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Energy Metabolism After Ischemic Preconditioning in Streptozotocin-Induced Diabetic Rat Hearts

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Objectives. **The aim of this study was to compare the cardioprotective effects of preconditioning in hearts from streptozotocin-induced diabetic rats with its effects in normal rat hearts.**

Background. **The protective effect of ischemic preconditioning against myocardial ischemia may come from improved energy balance. However, it is not known whether preconditioning can also afford protection to diabetic hearts.**

Methods. **Isolated perfused rat hearts were either subjected (preconditioned group) or not subjected (control group) to preconditioning before 30 min of sustained ischemia and 30 min of reperfusion. Preconditioning was achieved with two cycles of 5 min of ischemia followed by 5 min of reperfusion.**

Results. **In the preconditioned groups of both normal and diabetic rats, left ventricular developed pressure, high energy phosphates, mitochondrial adenosine triphosphatase and adenine nucleotide translocase activities were significantly preserved after ischemia-reperfusion; cumulative creatine kinase release was smaller during reperfusion; and myocardial lactate content was significantly lower after sustained ischemia. However, cumulative creatine kinase release was less in the preconditioned group of diabetic rats than in the preconditioned group of normal rats. Under ischemic conditions, more glycolytic metabolites were produced in the diabetic rats (control group) than in the normal rats, and preconditioning inhibited these metabolic changes to a similar extent in both groups.**

Conclusions. **The present study demonstrates that in both normal and diabetic rats, preservation of mitochondrial oxidative phosphorylation and inhibition of glycolysis during ischemia can contribute to preconditioning-induced cardioprotection. Furthermore, our data suggest that diabetic myocardium may benefit more from preconditioning than normal myocardium, possibly as a result of the reduced production of glycolytic metabolites during sustained ischemia and the concomitant attenuation of intracellular acidosis.**

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A brief ischemic insult followed by reperfusion renders the heart more resistant to myocardial infarction from a subsequent ischemic insult. Since the initial description by Murry et al. (1), the beneficial effects of ischemic preconditioning have been observed in dogs (1,2), rabbits (3), pigs (4) and rats (5). Recently, evidence was also presented for preconditioning in the human myocardium, thereby emphasizing the potential clinical significance of its cardioprotective effects (6). These protective effects may involve several receptor-mediated mechanisms triggered by adenosine (7), catecholamines (8) and bradykinin (9), as well as signal transduction through Gi proteins or protein kinase C (10). The effector site of this cardioprotection has not been clearly elucidated. However, one of the most likely mechanisms may be an alteration in myocardial energy metabolism.

Diabetes mellitus is a disorder of carbohydrate, lipid and protein metabolism that affects many organs. In addition to contractile abnormalities, this disease causes disturbances in the function of cardiac subcellular organelles, including the sarcolemma, sarcoplasmic reticulum and mitochondria (11). In particular, diabetes is associated with several abnormalities in energy metabolism (12). In view of the well established cardioprotective effects of ischemic preconditioning, it is of interest to determine whether preconditioning can also afford similar cardioprotection in diabetic hearts. In the present study, we therefore examined the cardioprotective effects of ischemic preconditioning on myocardial contractile function, high energy phosphate (HEP) levels and mitochondrial enzyme function in streptozotocin-induced diabetic rats and compared them with those in age-matched normal rats. We also measured myocardial glycogen and lactate content to evaluate the effect of anaerobic glycolysis on energy production and myocardial damage.

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Abbreviations and Acronyms

ADP = adenosine diphosphate
AMP = adenosine monophosphate
ANOVA = analysis of variance
ATP = adenosine triphosphate
CK = creatine kinase
CP = creatine phosphate
HEP = high energy phosphate
LVDP = left ventricular developed pressure
NADH = reduced form of nicotinamide adenine dinucleotide

Methods

Animal preparation. Male Sprague-Dawley rats weighing 140 to 160 g were made diabetic by a single intraperitoneal injection of streptozotocin (70 mg/kg body weight), which was dissolved in citrate buffer, pH 4.5. Diabetic animals were maintained on normal chow throughout the experimental period. Four weeks after streptozotocin injection, diabetic rat hearts were used as the diabetic group characterized in Table 1 (13). Age-matched normal male Sprague-Dawley rats were used as a control group. The isolated heart preparation was described previously (14). Briefly, rats were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg) and heparinized. The hearts were excised rapidly and retrogradely perfused through the aorta at a constant perfusion pressure of 80 mm Hg with Krebs-Henseleit buffer, consisting of 118 mmol/liter NaCl, 4.7 mmol/liter KCl, 1.2 mmol/liter $MgSO_4$, 1.2 mmol/liter KH_2PO_4, 1.8 mmol/liter $CaCl_2$, 25 mmol/liter $NaHCO_3$ and 11 mmol/liter glucose, at pH 7.4, oxygenated with 95% oxygen and 5% carbon dioxide at 37°C. The atria were removed and the hearts were paced at 270 beats/min. A water-filled latex balloon inserted into the left ventricle was used to measure left ventricular developed pressure (LVDP). The left ventricular end-diastolic pressure was adjusted to 7 mm Hg during the initial equilibration.

