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Difference in the Cardioprotective Mechanisms Between Ischemic Preconditioning and Pharmacological Preconditioning by Diazoxide in Rat Hearts

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Background Recent studies have implicated the opening of mitochondrial KATP (mitoKATP) channels and the production of reactive oxygen species (ROS) in the cardioprotective mechanism of ischemic preconditioning (IPC).

Methods and Results The involvement of mitoKATP channels and ROS in the cardioprotective effects of both IPC and the mitoKATP channel opener diazoxide (DZ) was investigated in ischemic/reperfused rat hearts. The effects of IPC and DZ on myocardial high-energy phosphate concentrations and intracellular pH (pH_i) were also examined using ^{31}P nuclear magnetic resonance spectroscopy. Although both the mitoKATP channel inhibitor 5-hydroxydecanoate and the antioxidant *N*-acetylcysteine abolished the postischemic recovery of contractile function by DZ, neither of them inhibited that by IPC. IPC attenuated the decline in pH_i during ischemia, but DZ did not (6.28±0.04 in IPC, $p<0.05$, and 6.02±0.05 in DZ vs 6.02±0.06 in control hearts). DZ, but not IPC, reduced the decrease in ATP levels during ischemia (ATP levels at 20-min ischemia: 26.3±3.4% of initial value in DZ, $p<0.05$, and 8.1±3.0% in IPC vs 15.1±1.3% in control hearts).

Conclusions These results suggest that DZ-induced cardioprotection is related to ROS production and reduced ATP degradation during ischemia, whereas attenuated acidification during ischemia is involved in IPC-induced cardioprotection, which is not mediated through mitoKATP channel opening or ROS production. (*Circ J* 2004; **68:** 156 – 162)

Key Words: ATP-sensitive potassium channel; High-energy phosphates; Intracellular pH; Mitochondria; Reactive oxygen species

Ischemic preconditioning (IPC) is a phenomenon whereby brief intermittent periods of ischemia protect the myocardium against subsequent lethal ischemia[1] Although the mechanisms responsible for IPC still remain elusive, several factors, such as adenosine, acetylcholine and opioids, which activate protein kinase C, have been implicated in the cardioprotective effect of IPC[2]

Recently, ATP-sensitive potassium (KATP) channels and reactive oxygen species (ROS), among the signaling pathways mediating IPC, have attracted considerable attention. Though the involvement of sarcolemmal KATP channels in IPC was first suggested[3] subsequent studies have reported that mitochondrial KATP (mitoKATP) channels play a potential role for the cardioprotective effects of KATP channel openers[4] More recent studies have indicated that the opening of mitoKATP channels induces cardioprotection by generating ROS, which may act as intracellular messengers[5,6]

It has been reported that pretreatment with mitoKATP channel openers[4] or exogenous H_2O_2[7,8] induces IPC-like protective effects, and that IPC-induced cardioprotection is antagonized by mitoKATP channel inhibitors[9,10] or ROS scavengers[11,12] These findings support the hypothesis that IPC is mediated through the opening of mitoKATP channels and ROS production. Nevertheless, several investigators have argued against the contribution of mitoKATP channels[13–15] and ROS[16,17] to IPC.

The protective effects of IPC and the mitoKATP channel opener diazoxide (DZ) in whole heart models have been generally evaluated as reduced infarct size and improved contractile function during reperfusion. IPC also has beneficial effects on energy metabolism, such as reduced intracellular acidification during ischemia and postischemic preservation of high-energy phosphates[18–20] Measurement of these parameters may reveal the difference in the cardioprotective mechanisms between IPC and DZ. Moreover, a comparison of IPC and DZ under the same experimental condition would minimize the discrepancy in the results caused by the experimental conditions and models used. However, few studies have compared the effects of IPC and DZ on energy metabolism under the same experimental conditions.

Therefore, in the present study we investigated the involvement of the opening of mitoKATP channels and ROS production in IPC- and DZ-induced cardioprotection, using the mitoKATP channel inhibitor 5-hydroxydecanoate (5-HD) and the antioxidant *N*-acetylcysteine (NAC), in perfused

