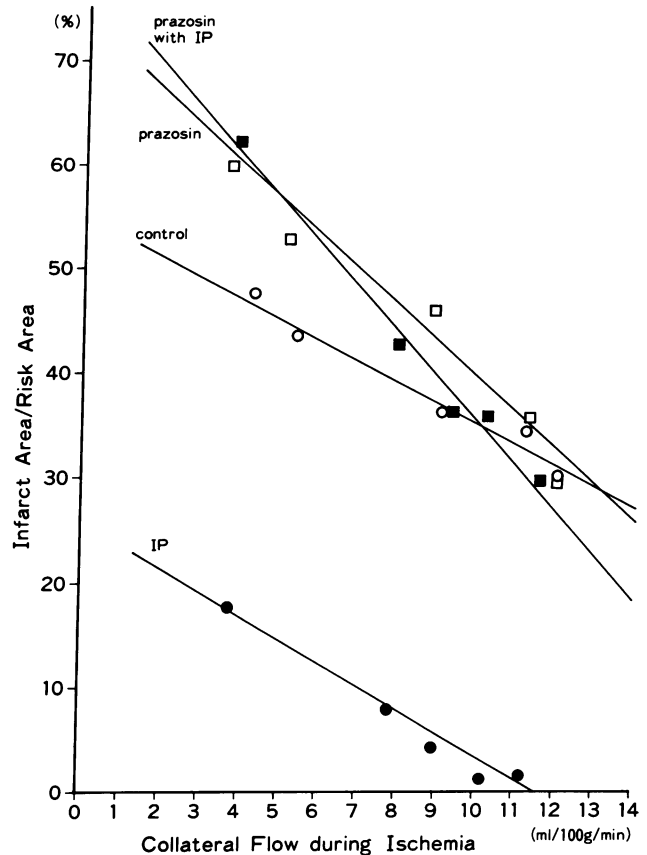


Figure 10. Plot of infarct size expressed as a percentage of the risk area and regional collateral flow during ischemia. In the control group (open circles, $n = 8$), the IP group (closed circles, $n = 10$), the AOPCP treatment with IP group (closed squares, $n = 8$), the AOPCP treatment group (open squares, $n = 9$), the AOPCP pretreatment with IP group (open triangles, $n = 6$), and the AOPCP during reperfusion with IP group (closed triangles, $n = 6$) there are inverse relations between normalized infarct area and collateral flow. Infarct size in the preconditioned hearts was smaller ($P < 0.001$) with respect to any given value for collateral blood flow than that in the control hearts. Infarct size was markedly increased ($P < 0.001$) in the prazosin with IP group compared with the IP group. Infarct size in the prazosin with IP group and the prazosin group were not different. Statistical significance was tested by ANCOVA.

tively, prazosin or methoxamine may affect collateral flow. Indeed, it is reported that α_2 -adrenoceptor activity regulates the function of collateral vessels (37). However, in the present study, neither prazosin nor methoxamine altered endomyocardial flow during ischemia. On the other hand, α_1 -adrenoceptor activation is known to increase the sensitivity of contractile proteins to Ca^{2+} in cardiomyocytes (38) and thus exert positive inotropism (39). However, this effect alone may not account for our data because an increase in myocardial oxygen demand would be expected to increase infarct size.

According to the data of Ytrehus et al. (19), protein kinase C is tightly linked to IP. We also have reported that activation of protein kinase C increases ectosolic and cytosolic 5'-nucleotidase activity in vitro (21, 22). Since protein kinase C is activated by ischemia per se (24) and by norepinephrine (23) released during brief periods of ischemia, IP may increase ectosolic and cytosolic 5'-nucleotidase activity via protein kinase C

activation. Activation of protein kinase C due to endogenous norepinephrine and translocation of protein kinase C from the cytosol to the cell membrane during ischemia may also increase 5'-nucleotidase activity. Although we have not elucidated the mechanisms whereby protein kinase C increases 5'-nucleotidase activity, we speculated that protein kinase C may change the characteristics of active site of ectosolic 5'-nucleotidase or induce a conformational change in the structure of 5'-nucleotidase.