Experimental design. All hearts were equilibrated for 20 min, subjected to 30 min of global normothermic (37°C) ischemia by clamping the aortic line and then reperfused for 30 min. The hearts were divided into four groups: 1) normal control group; 2) normal preconditioned group; 3) diabetic control group; and 4) diabetic preconditioned group. In the preconditioned group, preconditioning was achieved with two cycles of 5 min of global ischemia followed by 5 min of reperfusion before 30 min of sustained ischemia (14).

Table 1. Comparison of Diabetic and Normal Rats

	Normal Rats	Diabetic Rats
Body weight (g)	280 ± 8.9	197 ± 9.8*
Heart weight (g)	1.02 ± 0.05	0.75 ± 0.04*
Heart weight/body weight (mg/g)	3.71 ± 0.28	3.86 ± 0.24
Plasma glucose (mg/dl)	106 ± 2.5	449 ± 9.0*

*$p < 0.001$ compared with group of normal rats (n = 12). Data are presented as mean value ± SEM.

Creatine kinase release. Creatine kinase (CK) activity in the coronary effluent was measured after equilibration and preconditioning, as well as after 5 min, 10 min, 20 min and 30 min of reperfusion in each group. In addition, coronary effluent during the 30 min of reperfusion was pooled and cumulative CK release was determined for an estimation of myocardial damage. Creatine kinase activity was assayed spectrophotometrically at 37°C according to Rosalki's procedure (14).

Measurement of adenine nucleotides, creatine phosphate and creatine. Hearts were freeze-clamped with Wollenberger tongs, prechilled in liquid nitrogen, after equilibration (baseline), 30 min of ischemia or 30 min of reperfusion. The hearts were kept in liquid nitrogen and then lyophilized overnight. Forty-sixty milligrams of lyophilized tissue was homogenized in 0.6 N ice-cold perchloric acid and centrifuged at 2,000 rpm for 10 min at 4°C. The supernatants were neutralized with KOH to pH 5.0 to 7.0. After 10 min, the extracts were centrifuged to remove the $KClO_4$, and the supernatants were used for the assays. Creatine, creatine phosphate (CP), adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were measured by high performance liquid chromatography (LC-9A liquid chromatograph, Shimadzu, Kyoto, Japan) with a column of STR ODS-M (Shimadzu) and are presented as μmol/g dry weight (14). Energy charge was calculated from the formula (ATP + 0.5 × ADP)/(ATP + ADP + AMP).

Glycogen content. Myocardial glycogen content was measured enzymatically, according to the methods reported by Brodal and Gehrken (15), with some modifications. Briefly, 10 mg of lyophilized heart tissue, which was prepared for adenine nucleotide assay after equilibration (baseline) and 30 min of ischemia, was dissolved in 500 μl of 5.4 N KOH at 100°C for 20 min. The sample was neutralized with HCl to pH 7.0, and 100 μl of the sample was incubated with 80 μl of 50 mmol/liter acetate buffer (pH 4.6) and 20 μl of 2 IU/liter amyloglucosidase at 37°C for 60 min. The glycogen content of the sample was estimated by measuring the glucose concentration in the reaction mixture.

Lactate production. Myocardial lactate content was measured enzymatically using the Detaminer LA kit (Kyowa Medics Co. Ltd., Tokyo, Japan). Aliquots of the supernatant prepared for the adenine nucleotides assay (see above description) were added to a phosphate-buffered reaction mix containing 0.9 mmol/liter *N*-ethyl-*N*-(3-methylphenyl)-*N′*-acetylethylenediamine, 0.7 IU/ml lactate oxidase, 3.4 IU/ml peroxidase and 0.4 mmol/liter 4-aminoantipyrine, at pH 6.4. The lactate content was determined spectrophotometrically at 37°C by measuring the quinone content at 555 nm.

Mitochondrial isolation. Heart mitochondria were isolated from hearts different from those used for the adenine nucleotides assay, 20 min after equilibration or 30 min after ischemia-reperfusion. The isolation method was similar to that reported by Sordahl and Stewart (16), with some modification. At the

end of the experimental protocol, the heart was immediately immersed in ice-cold phosphate-buffered saline. The ventricular myocardium was isolated, rinsed in ice-cold potassium phosphate buffer consisting of 25 mmol/liter K_2HPO_4, 25 mmol/liter KH_2PO_4 and 1 mmol/liter EDTA, at pH 7.2, and minced into very small pieces with scissors in 1.6 ml of potassium-phosphate buffer containing 0.5 mmol/liter phenylmethanesulfonyl fluoride. The minced heart tissue was homogenized with a Polytron PT 10-35 tissue processor (Kinematica Instrument) three times at a setting of 7 for 10 s, and the homogenate was centrifuged at 27,000*g* for 10 min. The supernatant was discarded and the pellet was suspended in isolation medium (5% wt/vol) consisting of 0.18 mol/liter KCl, 5 mmol/liter Tris-HCl and 10 mmol/liter EGTA, at pH 7.2, using a Teflon-glass homogenizer. The suspension was centrifuged at 1,000*g* for 10 min, and the supernatant was centrifuged at 12,000*g* for 10 min. The mitochondrial pellet was washed once and resuspended in a 550-μl low salt medium consisting of 20 mmol/liter HEPES, 1.0 mmol/liter $MgCl_2$ and 2.0 mmol/liter EGTA, at pH 7.0. The protein concentration in the mitochondrial suspension was determined by the Lowry method (17).

