Ischemic Preconditioning Preserves Creatine Phosphate and Intracellular pH

Mitsugu Kida, MD; Hisayoshi Fujiwara, MD; Moriharu Ishida, MD; Chuichi Kawai, MD; Makoto Ohura, PhD; Iwao Miura, PhD; and Yoichi Yabuuchi, MD

Background. Ischemic preconditioning slows ATP depletion and ultrastructural damage during the final episode of ischemia. To define the influence of creatine phosphate (CP) and intracellular pH (pHi) on this effect, CP and pH were serially measured in porcine hearts without collateral circulation by using 31P-NMR spectroscopy and ultrastructural examination.

Methods and Results. Farm pigs weighing 12–15 kg were anesthetized with Fluothane. The control group underwent a single occlusion (20 minutes or 60 minutes); the preconditioned group underwent four episodes of 5-minute occlusion and 5-minute reperfusion followed by a sustained occlusion (20 minutes or 60 minutes). After ischemic preconditioning, CP increased to 115±11% (p < 0.05) of preischemic value and ATP decreased to 84±8% (p < 0.05) of preischemic value, but pH, returned to preischemic value. At 5 and 10 minutes of sustained ischemia, CP was significantly preserved in the preconditioned group (control group, 19±3%; versus preconditioned group, 29±4% at 5 minutes; control group, 5±3% versus preconditioned group, 11±3% at 10 minutes; p < 0.05). At 15 and 20 minutes of sustained ischemia, ATP was significantly preserved in the preconditioned group (control group, 64±3% versus preconditioned group, 73±3% at 15 minutes; control group, 51±7% versus preconditioned group, 62±2% at 20 minutes; p < 0.05). At 10, 15, 20, and 25 minutes of sustained ischemia, pH, was significantly higher in the preconditioned group (control group, 6.5±0.05 versus preconditioned group, 6.7±0.1 at 10 minutes; control group, 6.3±0.05 versus preconditioned group, 6.6±0.06 at 15 minutes; control group, 6.1±0.1 versus preconditioned group, 6.4±0.1 at 20 minutes; control group, 6.0±0.2 versus preconditioned group, 6.3±0.1 at 25 minutes; p < 0.05). Ultrastructural changes were milder in the preconditioned group at 20 minutes of sustained ischemia.

Conclusions. In addition to ATP and ultrastructure, preconditioning preserved CP and pH, during sustained ischemia. These protective effects might be due to overshoot phenomenon of CP and/or reduced ATP consumption. The relatively longer period of preservation of pH, which probably is the result of reduced ATP consumption, indicates its greater contribution to reducing infarct size than that of CP and ATP. (Circulation 1991;84:2495–2503)

Myocardial infarction is often preceded by multiple episodes of angina pectoris. With recent advances in thrombolytic therapy and coronary angioplasty, it is of clinical interest whether preceding ischemic episodes (ischemic preconditioning) alter the time course of myocardial injury in sustained final ischemia in humans.

Ischemic preconditioning has been reported to reduce infarct size after 40–60 minutes of sustained ischemia in dogs, and in pigs. Recently, Murry et al measured ATP and its metabolites by high-performance liquid chromatography to clarify the mechanisms of the protective effect of ischemic preconditioning in canine hearts. Their data revealed reduction of glycolysis, preservation of ATP, and preservation of ultrastructure during sustained ischemia. However, creatine phosphate (CP) and intracellular pH (pHi) were not analyzed. Because myocardial ischemic injury is closely related to CP and intracellular acidosis, in addition to ATPs we used 31P-NMR spectroscopy (MRS), which enabled us to analyze the time course change not only of ATP but also CP and pH, noninvasively in the same heart.