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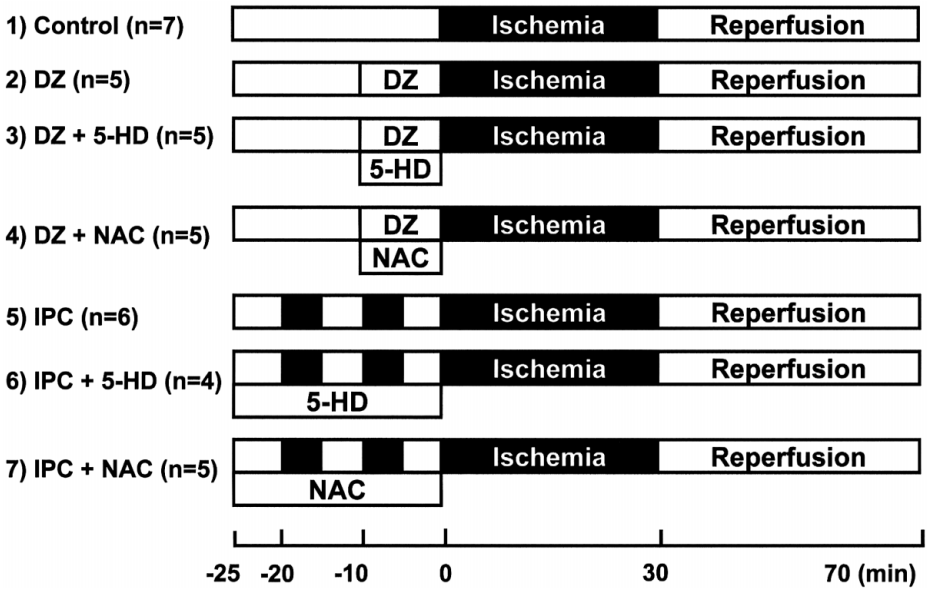

Fig 1. Perfusion protocols. All hearts were subjected to 30 min of no-flow ischemia, followed by 40 min of reperfusion. Diazoxide (DZ; 100 μmol/L) was pretreated for 10 min just before ischemia in the presence or absence of 5-hydroxydecanoate (5-HD; 200 μmol/L) or *N*-acetylcysteine (NAC; 4 mmol/L). Ischemic preconditioning (IPC) was achieved by 2 cycles of 5-min ischemia and 5-min reperfusion before 30 min of ischemia. Either 5-HD or NAC was administered for 25 min of the preconditioning period.

rat hearts. In addition, we used ^{31}P nuclear magnetic resonance (^{31}P NMR) spectroscopy to examine the effects of IPC and DZ on high-energy phosphate levels and intracellular pH (pH_i) during ischemia and reperfusion.

Methods

Heart Perfusion and Measurements of Function

The investigation conformed to *The Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats weighting 280–340 g were anesthetized with pentobarbital sodium (50 mg/kg, ip) and heparinized (200 U, iv). The hearts were rapidly excised, cannulated and perfused with phosphate-free Krebs-Henseleit buffer through the aorta in a non-circulating Langendorff apparatus at a constant perfusion pressure of 70 mmHg. Krebs-Henseleit buffer contained (in mmol/L) NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25, and glucose 11. The perfusate was continuously gassed with 95% O_2 and 5% CO_2 to a pH of 7.4 and maintained at 37°C. The hearts were not paced. A water-filled latex balloon was introduced into the left ventricle (LV) through the mitral valve. The balloon was connected with non-compliant tubing to a pressure transducer (Nihon Kohden Co, Japan) and inflated to set the end-diastolic pressure (LVEDP) at 5–10 mmHg. LV developed pressure (LVDP) was calculated as the difference between the peak-systolic pressure and LVEDP. LV peak-systolic pressure, LVEDP and heart rate were monitored on a polygraph. Coronary flow rate was measured by the collection of effluent in a cylinder.

^{31}P Nuclear Magnetic Resonance (NMR) Spectroscopy

The hearts were placed in a 17-mm NMR tube, which was mounted in a saddle-shaped Helmholz coil. Temperature was maintained at 37°C by warming the sample tube with a heat-controlled water jacket. ^{31}P NMR spectra were obtained at 109.16 MHz using a JEOL GSX 270 wide-bore NMR spectrometer (6.35 tesla field strength; Nihon Denshi, Tokyo, Japan). The proton signal from the heart was used for shimming. Spectra were acquired every 5 min using a 1-s interpulse delay and a pulse width of 19.5 μs (a flip angle of 45°). Spectral width was 6,000 Hz and 1 K data points were collected. The free induction decays were multiplied by an exponential function corresponding to a 30-Hz line broadening before Fourier transformation. The intracellular contents of phosphocreatine (PCr), ATP and inorganic phosphate (Pi) were determined from their respective peak areas. The pH_i was calculated from the difference in the chemical shift between the Pi and PCr peaks.

Experimental Protocols

As shown in Fig 1, 7 sets of experiments were performed. After the hearts were excised and cannulated, they were allowed to stabilize for 30 min before treatment. After the treatment period, all hearts were subjected to 30 min of no-flow ischemia and then reperfused for 40 min. The differences in the treatment period are summarized as follows. Hearts were perfused with Krebs-Henseleit buffer alone (control; n=7). For 10 min just before ischemia, 100 μmol/L DZ was added to the perfusate (DZ; n=6). DZ was administrated in the presence of either 200 μmol/L 5-HD (DZ+5-HD; n=5) or 4 mmol/L NAC (DZ+NAC; n=5). IPC was achieved by 2 cycles of 5-min ischemia followed by 5-min reperfusion (IPC; n=6). 5-HD (IPC+5-HD; n=4) or NAC (IPC+NAC; n=5) was administered throughout the preconditioning period. NAC was used as an antioxidant because it has been shown to interact with superoxide anion, hydrogen peroxide and hydroxyl radical[21]

DZ, 5-HD and NAC were purchased from Sigma Chemical Co (St Louis, MO, USA). DZ was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the solution was less than 0.05%.