Measurement of mitochondrial enzyme activity. The mitochondrial NADH cytochrome *c* oxidoreductase activity was assayed spectrophotometrically at 30°C by measuring the initial rate of cytochrome *c* reduction at 550 nm in the presence and absence of rotenone (15 μmol/liter) (18). Briefly, the mitochondria in the low salt medium were sonicated twice for 5 s using an ultrasonic disrupter (model UR200P, Tomy Seiko Co. Ltd., Tokyo, Japan). Aliquots of the sonicated mitochondria were added to 1 ml of a reaction buffer containing 0.1 mmol/liter cytochrome *c*, 0.3 mmol/liter KCN and 50 mmol/liter KH_2PO_4, at pH 7.5.

The mitochondrial ATPase activity was measured spectrophotometrically by coupling the reaction to a pyruvate kinase and lactate dehydrogenase system, according to the method of Lowe (19). Briefly, aliquots of the sonicated mitochondria were added to 1 ml of a reaction buffer containing 5 mmol/liter phosphoenol pyruvate, 50 mg pyruvate kinase, 50 mg lactate dehydrogenase, 25 mmol/liter Tris/H_2SO_4, 5 mmol/liter $MgSO_4$, 0.35 mmol/liter NADH and 2.5 mmol/liter ATP, at pH 8.0. Mitochondrial ATPase activity was determined at 30°C by monitoring the decline of NADH oxidation at 340 nm in the presence and absence of oligomycin (10 ng/ml).

A modified procedure of Duan and Karmazyn was used for the measurement of adenine nucleotide translocase (20). [^{14}C]Adenosine disphosphate (50 nmol, specific activity 57.1 mCi/mmol, New England Nuclear Corp.) was added to a reaction buffer consisting of 110 mmol/liter KCl, 1 mmol/liter EDTA and 20 mmol/liter Tris-HCl, at pH 7.4, and 500 μg mitochondrial protein. The reaction was carried out at 0°C and stopped after 60 s by the addition of 0.1 mmol atractyloside. The reaction mixture was centrifuged at 25,000*g* for 10 min and the supernatant was discarded. The pellet was washed twice with ice-cold reaction buffer and then dissolved in 500 μl of 0.012 N NaOH, 1.2% mercaptoethanol and 12% sodium dodecylsulfate, by incubating at 50°C for 30 min. After addition of Clearsol-I (Nacalai Tesque Co. Ltd., Kyoto, Japan), the radioactivity was determined in a liquid scintillation counter (Packard TRI-CARB 460, Packard Co. Ltd.). Adenine nucleotide translocase activity was measured as atractyloside-sensitive ADP uptake per milligram of mitochondrial protein, according to the method reported previously (21).

Statistical analysis. The data in Figures 1 and 2 were analyzed by two-way analysis of variance (ANOVA), and the data at each time point were analyzed by one-way ANOVA and Tukey's multiple comparison. The data in Figures 3, 4 and 5 and Table 2 were also analyzed by one-way ANOVA and Tukey's multiple comparison, and the data in Table 1 were analyzed by the Wilcoxon two-sample test. Data are presented as the mean value ± SE, and a p value <0.05 was considered to be statistically significant.

Results

Experimental rats. The normal and diabetic rats used in this study are compared in Table 1. Both body weight and heart weight in the diabetic rats were significantly lower than those weights in normal rats. Plasma glucose in diabetic rats was routinely >400 mg/dl.

Myocardial function. The changes in cardiac performance of control and preconditioned hearts from normal or diabetic rats are illustrated in Figure 1. There was no significant difference in initial LVDP between the control and preconditioned groups in either normal or diabetic rats, but LVDP in the diabetic rats was smaller than that in the normal rats ($p < 0.01$). In the control hearts of either the normal or diabetic rats, LVDP was markedly depressed after 30 min of sustained ischemia and showed little recovery during 30 min of reperfusion. In contrast, LVDP in the preconditioned hearts from both groups showed significantly better recovery than that in the control groups, although LVDP in the normal and diabetic rats was reduced to 67% and 63% of the initial value after two cycles of 5 min of ischemia followed by 5 min of reperfusion, respectively.

Release of CK. The time-dependent changes in CK activity in the coronary effluent, as well as the cumulative CK release, during 30 min of reperfusion in the control and preconditioned groups are illustrated in Figure 2. There was no significant increase in CK release immediately after ischemic preconditioning in either the normal or diabetic group. However, a significant increase was observed in both groups on reperfusion, with maximal CK activity detected at 5 to 10 min of reperfusion, followed by a gradual decline. There was a significantly greater cumulative release of CK from the control groups than from the preconditioned groups. It is noteworthy that cumulative CK release was significantly smaller in the preconditioned group of diabetic rats than in the normal rats ($p < 0.05$), although there was no significant difference between the control groups of normal and diabetic rats.