The purpose of this study is to assess the influence of CP and pH, on the protective effect of ischemic preconditioning by using 31P-MRS in porcine hearts without significant collateral circulation.
Methods

Animal Model

The experiments conformed to the guiding principles of the American Physiological Society regarding the use of laboratory animals. Twenty-five farm pigs weighing 12–15 kg were sedated with ketamine (15 mg/kg), anesthetized with sodium pentobarbital (15 mg/kg), and divided randomly into a control group (n=14) and an ischemic preconditioned group (n=11). Via tracheotomy, the pigs were intubated and connected to a mechanical respirator. Respiratory rate was fixed to 18 respirations per minute and tidal volume was adjusted from 13 to 17 ml/kg using a mixed inhalation gas of oxygen, nitrogen, and carbon dioxide (0.24:0.75:0.01) to keep arterial blood gas within physiological range. Anesthesia was maintained with Fluothane (0.5–1.5%). The carotid artery was cannulated, and aortic pressure and heart rate were recorded continuously on a polygraph system (Nihon Kohden Inc., Tokyo). Via a median sternotomy, the heart was exposed and suspended in a pericardial cradle. A 3-mm segment of the distal one third of the left anterior descending coronary artery (LAD) was dissected free from the surrounding tissue and an air occluder was positioned around it. A brief test occlusion was done for a few seconds by inflating the air occluder, and the risk area was confirmed by the cyanotic color. A 17-mm-diameter surface coil tuned to 32.7 MHz was placed in the center of the risk area, and the pigs were moved into an in vivo spectrometer.

$^{31}$P-NMR Spectroscopy

$^{31}$P spectra were obtained using a BEM-250/80 in vivo spectrometer (Otsuka Electronics Inc., Philadelphia, Pa.) with a 1.9-T, 31-cm bore superconducting magnet. The spectrometer was interfaced with the surface coil. The magnetic field homogeneity in the region of the surface coil was optimized by shimming on the proton signal, using 12-channel shim supply to make the width less than 0.5 ppm. Respiration and arterial pressure–gated spectra were obtained at end inspiration and peak systole, accumulating 90 free induction decays (FID) for each spectrum over 5 minutes. The pulse was 90° broad band (15 μsec) with a 3.3±0.2-second cycle time; sweep width was 3 kHz. The FID was multiplied by an exponential to 10-Hz line broadening to improve the signal-to-noise ratio. After baseline measurement (for 70 minutes), the control group (n=14) underwent a single sustained occlusion of the LAD by inflation of the air occluder. After baseline measurement (for 30 minutes), the preconditioned group (n=11) underwent four episodes of 5-minute occlusion and 5-minute reperfusion followed by a sustained occlusion of the LAD. $^{31}$P-NMR spectroscopy was continued throughout the experiment. Each group was subdivided into 20-minute and 60-minute sustained ischemia subgroups.

Postmortem Tissue Preparation and Sectioning

In each pig, within 2 minutes after excision of the heart, three transmural tissues of the left ventricular wall were taken from the center of the ischemic areas and divided into the endocardial and epicardial halves. Each tissue was cut into thicknesses of about 1 mm in a plane parallel to the atrioventricular groove, and large sections of 2.0×4.0×1.0 mm were made. In the same way, three nonischemic tissue sections were also made to serve as a control. Twelve sections per pig were fixed in cold 2% glutaraldehyde for 3 hours, postfixed in osmium tetroxide for 8 hours, dehydrated in graded series of ethanol and propylene oxide, embedded in Spurr medium, and cut into semithin (1-μm-thick) sections with the use of an Ultracut N (Reichert-Jung Co., Vienna) ultramicrotome. These sections were stained with toluidine blue; each block was trimmed, and each representative portion served for electron microscopy. Ultrathin sections (approximately 0.08 μm thick) were mounted on plain copper grids and stained with uranyl acetate and lead citrate and examined on an H-600 transmission electron microscope (Hitachi, Tokyo).

Identification and Quantification of Risk Area

After tissue sampling, the right and left coronary arteries were cannulated. Keeping the air occluder inflated, 1% monastral blue dye (Sigma) was injected into the coronary artery at a pressure of 90 mm Hg. The hearts were then fixed in 10% formalin. After that, the hearts were sliced into 5-mm serial sections in a plane parallel to the atrioventricular groove. Trimmed of the right ventricular tissue, the slices were then weighed and the apical surface was photographed. The risk area was identified, traced from enlarged projections of the photographic slide of each ventricular slice, and calculated with a digitizer. The percent risk area (risk area divided by area of slice) was calculated for each slice, and the average value for each slice was multiplied by the weight. The weight of the risk area was summed and divided by the weight of the left ventricle (LV) to yield the percent of the risk area of LV.