Table 1 Heart Rate, Coronary Flow Rate and Left Ventricular Developed Pressure at Baseline, the End of Preischemia, and at the End of Reperfusion

Group	Heart rate (beats/min)			Coronary flow rate (ml/min)			LDVP (mmHg)	
	Baseline	Preischemia	Reperfusion	Baseline	Preischemia	Reperfusion	Baseline	Preischemia
Control	321±9	311±9	310±9	13±0.2	12±0.3	7±0.4‡	91±2	89±3
DZ	328±8	324±5	328±7	13±0.6	15±0.3*‡	8±0.6‡	93±2	87±4
DZ+5-HD	320±10	320±9	298±13	13±0.6	15±0.5*‡	5±0.4†‡	93±3	95±3
DZ+NAC	311±5	288±5	308±9	12±0.5	15±0.7*‡	6±0.4†‡	90±3	85±2
IPC	326±10	321±8	313±7	12±0.3	15±0.3*‡	11±0.4*†	91±2	66±3*†‡
IPC+5-HD	316±10	302±11	321±11	12±0.3	14±0.1‡	10±0.6*‡	96±7	73±6*‡
IPC+NAC	322±10	312±13	335±16	13±0.6	15±0.5*‡	10±0.2*‡	92±3	65±3*†‡

*DZ, diazoxide; IPC, ischemic preconditioning; 5-HD, 5-hydroxydecanoate; NAC, N-acetylcysteine; LVDP, left ventricular developed pressure; Values are means±SEM. *p<0.05 vs control hearts, †p<0.05 vs DZ-treated hearts, ‡p<0.05 vs the baseline values by ANOVA.*

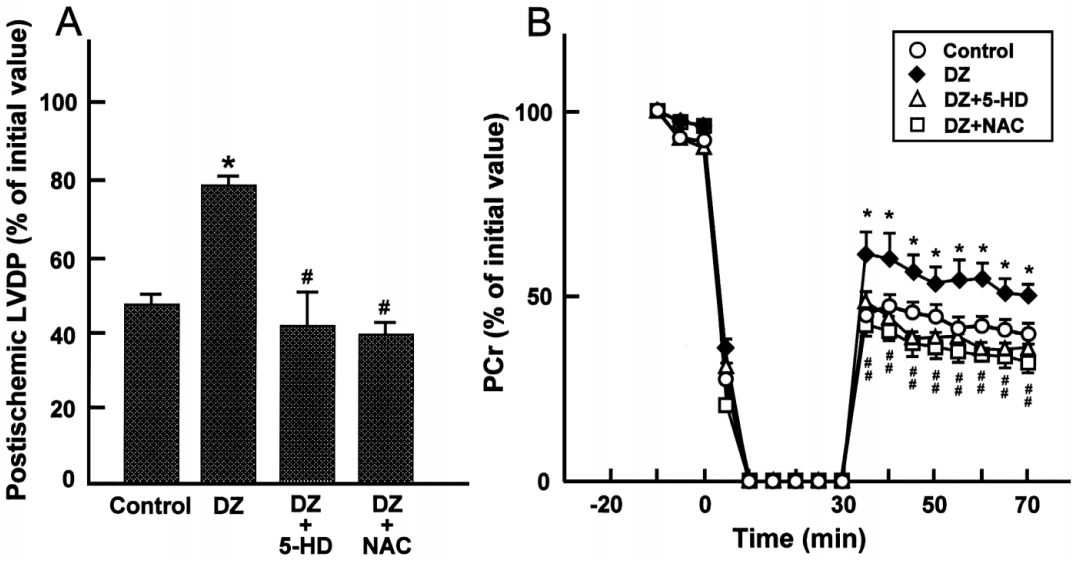

Fig 2. (A) Recovery of left ventricular developed pressure (LVDP) at the end of reperfusion. (B) Time course of the changes in phosphocreatine (PCr) levels. Values are expressed as the percentage (%) of initial value. DZ, diazoxide; 5-HD, 5-hydroxydecanoate; NAC, *N*-acetylcysteine. Values are means ± SEM. *p<0.05 vs control hearts, #p<0.05 vs DZ-treated hearts.

Statistics

Data are expressed as means ± SEM. Differences in data among the experimental groups were analyzed using ANOVA. The time-dependent data were tested by ANOVA for repeated measures. If ANOVA indicated a significant difference, a Tukey-Kramer post hoc test was performed. A value of p<0.05 was considered statistically significant.