Adenine nucleotides, CP, creatine and energy charge. Table 2 shows the absolute values of adenine nucleotides, CP,

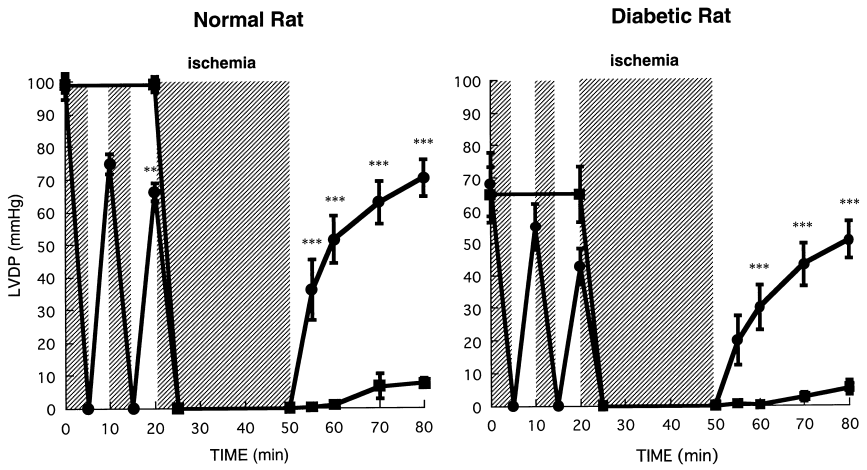

Figure 1. Left ventricular developed pressure in control or preconditioned hearts in response to sustained ischemia and reperfusion. The hearts were isolated from normal or diabetic rats and were either perfused (control) or preconditioned according to the protocol described in Methods. The hearts were then subjected to ischemia and reperfusion, and the LVDP was monitored as described in Methods. **Squares** = control hearts; **circles** = preconditioned hearts; **shaded areas** = global ischemia. The interaction between four groups and time course was significant ($p = 0.0001$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group ($n = 8$).

creatine and energy charge. In both normal and diabetic rats, the ATP content decreased only slightly after ischemic preconditioning, but was markedly lowered in both the control and preconditioned groups after 30 min of ischemia. However, after 30 min of reperfusion, ATP was significantly higher in the preconditioned groups than in the control groups.

The time-dependent change in the ADP content was similar to that of ATP in both the control and preconditioned groups of either normal or diabetic rats. The ADP content declined after sustained ischemia and recovered after reperfusion. Thirty minutes of ischemia markedly increased the AMP level by 17.8- and 8.9-fold in the control and preconditioned groups of normal rats, and 10.4- and 6.4-fold in those of diabetic rats, respectively. After reperfusion, AMP was depleted in both groups. The sharp decline in the total adenine nucleotide pool observed in the reperfused control groups was greatly attenuated by preconditioning (Table 2).

Creatine phosphate was markedly decreased after 30 min of sustained ischemia in both control and preconditioned groups of either normal or diabetic rats. However, after reperfusion, CP in the preconditioned hearts was significantly higher than that in the control hearts. Immediately after preconditioning, the CP content was greater than preischemic values in both normal (123.4 ± 1.5%) and diabetic (117.1 ± 3.7%) rats. Creatine content increased during sustained ischemia and subsequently decreased during reperfusion in both groups.

The energy charge decreased after ischemia and recovered significantly during reperfusion in both the control and preconditioned groups of either normal or diabetic rats. However, the energy charge was better preserved in the preconditioned groups after 30 min of reperfusion, as shown in Table 2.

Myocardial glycogen content. The glycogen content at baseline, immediately after preconditioning and 30 min after sustained ischemia in both normal and diabetic rats is shown in Figure 3. The preischemic glycogen content in diabetic hearts was significantly greater than that in normal hearts (3.13 ± 0.30 [normal] vs. 5.50 ± 0.27 [diabetic] mg/g wet weight, $p < 0.001$). The glycogen content was significantly reduced immediately after preconditioning and was further decreased 30 min after sustained ischemia in the preconditioned group of either normal or diabetic rats. The glycogen content was also markedly reduced 30 min after sustained ischemia in the control group of either normal or diabetic rats.

Myocardial lactate content. In both normal and diabetic rats, the myocardial lactate content was significantly lower in the preconditioned groups after 30 min of sustained ischemia, as shown in Figure 4. There was no significant difference in the lactate content before sustained ischemia among the four groups (data not shown).

Mitochondrial enzyme activities. The initial preischemic activity of rotenone-sensitive NADH cytochrome *c* oxidoreductase, oligomycin-sensitive ATPase and adenine nucleotide translocase tended to be lower in diabetic than in normal rats (NADH cytochrome *c* oxidoreductase: 0.44 ± 0.03 [normal] vs. 0.35 ± 0.04 [diabetic] μmol cytochrome *c* reduced/min per mg protein; ATPase: 2.18 ± 0.06 [normal] vs. 1.04 ± 0.04 [diabetic] μmol NADH oxidized/min per mg protein, $p < 0.05$; adenine nucleotide translocase: 0.95 ± 0.06 [normal] vs. 0.86 ± 0.11 [diabetic] nmol ADP uptake/min per mg protein). Figure 5 shows the percent decline in the activity of these enzymes after sustained ischemia followed by reperfusion. At the end of the 30 min of reperfusion, NADH cytochrome *c* oxidoreductase activity was decreased to a similar extent in the control and preconditioned groups of both normal and diabetic rats when compared with preischemic values. In contrast, ATPase activity was not significantly decreased after reperfusion in the preconditioned group in either the normal or diabetic rats, but was significantly lower in control hearts of both groups. Adenine nucleotide translocase activity declined after reperfusion in both the control and preconditioned groups, but the activity in the preconditioned hearts was significantly higher than that in control hearts in both the normal and diabetic groups.