Assessment of $^{31}$P Spectra

Each spectrum was an accumulation of 90 FID over 5 minutes; therefore, it represented the mean value of each metabolite over the 5 minutes. Tissue levels of ATP, CP, and inorganic phosphate (P$_i$) were estimated by integrating areas under individual peaks by using a computer program (MEAS1, Graphtec Co., Tokyo) and digitizer. Time course changes after ischemia were expressed as percentages relative to preischemic values. Intracellular pH was calculated from the chemical shifts of the major P$_i$ peak through the Flaherty equation. CP was used as a reference for chemical shift of P$_i$, and after disappearance of CP, a small glass tube containing hexamethylenephosphoric triamide just above the surface coil was used as a reference.
Additional Experiment

To determine whether ischemic preconditioning itself might cause necrosis in the porcine heart, an additional five pigs underwent the same preconditioning procedure (four episodes of 5-minute ischemia), with the fourth reperfusion period extended to 2 hours. The heart was then excised, and 1% monastral blue dye was injected into the right and left coronary arteries at a pressure of 90 mm Hg. Simultaneously, the distal one third of the LAD, cannulated at the level of the air occluder, was perfused with 1% triphenyltetrazolium chloride (TTC) at the same pressure. After fixation in formalin, the heart was sliced and the percent risk area of LV was calculated as mentioned above. The slices were also examined histologically with Masson’s trichrome stain and hematoxylin-eosin stain.

Statistical Analysis

Comparisons between the two groups were made by Student’s t test for each metabolite and pH. Comparisons across time in the same animals were made by repeated-measures analysis of variance. Comparisons among the groups were made by one-way ANOVA for hemodynamics and percent risk area. Statistical significance was achieved at a value of \( p<0.05 \). Data are presented as mean±SD.

Results

Mortality

In the control group (\( n=14 \)), one of six pigs in the 20-minute ischemia subgroup and three of eight pigs in the 60-minute ischemia subgroup died prematurely. In the preconditioned group (\( n=11 \)), none of five pigs in the 20-minute ischemia subgroup and one of six pigs in the 60-minute ischemia subgroup died prematurely. The cause of premature death was ventricular fibrillation in all cases. These animals were excluded from analysis.

Identification and Quantification of Risk Area

The risk area of the anterior wall of the LV lacked monastral blue dye, indicating that there was no significant collateral circulation in the risk area (Figure 1). The percent of risk area of the LV was 13.7±1.3%, 12.9±1.4%, 13.6±1.2%, and 13.1±1.3% for 20-minute ischemia control, 60-minute ischemia control, 20-minute ischemia preconditioned, and 60-minute ischemia preconditioned groups, respectively. There was no significant difference of the percent of risk area among the groups.

Hemodynamic Changes

Heart rate, systolic aortic pressure, and diastolic aortic pressure were expressed as mean values for 5 minutes (Table 1). There was no significant difference of hemodynamic data among the groups, so comparison of each metabolite and pH was made between the 60-minute ischemia subgroups.

\(^{31}\)P-NMR Spectroscopy

During baseline measurement, CP, ATP, \( P_i \), and pH did not change significantly, so the time course...
TABLE 1. Hemodynamic Data of Pigs

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
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<td>58±7</td>
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There were no significant differences in hemodynamics among the groups. SAP, systolic aortic pressure; DAP, diastolic aortic pressure; C60/C20, 60/20 minutes of ischemia in control group; P60/P20, 60/20 minutes of ischemia in preconditioned group; ADD, additional experiment group (data in parentheses are those 5 minutes after fourth occlusion).

Results are expressed as mean±SD; p<0.05.

Changes of CP, ATP, and P1 were expressed as percentages relative to the values just before ischemia. Typical time course changes of 31P-NMR spectra during sustained ischemia in the control group are shown in Figure 2.

CP decreased to 19±3% and 5±3% after 5- and 10-minute ischemia, respectively, and disappeared early in the control group. In the preconditioned group, CP decreased to 18±6% (p<0.01) during the first 5-minute occlusion. The CP level during the second (21±9%), third (23±5%), and fourth (22±5%) 5-minute occlusions was not different from that during the first occlusion. CP returned to the preischemic value at the first and second 5-minute reperusions. CP then returned over the preischemic value at the third and fourth 5-minute reperusions (113±8% and 115±11%, respectively, p<0.05). CP decreased to 28±4% and 11±3% at 5- and 10-minute sustained ischemia, respectively, and disappeared early (Figure 3). For the first 10 minutes of sustained ischemia, CP was significantly (p<0.05) higher in the preconditioned group.