Results

Preischemic and Postischemic Hemodynamics

As shown in Table 1, there were no differences in heart rate, coronary flow rate or LVDP among the 7 groups at baseline. LVDP in hearts treated with DZ did not change at the end of the treatment period (87±4 mmHg), whereas in the preconditioned hearts it decreased (66±3 mmHg in IPC vs 89±3 mmHg in control, p<0.05). DZ and IPC increased coronary flow rate at the end of the treatment period (15±0.3 ml/min in DZ and 15±0.3 ml/min in IPC vs 12±0.3 ml/min in control, p<0.05). At the end of reperfusion, IPC preserved the coronary flow rate in the presence or absence of 5-HD or NAC during the preconditioning period (11±0.4 ml/min, 10±0.6 ml/min, 10±0.2 ml/min in IPC, IPC+5-HD and IPC+NAC vs 7±0.4 ml/min in control; p<0.05, respectively).

Effects of 5-HD and NAC on Postischemic Recovery of Contractile Function and High-Energy Phosphates in Hearts Treated With DZ

Fig 2A shows that the postischemic recovery of LVDP at the end of reperfusion was significantly better in hearts treated with DZ, which recovered 77±4% of LVDP at baseline, than in control hearts (48±3%, p<0.05). We then tested whether DZ-induced cardioprotection is blocked by 5-HD and NAC. Postischemic recovery of LVDP in hearts treated with DZ plus 5-HD (42±11%) was significantly worse than that in hearts treated with DZ alone (p<0.05). DZ plus NAC also resulted in a significant deterioration of postischemic LVDP compared with DZ alone (39±4%, p<0.05). Our preliminary data showed that pretreatment with 5-HD or NAC alone did not affect postischemic recovery of LVDP in control hearts (data not shown).

The time course of the changes in PCr levels is shown in Fig 2B. At the end of reperfusion the PCr levels in hearts treated with DZ (50±3%, p<0.05) were significantly higher than those in control hearts (40±3%). The restoration of

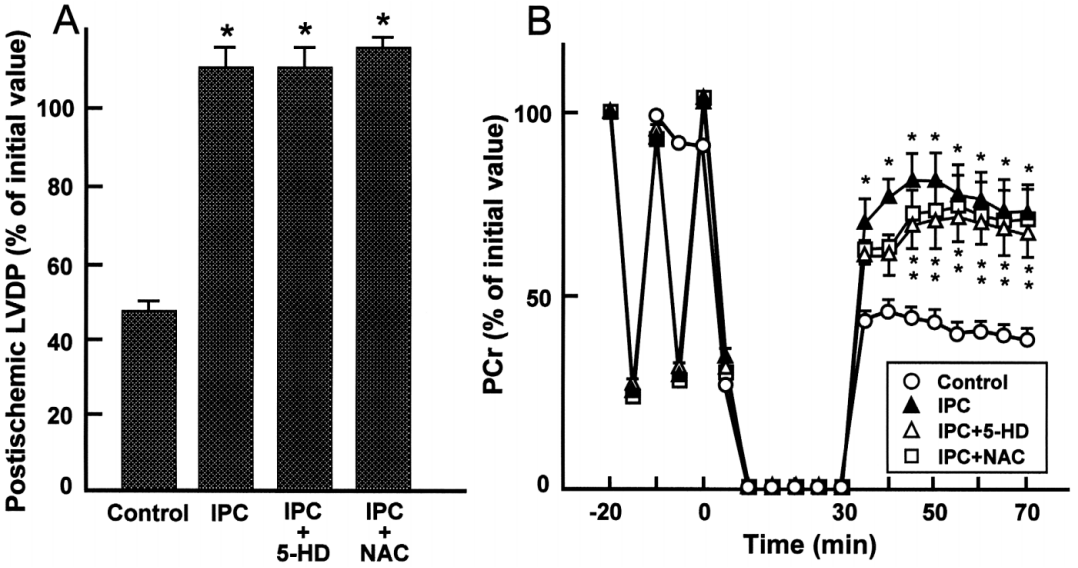

Fig 3. (A) Recovery of left ventricular developed pressure (LVDP) at the end of reperfusion. (B) Time course of the changes in phosphocreatine (PCr) levels. Values are expressed as the percentage (%) of initial value. IPC, ischemic preconditioning; 5-HD, 5-hydroxydecanoate; NAC, *N*-acetylcysteine. Values are means ± SEM. *p<0.05 vs control hearts.