Discussion

In the present study, we show that ischemic preconditioning can protect diabetic rat hearts against ischemia-reperfusion.

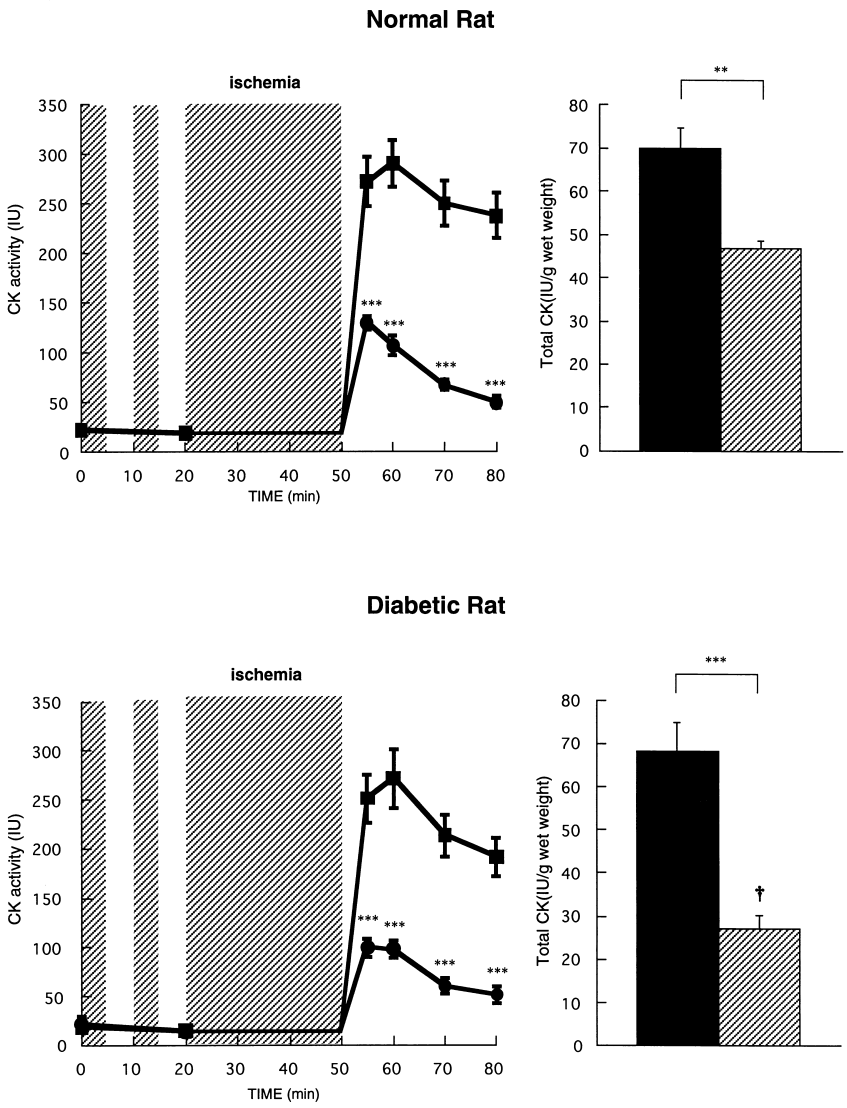

Figure 2. Time-dependent as well as cumulative CK release in the effluent of control and preconditioned rats in response to sustained ischemia and reperfusion. Coronary effluent was collected, and CK activity was determined at the indicated time points for both normal and diabetic rats according to the protocol described in Methods. In the time-dependent graphs, the **hatched areas** represent global ischemia. **Squares** = control hearts; **circles** = preconditioned hearts. In the cumulative CK histograms, **solid bars** represent control rats, and **hatched bars** represent preconditioned rats. The interaction between the four groups and time course was significant ($p = 0.0001$). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group. †$p < 0.05$ compared with the normal preconditioned group ($n = 8$).

Our major findings with the preconditioned hearts are that, when compared with control hearts, 1) HEP, such as ATP and CP, was significantly better preserved after ischemia-reperfusion; 2) the myocardial lactate content was significantly lower after sustained ischemia; and 3) mitochondrial ATPase and adenine nucleotide translocase activities were significantly better preserved after 30 min of reperfusion. In addition, we have observed for the first time several significant similarities and differences between normal and diabetic rats: 1) although the baseline values of LVDP, ATP, CP and mitochondrial enzyme activities were smaller in diabetic rat hearts, their recovery rates after ischemia-reperfusion were almost identical in both normal and diabetic rat hearts; 2) a CP overshoot was seen immediately after preconditioning in both normal and diabetic hearts; 3) myocardial damage, estimated by cumulative CK release, was smaller in the preconditioned diabetic hearts than in the normal hearts; 4) initial glycogen content in diabetic hearts was significantly greater than in normal hearts; and 5) although myocardial lactate levels immediately after sustained ischemia tended to be higher in the control diabetic hearts than in the control normal hearts, lactate content in the preconditioned hearts of diabetic rats declined to a similar extent as that in normal rats. In addition, there were no significant differences in the LVDP, cumulative CK release, adenylates and glycogen content between pair weighted rats (180 to 200 g) and normal rats, suggesting that the difference in the weight between the normal and diabetic rats was not a contributing factor in the present study (data not shown).