ATP decreased to 90±3%, 79±5%, 64±7%, and 51±6% after 5, 10, 15, and 20 minutes of ischemia, respectively, and disappeared after 60-minute ischemia in the control group. In the preconditioned group, ATP decreased to 91±7% (p<0.05) during the first 5-minute occlusion. ATP level during the second (89±13%), third (85±16%), and fourth (80±12%) 5-minute occlusions was not different from that during the first occlusion. During each 5-minute reperfusion, ATP did not change significantly compared with that during the first 5-minute occlusion. After the fourth 5-minute reperfusion, ATP was 84±8% and then decreased to 83±4%, 80±3%, 73±3%, and 62±2% after 5, 10, 15, and 20 minutes of final ischemia, respectively, and disappeared after 60-minute ischemia. In sustained ischemia, ATP decreased more slowly in preconditioned hearts and became significantly higher at 15 and 20 minutes of ischemia than in the control hearts, though it was lower at 5 minutes of sustained ischemia. In the latter half of sustained 60-minute ischemia, there was no significant difference of ATP levels between the two groups (Figure 4).

P1 increased rapidly and reached over three times the baseline level during sustained 60-minute isch-
emia in the control group. In the preconditioned group, P, increased to 243±44% (p<0.01) during the first 5-minute occlusion. P, during the second, third, and fourth 5-minute occlusions was not significantly different from that during the first 5-minute occlusion. During each 5-minute reperfusion, it was not significantly different from preischemic value, and was 126±27% of preischemic value during the fourth reperfusion. During 60-minute sustained ischemia, though it tended to increase somewhat more slowly in the preconditioned group than in the control group, there was no significant difference between the two groups (Figure 5).

Intracellular pH was 7.2±0.1 before ischemia and gradually fell to approximately 6.0 within 40 minutes of sustained ischemia in the control group. In the preconditioned group, pH, was 7.1±0.1 before ischemia and fell to 6.9±0.1, 6.8±0.1, 6.9±0.1, and 6.9±0.1 during the first, second, third, and fourth 5-minute occlusions, respectively, and returned to the preischemic value during each reperfusion. It was 7.2±0.1 after the fourth reperfusion and fell toward 6.0 more gradually than in the control group during 60 minutes of sustained ischemia. At 10, 15, 20, and 25 minutes of sustained ischemia, pH, was significantly higher in the preconditioned group (control group, 6.5±0.05 versus preconditioned group, 6.7±0.1 at 10 minutes; control group, 6.3±0.05 versus preconditioned group, 6.6±0.1 at 15 minutes; control group, 6.1±0.1 versus preconditioned group, 6.4±0.1 at 20 minutes; control group, 6.0±0.2 versus preconditioned group, 6.3±0.1 at 25 minutes; p<0.05; Figure 6).

Electron Microscopic Findings

In each group, there was no significant difference of hemodynamic and metabolic data between 20-minute and 60-minute sustained ischemia subgroups for 20 minutes after occlusion of the LAD; therefore, ultrastructural changes were compared between the two subgroups.

![Graph showing time course change of ATP in porcine hearts. ATP decreased to 90±3%, 79±5%, 64±7%, and 51±6% after 5, 10, 15, and 20 minutes of ischemia, respectively, and disappeared after 60 minutes of ischemia in the control group (closed circles). In the preconditioned group, ATP decreased to 84±8% (p<0.05) after four episodes of 5-minute occlusion (O1, O2, O3, O4) and reperfusion, and then decreased to 83±4%, 80±3%, 73±3%, and 62±2% after 5, 10, 15, and 20 minutes of final ischemia, respectively, and disappeared after 60 minutes of ischemia (open circles). In sustained ischemia, ATP decreased more slowly in preconditioned hearts than in control hearts. ATP became significantly higher at 15 and 20 minutes of ischemia in preconditioned hearts than in control hearts, though it was lower at 5 minutes of sustained ischemia. In the latter half of sustained 60-minute ischemia, there was no significant difference of ATP levels between the two groups.](http://circ.ahajournals.org/content.figshare/cimages/579x765_50953.png)
At 20 minutes of sustained ischemia in the control group, mild clumping and margination of nuclear chromatin, mild swelling and relatively clear matrix in mitochondria, and moderate decrease in glycogen were observed in the endocardial half (Figure 7, panel A1). In the epicardial half, minimal clumping and margination of nuclear chromatin, minimal change in mitochondria, and mild decrease in glycogen were observed (Figure 7, panel A2). Morphological changes were milder in the epicardial half than in the endocardial half.