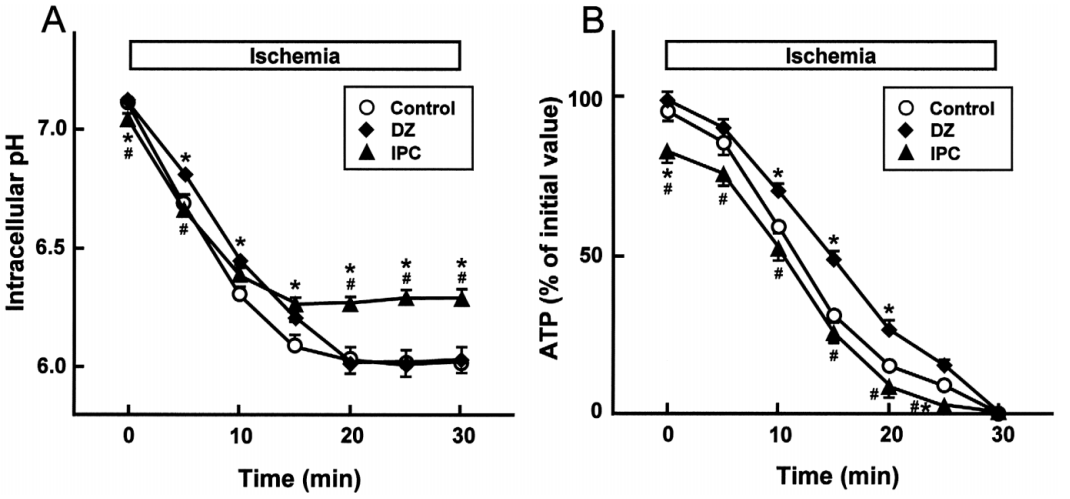

Fig 4. Time course of the changes in pH_i (A) and ATP levels (B) during ischemia. DZ, diazoxide; IPC, ischemic preconditioning. Values are means ± SEM. *p<0.05 vs control hearts, #p<0.05 vs DZ-treated hearts.

PCr levels by DZ was almost completely prevented by adding 5-HD or NAC (32±3% and 39±4% in DZ+5-HD and DZ+NAC, respectively).

Effects of 5-HD and NAC on Postischemic Recovery of Contractile Function and High-Energy Phosphates in Hearts Treated With IPC

IPC remarkably improved the postischemic recoveries of both LVDP and PCr levels compared with control hearts (110±6% in LVDP and 73±8% in PCr levels, p<0.05) (Fig 3A,B). In contrast to DZ, neither 5-HD nor NAC abolished the postischemic recovery of LVDP (110±6% and 114±4% in IPC+5-HD and IPC+NAC, respectively) or PCr levels (72±8% and 68±6% in IPC+5-HD and IPC+NAC, respectively) induced by IPC.

Effects of DZ and IPC on Intracellular pH During Sustained Ischemia

In addition to examining the signal pathways in IPC and DZ by using the pharmacological inhibitors, we investigated the effects of IPC and DZ on energy metabolism during ischemia.

As shown in Fig 4A, the pH_i in DZ-treated hearts, as well as control hearts, reached a plateau at 20 min of ischemia, and pH_i in preconditioned hearts reached a plateau at 15 min of ischemia. At the end of ischemia, the pH_i in preconditioned hearts was significantly higher than those in control and DZ-treated hearts (6.28±0.04 in IPC, p<0.05, and 6.02±0.05 in DZ, vs 6.02±0.06 in control). In contrast, DZ slightly increased the pH_i at 5–10 min of ischemia, but did not reduce intracellular acidification at the end of ischemia compared with control hearts.