Mitochondrial function and energy metabolism in preconditioned hearts. It has been proposed that the depression of mitochondrial ATPase during preconditioning may account for the reduced rate of ATP consumption and increased cardioprotection in animals with slow (2,22) as well as fast (23) heart rates. However, in our previous studies of preconditioned rat hearts, we observed that ATPase activity was not significantly depressed either after 5 min of ischemic preconditioning, 30 min of sustained ischemia or 30 min of reperfusion (14). Moreover, there was no significant difference in either the ATP

Table 2. Energy Level Changes in Control and Preconditioned Hearts in Response to Sustained Ischemia and Reperfusion

Time	n	ATP	ADP	AMP	CP	Cr	EC
				Normal Rats			
Baseline	12	13.8 ± 0.15	3.70 ± 0.13	0.60 ± 0.03	32.5 ± 0.32	30.4 ± 0.89	0.86 ± 0.004
Control group							
Ischemia	12	3.72 ± 0.16	1.85 ± 0.19	10.7 ± 0.44	10.5 ± 0.30	51.2 ± 1.51	0.29 ± 0.005
Reperfusion	12	3.40 ± 0.10	2.10 ± 0.06	1.60 ± 0.14	13.4 ± 0.73	25.8 ± 0.85	0.63 ± 0.014
After IP	12	13.3 ± 0.21	3.40 ± 0.09	0.42 ± 0.01	40.1 ± 0.50	21.0 ± 0.19	0.88 ± 0.001
IP group							
Ischemia	12	3.54 ± 0.11	1.20 ± 0.04*	5.34 ± 0.16†	7.40 ± 0.32	49.6 ± 0.98	0.41 ± 0.009†
Reperfusion	12	5.91 ± 0.23†	2.92 ± 0.09*	0.90 ± 0.07	29.8 ± 1.56†	20.2 ± 0.66†	0.76 ± 0.010†
				Diabetic Rats			
Baseline	12	13.9 ± 0.43	3.62 ± 0.14	1.04 ± 0.14	24.2 ± 1.01	35.8 ± 1.26	0.85 ± 0.004
Control group							
Ischemia	12	2.89 ± 0.10	1.92 ± 0.07	10.8 ± 0.20	6.50 ± 0.49	53.8 ± 1.59	0.25 ± 0.006
Reperfusion	12	3.02 ± 0.11	1.88 ± 0.05	1.62 ± 0.14	9.62 ± 0.43	28.9 ± 1.14	0.61 ± 0.016
After IP	12	13.2 ± 0.88	3.24 ± 0.20	0.65 ± 0.03	28.6 ± 0.90	30.8 ± 1.80	0.86 ± 0.008
IP group							
Ischemia	12	2.68 ± 0.11	1.34 ± 0.10‡	6.68 ± 0.20†	5.18 ± 0.32	51.3 ± 2.16	0.31 ± 0.008†
Reperfusion	12	5.68 ± 0.24†	2.02 ± 0.07	0.88 ± 0.04*	21.8 ± 0.54†	22.6 ± 1.26	0.78 ± 0.006†

*$p < 0.01$, †$p < 0.001$, ‡$p < 0.05$ compared with the control group (n = 12). Isolated hearts from normal or diabetic rats were subjected to ischemic preconditioning (IP) or simple perfusion (Control) protocols, followed by sustained ischemia and reperfusion, as described in Methods, and the values of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), creatine phosphate (CP), creatine (Cr) and energy charge (EC) were determined. Initial values are compared with those obtained immediately after ischemic preconditioning in the preconditioned group and immediately after sustained ischemia (Ischemia) or 30 min of reperfusion (Reperfusion) for all groups. Data are presented as mean value ± SEM (μmol/g dry weight).

content or the decline in the rate of ATP hydrolysis between the control and preconditioned hearts, suggesting that mitochondrial ATPase has only a relatively small effect on ATP depletion in ischemic rat hearts (14). The fact that the inhibition of mitochondrial ATPase by oligomycin also has a negligible ATP-sparing effect in the ischemic rat heart is consistent with our data (24). Thus, there remains some uncertainty concerning the role of mitochondrial ATPase in maintaining intracellular ATP content in rats.