The subcellular mechanisms of the infarct size-limiting effects in IP: a role of activation of ectosolic 5'-nucleotidase. As a potential subcellular mechanism of IP. Murry et al. (1, 5) of Jennings' laboratory proposed the hypothesis that reduced ATP depletion plays an essential role. Interestingly, Liu et al. (11) and Thornton et al. (12, 16) of Downey's laboratory reported that exposures to adenosine before and during sustained ischemia are responsible for the infarct size-limiting effect and that activation of adenosine A₁ receptors mediates IP. These two lines of hypothesis may culminate in the present result. IP activates ectosolic and cytosolic 5'-nucleotidase, which may make myocardium resistant to the sustained ischemia (40–43). Ectosolic and cytosolic 5'-nucleotidase are different enzymes, and we showed in this study that activation of ectosolic 5'-nucleotidase plays a more important role for cardioprotection in IP, because the infarct size-limiting effect of IP is attenuated by AOPCP, a selective inhibitor of ectosolic 5'-nucleotidase. However, present results do not deny the role of cytosolic 5'-nucleotidase (44, 45) in cardioprotection. This is because even with AOPCP, adenosine release is not completely attenuated in ischemic myocardium (46). If release of adenosine through cytosolic 5'-nucleotidase is also inhibited, infarct size would be increased compared with the untreated condition.

The present results do not necessarily indicate an increase in the whole amount of adenosine concentration in the myocardium and interstitial space. This is because the myocardial adenosine production during ischemia is determined by (a) the rate of enzymatic synthesis, i.e., activity of 5'-nucleotidase and S-adenosylhomocysteine (SAH)-hydrolase; (b) the rates of enzymatic degradation to inosine and enzymatic resynthesis to 5'-AMP, i.e., activity of adenosine deaminase and adenosine kinase; (c) the availability of substrates, i.e., 5'-AMP and SAH; and (d) the reuptake of adenosine into myocytes for substrate of the resynthesis of ATP (40–43). These factors aside from activation of 5'-nucleotidase may affect the whole amount of adenosine concentration in the myocardium. In the nonischemic myocardium, adenosine is generated by SAH-hydrolase (47), while adenosine production in the ischemic myocardium has been reported to be mainly attributable to 5'-nucleotidase (40–43). Although adenosine produced through ectosolic 5'-nucleotidase during sustained ischemia is increased due to activation of ectosolic 5'-nucleotidase in the IP group, the total amount of adenosine may not be increased if adenosine kinase and adenosine deaminase are activated and/or adenosine uptake rate is increased. Indeed, adenosine concentrations in the interstitial space (48) and the myocardial tissue (5) are decreased in the early phase of sustained ischemia in the IP group. Adenosine concentration of microenvironment around ectosolic 5'-nucleotidase attached to the myocytes may be increased. However, locally increased adenosine can be degraded immediately by the other factors, e.g., adenosine kinase, adenosine deaminase, and adenosine uptake rate. Furthermore, increases in ectosolic 5'-nucleotidase may play another role different from the augmentation of adenosine production. Ecto-

solic 5'-nucleotidase activity produces adenosine using extracellular AMP, and this conversion of extracellular AMP to adenosine may make it possible to convey endogenous adenosine into cardiomyocytes for the substrates of resynthesis of high energy phosphates. Adenosine A₁ receptor stimulation has been reported to inhibit norepinephrine release from the presynaptic vesicles (49) and to attenuate the beta-adrenoceptor-mediated inotropic response (50, 51), both of which are thought to be cardioprotective against ischemia. Besides, adenosine also inhibits Ca²⁺ influx through Ca²⁺ channels and Na⁺/Ca²⁺ exchanges (52, 53) into cardiomyocytes. Adenosine A₁ receptor activation has been reported to open K⁺ channels in cardiomyocytes (54), which hyperpolarizes cellular membrane and inhibits Ca²⁺ overload. Because Ca²⁺ overload during reperfusion is known to produce contractile dysfunction (55, 56) and even myocardial necrosis, attenuation of Ca²⁺ overload by adenosine may help to limit infarct size.

Clinical relevance. The findings in this study suggest two clinical applications for treatment of acute myocardial infarction. One strategy to limit infarct size is to find a method to increase 5'-nucleotidase activity. As we observed, protein kinase C activation induced by administration of alpha-adrenoceptor stimulants or phorbol esters may increase both 5'-nucleotidase activity (21–23, 35) and may attenuate contractile dysfunction (3, 37, 57) and infarct size (17, 19).

Another possibility is to enhance adenosine release. Administration of adenosine (58) or a potentiator of adenosine production, e.g., acadesine, dilazep, or dipyridamole (13, 59), may limit infarct size. Both strategies merit clinical investigation, although an enhanced understanding of the basic process involved in IP is necessary.

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