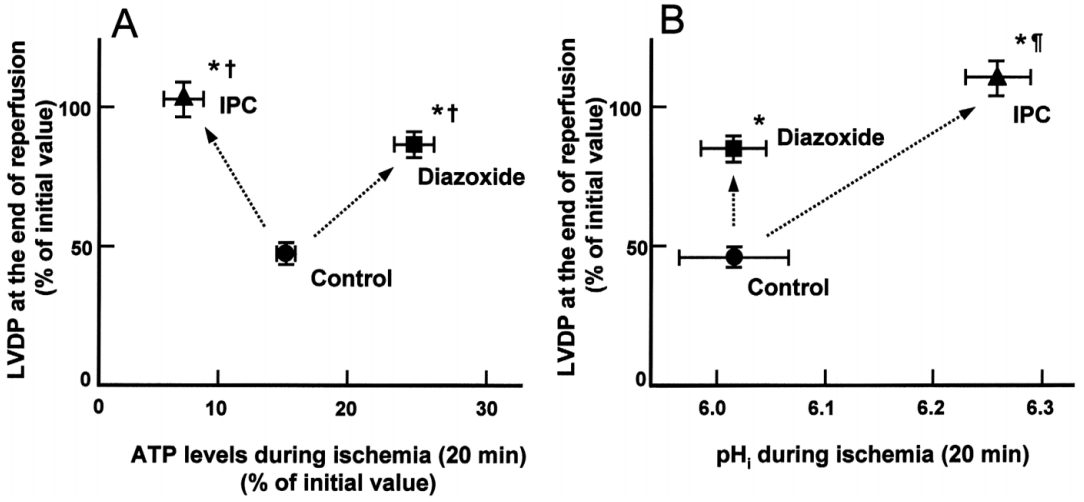

Fig 5. The relationship between postischemic recovery of left ventricular developed pressure (LVDP) and ATP levels (A) or pH_i (B) at 20 min of ischemia in diazoxide- and IPC- treated hearts. Data are presented as means ± SEM. *$p<0.05$ vs LVDP in control hearts, †$p<0.05$ vs ATP levels in control hearts, ¶$p<0.05$ vs pH_i in control hearts. The arrows indicate the distinctive effects of the ATP levels and pH_i during ischemia on postischemic recovery of contractile function in diazoxide- and IPC- treated hearts.

Effects of DZ and IPC on ATP Levels During Sustained Ischemia

Fig 4B shows the time course of the changes in ATP levels during ischemia. In all groups the ATP levels decreased gradually during ischemia and was undetectable at 30 min of ischemia. ATP levels in the preconditioned hearts were slightly lower than those in control hearts throughout ischemia, but there were no differences in ATP levels between preconditioned and control hearts except at the onset of ischemia. DZ delayed the reduction in ATP levels during ischemia, and at 20 min of ischemia the ATP levels in the DZ-treated hearts were significantly higher than those in preconditioned and control hearts (26.3±3.4% in DZ vs 8.1±3.0% in IPC and 15.1±1.3% in control, $p<0.05$).

Relationship Between Postischemic Recovery of Contractile Function and ATP Levels or pH_i During Ischemia in DZ- and IPC-Treated Hearts

Fig 5 shows the relationships between postischemic recovery of LVDP, and ATP levels or pH_i during ischemia. Although IPC and DZ resulted in a recovery of postischemic contractile function, the changes in ATP levels and pH_i during ischemia were different between the 2 treatments. DZ improved the postischemic LVDP with increased ATP levels at 20 min of ischemia, but IPC restored the postischemic LVDP without preservation of ATP levels (Fig 5A). In addition, the improvement in the postischemic LVDP by IPC was associated with a higher level of pH_i during ischemia, whereas the restoration of LVDP by DZ was not accompanied by attenuation of ischemic acidification (Fig 5B).

Discussion

In the present study, we demonstrated that although both 5-HD and NAC abolished DZ-induced cardioprotection, neither inhibited IPC-induced cardioprotection. DZ reduced a decline in ATP levels during ischemia and IPC attenuated a fall in pH_i during ischemia. These findings suggest that the cardioprotective effects of DZ are associated with ROS production and reduced ATP degradation during ischemia, whereas attenuated acidification during ischemia could play an important role in the cardioprotective effect of IPC, which is not linked to either the opening of mitoKATP channels or ROS generation.

Involvement of MitoKATP Channels and ROS in DZ- and IPC-Induced Cardioprotection

It is controversial whether the opening of mitoKATP channels promotes or suppresses ROS production[5,6,22,23] In the present study, both 5-HD and NAC abolished DZ-induced cardioprotection, which is in agreement with the findings of Pain et al[5] and Forbes et al[6] who have suggested that the opening of the mitoKATP channels precedes ROS production, leading to cardioprotection. The mitochondrial respiratory chain is known to be a major source of ROS. Although the ability of KATP channel openers to generate ROS has been demonstrated[6,24] the mechanism of ROS production by DZ remains unclear. Krenz et al have proposed that alkalization in the mitochondrial matrix, which is caused by increased K^+ uptake through the mitoKATP channels, may be related to increased ROS production[25]

There is considerable evidence for the involvement of mitoKATP channels[9,10] and ROS[11,12] in IPC. However, in the present study neither 5-HD nor NAC inhibited the cardioprotection induced by IPC, suggesting that IPC is not mediated through the mitoKATP channel opening or ROS production during the preconditioning period. Several investigators have also documented that the administration of 5-HD[13–15] and ROS scavengers[16,17,26] could not cancel the cardioprotective effects of IPC. Lim et al have indicated that DZ and 5-HD have additional effects on mitochondrial function, independently of any effects on mitoKATP channels[15] Moreover, it has been reported that mitochondria can generate ROS by the inhibition of the electron transport chain, especially complexes I and III[27] or in state 4 respiration[28] although the mechanism by which ROS are generated during a preconditioning period is unclear. Recent studies have demonstrated that the mitochondrial respira-

tory rate of preconditioned hearts before sustained ischemia did not decrease compared with untreated hearts[15,29] These findings suggest that at least the inhibition of the electron transport chain might not be responsible for generating ROS during the period of preconditioning.

IPC increased the coronary flow rate and improved LVDP and PCr levels during reperfusion compared with DZ. de Albuquerque et al have demonstrated that improved LV contractile performance was associated with increased coronary flow rate during reperfusion, but matching the coronary flow rate of control hearts to that of preconditioned hearts did not improve contractile or metabolic performance during reperfusion[20] That finding suggests that the increased coronary flow rate during reperfusion is not responsible for the improvement in postischemic contractile function.