It has been reported that diabetic myocardium exhibits a variety of abnormalities that may contribute to the severity of the injury associated with ischemia-reperfusion, including a shift in myosin isozymes from predominantly V_1 to V_3 (25), depressed Na^+/Ca^{2+} exchange (11), Na^+/H^+ exchange (26) and sarcoplasmic reticulum ATPase activities (27), and a decrease in sensitivity to beta-adrenergic stimulation (28). Moreover, abnormalities in energy metabolism, glucose transport, as well as decreased phosphofructokinase, glycogen synthetase and pyruvate dehydrogenase activities, have also been described (12). It seems likely, therefore, that these metabolic dysfunctions could disrupt the energy balance during ischemic preconditioning. In the present study, however, we found that there are many similarities in the recovery rates of HEP, ATPase and adenine nucleotide translocase activities after sustained ischemia-reperfusion in both normal and diabetic rats. These findings suggest that in diabetic rats, as in normal

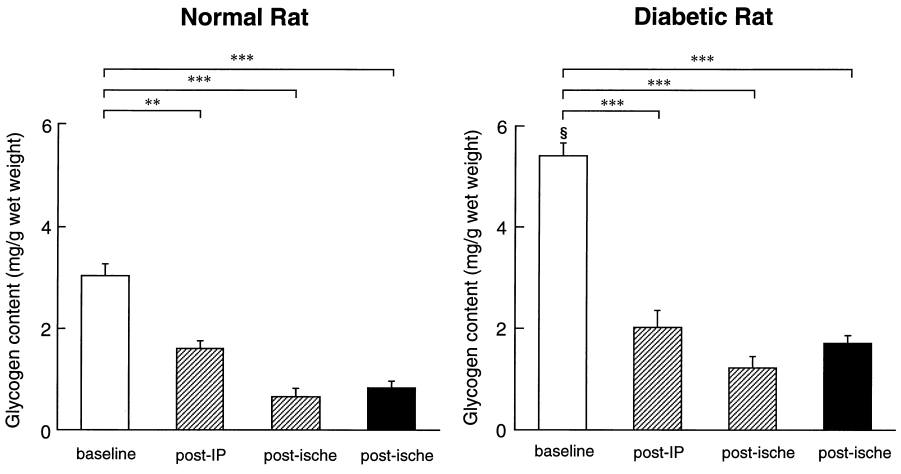

Figure 3. Myocardial glycogen content in control and preconditioned hearts. Isolated hearts from normal and diabetic rats were subjected to perfusion protocols as described in Methods. At preischemic baseline (baseline), immediately after preconditioning (post-IP) and 30 min after sustained ischemia (post-ische), the hearts were analyzed for glycogen content (see Methods). **Solid bars** = control group; **hatched bars** = preconditioned group. **$p < 0.01$, ***$p < 0.001$, §$p < 0.001$ compared with the group of normal rats (n = 8).

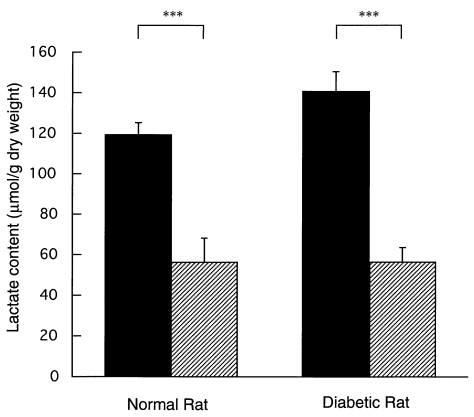

Figure 4. Myocardial lactate content in control and preconditioned hearts. Isolated hearts from normal and diabetic rats were subjected to perfusion protocols as described in Methods. At the end of sustained ischemia, the hearts were homogenized and analyzed for lactate content (see Methods). **Solid bars** represent the control group; **hatched bars** represent the preconditioned group. ***$p < 0.001$ ($n = 8$).

rats, mitochondrial ATPase activity may contribute to the restoration of HEP.

We observed that adenine nucleotide translocase activity recovered significantly in the preconditioned groups of both normal and diabetic rats at 30 min after reperfusion, when compared with the control groups. Previous studies have suggested that myocardial ischemia results in a significant depression in translocase activity (29), and that its activity and oxidative phosphorylation are closely related to contractile recovery after ischemia-reperfusion (20). Because alterations in carnitine level have been reported in diabetic myocardium (30), it seems possible that translocase activity may be affected in this tissue. Our present data, however, suggest that the preservation of translocase may contribute to the restoration of HEP and myocardial contractile function in the preconditioned diabetic as well as normal rat hearts. In addition, there was no significant difference in isolated mitochondrial protein among the four groups, although we did not determine the proportion of dead, inert or active mitochondria.

Cardioprotective efficiency of preconditioning in diabetic hearts. Our present data show that the cumulative CK release was significantly lower in the preconditioned group of diabetic hearts than in the normal hearts, although there was no significant difference in CK release in the control groups between both rats. Presently there are conflicting opinions concerning the tolerance of diabetic hearts to ischemic injury (31–33). The present results suggest that under our experimental conditions there is no difference in myocardial sensitivity to ischemia-reperfusion injury between the control groups of normal and diabetic rats, and that the cardioprotective efficiency of preconditioning is potentiated in diabetic rats.

It is well known that intracellular acidosis during ischemia is one of the most important factors affecting the extent of myocardial damage after reperfusion. In the present study, although lactate levels during sustained ischemia tended to be higher in the control group of diabetic hearts than in control normal hearts, its production in preconditioned hearts was inhibited to a similar extent in both diabetic and normal rats, suggesting a lower lactate production rate in these hearts during ischemia. Because glucose transport and phosphofructokinase are inhibited in diabetic hearts, it can be expected that lactate production would be lower in ischemic diabetic hearts. However, our data show that lactate production was not inhibited in the control group of diabetic rats. Our data are therefore in agreement with those of Tani and Neely (34), who have reported that lactate production was not inhibited at the end of ischemia in perfused diabetic rat hearts. However, the contradictory data of Khandoudi et al. (35) suggest that this issue is not yet resolved.