At 20 minutes of sustained ischemia in the preconditioned group, clumping and margination of nuclear chromatin and mitochondrial changes were milder than in the control group both in the endocardial and the epicardial halves (Figure 7, panels B1 and B2). At 60 minutes of sustained ischemia in both groups, marked clumping and margination of nuclear chromatin, depletion of glycogen granules, very wide I band, disruption of myofibrils, severe edema, and electron-dense granules in mitochondria were observed transmurally, indicating irreversible injury to the myocyte (Figure 7, panels A3 and B3).

Additional Experiment and Necrosis in Porcine Hearts

In the additional five pigs undergoing four episodes of 5-minute ischemia and a fourth reperfusion of 2 hours, the percent risk area of the LV was 13.7±1.3%, almost the same as those of the control and preconditioned groups. There was no TTC staining defect in the risk area. Histological examination using Masson’s trichrome stain and hematoxylin-eosin stain revealed neither coagulation necrosis nor contraction band necrosis in the risk area.

Discussion

The findings in the present study indicate that the ischemic preconditioning has a salutary effect not only on ATP and ultrastructure but also on CP and pH during subsequent sustained ischemia.

$^{31}$P-MRS in Regional Ischemia

$^{31}$P-MRS has been used to assess the energy metabolism of total ischemia in vitro$^{9,12}$ and of regional ischemia in vivo.$^{13-16}$

To take enough risk area for the 1.7-cm-diameter surface coil to collect signals only from the ischemic area, we used 12–15-kg pigs without collateral coronary circulation$^{10,11,17,18}$ in a larger-bore (31 cm in diameter) superconducting magnet.

We also used respiration and blood pressure gating because the heart changes its position in the thorax during the respiratory cycle, and the levels of CP and ATP change during the cardiac cycle.$^{19,20}$ This en-
abled us to keep the position of the heart constant to the surface coil.21–23

In contrast with our 31P-MRS data, earlier depletion of ATP after coronary artery ligation has been reported in the subendocardium of dogs4–7 and pigs17 by using high-performance liquid chromatography. In our study, the sampling was transmural and rather weighted toward the epicardium because of the position and the small diameter of the surface coil. Thus, the relatively slower depletion of ATP in our study may be caused by sampling epicardial tissue, which was damaged less profoundly than endocardial tissue even in porcine hearts, as shown by electron microscopy in this study and in our previous study.11,18 However, McDonough et al17 also demonstrated earlier depletion of ATP in the subepicardium of pigs than do our data. Their pigs were paced at 160 beats per minute and systolic aortic pressure was over 100 mm Hg during coronary artery occlusion, whereas in our pigs, heart rate was about 100 beats per minute and systolic aortic pressure was about 90 mm Hg. Therefore, rate pressure products (RPP) in their pigs and in ours were over 16,000 mm Hg/min and about 9,000 mm Hg/min, respectively. The relatively smaller depletion of ATP in our present study may be explained by the difference of RPP. Indeed, our previous 31P-MRS data under pentobarbital anesthesia22 showed higher RPP and earlier depletion of ATP than our present data under anesthesia with Fluothane.

Ischemic Preconditioning

Our data revealed that four episodes of 5-minute occlusion caused neither cumulative depletion of ATP and CP nor cumulative change in P$_i$ and pH$_i$. The lack of cumulative ATP depletion after intermittent brief episodes of ischemia is in agreement with other reports.24–25 After ischemic preconditioning, CP increased to 115±11% ($p<0.05$) of preischemic value (overshoot phenomenon8,26,27), and ATP de-
increased to 84±8% (p<0.05) of preischemic value, although little overshoot of CP was seen at the first and second 5-minute occlusions.