DZ and ATP Levels During Ischemia

There are conflicting results for ATP levels during ischemia in IPC[19,20,30–32] Our results indicated that ATP levels during ischemia in preconditioned hearts tended to decrease slightly faster than in control hearts, and the energy-sparing effect of IPC at the end of a preconditioning period and during ischemia was not observed. These findings imply that cardioprotection by IPC does not necessarily require higher levels of ATP during ischemia. In contrast, DZ kept ATP levels higher during ischemia. Preservation of ATP levels during ischemia has also been observed in a study using cromakalim, a non-selective KATP channel opener[33] Limited ATP degradation during ischemia may be the common effect of KATP channel openers. Dzeja et al recently found that DZ attenuates cellular and mitochondrial ATPase activities[34] It appears that reduced ATP degradation during ischemia because of the inhibitory effect of DZ on cellular and mitochondrial ATPase activities preserves the mitochondrial respiratory chain[35] and sarcolemmal integrity[36] and thereby is associated with cardioprotection in rat hearts.

IPC and Intracellular pH During Ischemia

IPC attenuates intracellular acidification during ischemia[18–20] Forbes et al indicated that DZ[6] as well as IPC[12] reduced the decrease in pH_i during ischemia. Our data showed that although IPC attenuated ischemic acidification, DZ did not affect the degree of ischemic acidification. It has also been documented that other KATP channel openers, such as cromakalim[33] or pinacidil[37] did not reduce the fall in pH_i at the end of ischemia. It is, therefore, likely that KATP channel openers have no effect on reducing the ischemic acidification as observed with IPC. Although the reason why the reduction in acidification contributes to cardioprotection remains elusive, ischemic acidification per se can cause increased fragility and permeability of the mitochondrial membrane[38] and impairment of the mitochondrial respiratory chain[39] On reperfusion, Ca^{2+} overload through the Na^+/H^+ and Na^+/Ca^{2+} exchangers[40] and increased opening probability of the mitochondrial permeability transition pores[41] may be involved in myocardial damage.

Because a decline in pH_i and a lack of ATP caused by ischemia are the critical changes in the cellular environment, reduced acidification and ATP degradation during ischemia can be directly linked to limited myocardial damage. Glycogen is the sole substrate during total ischemia, and its degradation supplies ATP, but leads to accumulation of protons and lactate during ischemia. The hypothesis that glycogen depletion before ischemia causes the attenuation of ischemic acidification and then contributes to the cardioprotective effect[32] is a promising candidate for the mechanism of IPC. However, King et al have suggested that when pre-ischemic glycogen content decrease or increase considerably, the improvement in postischemic contractile function by IPC is limited[42] Therefore, moderate depletion of glycogen before ischemia, which keeps the balance between intracellular acidification and the supply of ATP during ischemia, might be related to the cardioprotection induced by IPC.

Study Limitations

Most of in vivo studies have demonstrated that IPC-induced cardioprotection is abolished by 5-HD[10] However, in the present study, we did not observe the inhibitory effect of 5-HD on IPC-induced cardioprotection. This discrepancy may be caused by differences in the experimental model. The Krebs-Henseleit buffer used in the perfused heart model lacks the components of blood, such as fatty acids, pyruvate or insulin, that can affect myocardial protection or injury during ischemia and reperfusion[43,44] Because of that lack, signaling pathways other than the mitoKATP channel opening may have gained predominance in the protective effects of IPC in our experimental set-up. It should be also noted that the poor recovery of postischemic LVDP in the control hearts might have been caused partly by myocardial stunning rather than myocardial infarction, because we assessed the functional and metabolic recovery but did not measure infarct size or creatine kinase release on reperfusion. These issues could explain the inability of 5-HD to abolish IPC-induced cardioprotection in the present study.

In summary, we demonstrated that ROS production before sustained ischemia and attenuated ATP degradation during ischemia are associated with DZ-induced cardioprotection, whereas reduced acidification during ischemia is involved in IPC-induced cardioprotection, which is independent of mitoKATP channel opening or ROS production. Therefore, the cardioprotective mechanisms of IPC and DZ differ both in the signaling pathway and the metabolic aspects.

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References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; **74:** 1124–1136.
2. O'Rourke B. Myocardial KATP channels in preconditioning. *Circ Res* 2000; **87:** 845–855.
3. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992; **70:** 223–233.
4. Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, et al. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. *Circ Res* 1997; **81:** 1072–1082.
5. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, et al. Opening of mitochondrial KATP channels triggers the preconditioned state by generating free radicals. *Circ Res* 2000; **87:** 460–466.
6. Forbes RA, Steenbergen C, Murphy E. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism.