The possible contribution of stored glycogen is of interest here. In the present study, glycogen content in diabetic hearts was significantly greater than that in normal hearts. It is noteworthy that the difference in glycogen content between baseline levels and those after sustained ischemia was larger in the control group of diabetic rats than in normal rats, suggest-

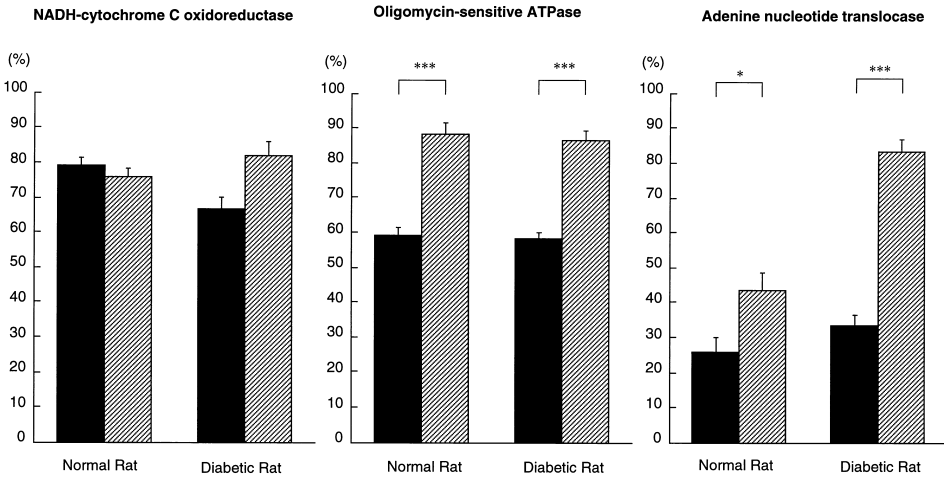

Figure 5. Mitochondrial enzyme activity. Control and preconditioned hearts were subjected to sustained ischemia and reperfusion, the mitochondria were isolated, and enzyme activity was assayed as described in Methods. The values for NADH cytochrome *c* oxidoreductase, ATPase and adenine nucleotide translocase were determined after 30 min of reperfusion and are expressed as a percent of preischemic values (see text) for both normal and diabetic rats. **Solid bars** = control group; **hatched bars** = preconditioned group. *$p < 0.05$, ***$p < 0.001$ ($n = 8$).

ing that glycolytic metabolites were produced in greater quantities in diabetic hearts under ischemic conditions. Neely and Morgan (12) also reported higher levels of glycogen in diabetic myocardium, suggesting that higher levels of lactate may accumulate in these hearts during ischemia. After preconditioning, myocardial glycogen content was markedly reduced in diabetic hearts, and the production of glycolytic metabolites during the sustained ischemia was inhibited to a similar extent as that in normal hearts, as indicated by the lactate content. Therefore, it seems reasonable to speculate that, in diabetic hearts, preconditioning may induce a more efficient depletion of myocardial glycogen content, thereby contributing to a lower lactate production during sustained ischemia.

The present data also suggest that intracellular acidosis during ischemia may be attenuated in the preconditioned hearts, because it has been reported that anaerobic glycolysis is the major source of H^+ production during myocardial ischemia (36), and that lactate production by glycolysis closely correlates with intracellular acidosis (37). Recently, Wolfe et al. (38) reported that glycogen depletion and the concomitant attenuation of intracellular acidosis during ischemia appear to be an important mechanism for cardioprotection in ischemic preconditioned rat hearts. Albuquerque et al. (39) have also reported that lowering of intracellular pH results in decreased HEP content and increased deleterious myocardial dysfunction. Although we did not measure intracellular pH, it appears reasonable to propose that similar effects would have been observed in our model. Taking all these factors into consideration, a greater depression of lactate production in the preconditioned diabetic heart may contribute to the attenuation of intracellular acidosis and may result in more efficient cardioprotection.

There are presently conflicting data with regard to the effect of ischemic preconditioning on diabetic hearts. Liu et al. (40) have demonstrated that preconditioning can significantly reduce the infarct size in the non–insulin-dependent diabetic rat hearts. In contrast, Tosaki et al. (41) have reported that preconditioning does not afford similar protection in streptozocin-induced diabetic rat hearts. Although our results suggest the possibility that preconditioning provides greater protection in isolated diabetic rat hearts, further studies will be required to resolve this issue.

Conclusions. We demonstrate in this study that in both normal and diabetic rats, the preservation of mitochondrial ATPase and adenine nucleotide translocase activities can contribute to the restoration of HEP after sustained ischemia-reperfusion in the ischemic preconditioned heart. Our data show an inhibition of glycolysis during ischemia in preconditioned hearts and suggest, therefore, that the attenuation of intracellular acidosis may be important for cardioprotection in the preconditioned heart. Moreover, we show that in the diabetic myocardium, preconditioning may offer greater protection than in normal myocardium, as illustrated by the lower cumulative CK release.

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