The additional experiment revealed that four episodes of 5-minute ischemia did not cause necrosis in porcine hearts without collateral circulation. Two hours of reperfusion was thought to be enough for detecting necrosis, according to previous studies. Moreover, preservation of ATP, 4.0 μmol/g wet wt; therefore, a 15% increase (overshoot) in CP may be sufficient to make up for the 16% loss of ATP. Therefore, the overshoot phenomenon of CP might contribute in part to the preservation of CP and ATP in sustained ischemia. However, it cannot solely account for the fact that ATP was significantly preserved in the preconditioned hearts at 15 and 20 minutes of sustained ischemia when most of the CP had been depleted. ATP production in such virtually zero-flow ischemia of porcine hearts depends entirely on anaerobic glycolysis. Therefore, preservation of ATP after disappearance of CP must have come from increased glycolysis or reduced consumption of ATP. Increased glycolysis is not likely; Murry et al reported reduced glycolysis in sustained ischemia after ischemic preconditioning.

In our present study, pH fell more slowly in preconditioned myocardium. This may be due to reduced production of protons. Gevers et al. described that in the presence of enough Mg++ to complex all of the ADP and ATP molecules, the following equations are most representative of intracellular events of glycolysis:

\[
glucose + 2MgADP^{-} + 2P_{i}^{2-} \rightarrow 2\text{lactates}^{-} + 2MgATP^{2-} + MgATP^{2-} \rightarrow MgADP^{-} + P_{i}^{2-} + H^{+}
\]

Thus, ATP hydrolysis itself is the principal direct means to produce protons. However, for ATP hydrolysis following CP hydrolysis, the net production of protons is zero, because CP hydrolysis consumes protons:

\[
H^{+} + CP^{2-} + MgADP^{-} \rightarrow MgATP^{2-} + \text{creatinine}
\]

\[
MgATP^{2-} \rightarrow MgADP^{-} + P_{i}^{2-} + H^{+}
\]

Therefore, preservation of pH in preconditioned myocardium may be due partly to increased CP hydrolysis after overshoot, but, after disappearance of most of the CP, that is, at 15, 20, and 25 minutes of sustained ischemia, it should be due to reduced ATP hydrolysis. Murry et al. revealed that ATP consumption from 5 to 20 minutes of sustained ischemia was less in the preconditioned group than in the control group, though it was not different between the two groups during the first 5 minutes of sustained ischemia. Thus, reduced ATP consumption appears to be the most important mechanism in the preservation of both ATP and pH after disappearance of CP.

By lowering pH, others observed relaxation of myofibrils, clumping of nuclear chromatin, and mitochondrial change. Preservation of ultrastructure at 20 minutes of sustained ischemia in preconditioned hearts might be secondary to preservation of pH as well as preservation of ATP. Preservation of pH and ATP might also be beneficial to maintain the activity of enzymes and organelles.

Possible Mechanisms of Reducing Infarct Size

The preservation of CP and ATP may not be the cause of reducing infarct size but the result of protection of an energy-generating system in the heart. Because Li et al. reported that a single 5-minute occlusion (when little overshoot of CP would be expected from our data and those of Hoffmeister et al.) was sufficient to fully precondition the heart, this might suggest little contribution of overshoot of CP. In addition, in the previous studies, preconditioning reduced infarct size dramatically and was equivalent to 15–30-minute reduction of ischemic time, whereas in our data and those of Murry et al., neither ATP nor CP curves were shifted horizontally anywhere near this much. They showed at best a 5-minute shift. These data seemed to provide a powerful argument against ATP and CP preservation as being the mechanism of protection. Only the pH curves showed a shift consistent with the magnitude of protection seen.

Preservation of pH, which probably is the result of reduced ATP consumption as discussed above, lasted for 20 minutes or so and might be able to retard and reduce necrosis dramatically through shortening the duration that myocytes were exposed under a critical level of pH. The basis of the mechanism of the protection against necrosis may be reduced ATP consumption, but other factors, such as reduced catabolite accumulation, must be taken into consideration.

Acknowledgments

We would like to thank Yumiko Yamamoto for assistance in preparing the manuscript and Maria Tanaka for reading it.

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**KEY WORDS** • myocardial ischemia • ATP • nuclear magnetic resonance • intracellular pH
Ischemic preconditioning preserves creatine phosphate and intracellular pH.
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Circulation. 1991;84:2495-2503
doi: 10.1161/01.CIR.84.6.2495

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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