Circ Res 2001; **88:** 802–809.
7. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* 1998; **273:** 18092–18098.
8. Yaguchi Y, Satoh H, Wakahara N, Katoh H, Uehara A, Terada H, et al. Protective effects of hydrogen peroxide against ischemia/reperfusion injury in perfused rat hearts. *Circ J* 2003; **67:** 253–258.
9. Toombs CF, Moore TL, Shebuski RJ. Limitation of infarct size in the rabbit by ischaemic preconditioning is reversible with glibenclamide. *Cardiovasc Res* 1993; **27:** 617–622.
10. Fryer RM, Eells JT, Hsu AK, Henry MM, Gross GJ. Ischemic preconditioning in rats: Role of mitochondrial KATP channel in preservation of mitochondrial function. *Am J Physiol Heart Circ Physiol* 2000; **278:** H305–H312.
11. Tanaka M, Fujiwara H, Yamasaki K, Sasayama S. Superoxide dismutase and N-2-mercaptopropionyl glycine attenuate infarct size limitation effect of ischaemic preconditioning in the rabbit. *Cardiovasc Res* 1994; **28:** 980–986.
12. Chen W, Gabel S, Steenbergen C, Murphy E. A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. *Circ Res* 1995; **77:** 424–429.
13. Grover GJ, Murray HN, Baird AJ, Dzwonczyk S. The KATP blocker sodium 5-hydroxydecanoate does not abolish preconditioning in isolated rat hearts. *Eur J Pharmacol* 1995; **277:** 271–274.
14. Schwartz LM, Welch TS, Crago MS. Cardioprotection by multiple preconditioning cycles does not require mitochondrial KATP channels in pigs. *Am J Physiol Heart Circ Physiol* 2002; **283:** H1538–H1544.
15. Lim KH, Javadov SA, Das M, Clarke SJ, Suleiman MS, Halestrap AP. The effects of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat heart mitochondrial volume and respiration. *J Physiol* 2002; **545:** 961–974.
16. Iwamoto T, Miura T, Adachi T, Noto T, Ogawa T, Tsuchida A, et al. Myocardial infarct size-limiting effect of ischemic preconditioning was not attenuated by oxygen free-radical scavengers in the rabbit. *Circulation* 1991; **83:** 1015–1022.
17. Richard V, Tron C, Thuillez C. Ischaemic preconditioning is not mediated by oxygen derived free radicals in rats. *Cardiovasc Res* 1993; **27:** 2016–2021.
18. Kida M, Fujiwara H, Ishida M, Kawai C, Ohura M, Miura I, et al. Ischemic preconditioning preserves creatine phosphate and intracellular pH. *Circulation* 1991; **84:** 2495–2503.
19. Asimakis GK, Inners-McBride K, Medellin G, Conti VR. Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart. *Am J Physiol* 1992; **263:** H887–H894.
20. de Albuquerque CP, Gerstenblith G, Weiss RG. Importance of metabolic inhibition and cellular pH in mediating preconditioning contractile and metabolic effects in rat hearts. *Circ Res* 1994; **74:** 139–150.
21. Dhalla NS, Elmoselhi AB, Hata T, Makino N. Status of myocardial antioxidants in ischemia–reperfusion injury. *Cardiovasc Res* 2000; **47:** 446–456.
22. Ozcan C, Bienengraeber M, Dzeja PP, Terzic A. Potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation. *Am J Physiol Heart Circ Physiol* 2002; **282:** H531–H539.
23. Ferranti R, da Silva MM, Kowaltowski AJ. Mitochondrial ATP-sensitive K^+ channel opening decreases reactive oxygen species generation. *FEBS Lett* 2003; **536:** 51–55.
24. Obata T, Yamanaka Y. Block of cardiac ATP-sensitive K^+ channels reduces hydroxyl radicals in the rat myocardium. *Arch Biochem Biophys* 2000; **378:** 195–200.
25. Krenz M, Oldenburg O, Wimpee H, Cohen MV, Garlid KD, Critz SD, et al. Opening of ATP-sensitive potassium channels causes generation of free radicals in vascular smooth muscle cells. *Basic Res Cardiol* 2002; **97:** 365–373.
26. Klawitter PF, Murray HN, Clanton TL, Angelos MG. Reactive oxygen species generated during myocardial ischemia enable energetic recovery during reperfusion. *Am J Physiol Heart Circ Physiol* 2002; **283:** H1656–H1661.
27. Lenaz G. The mitochondrial production of reactive oxygen species: Mechanisms and implications in human pathology. *IUBMB Life* 2001; **52:** 159–164.
28. Korshunov SS, Skulachev VP, Starkov AA. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* 1997; **416:** 15–18.
29. Crestanello JA, Doliba NM, Babsky AM, Niibori K, Osbakken MD, Whitman GJ. Mitochondrial function during ischemic preconditioning. *Surgery* 2002; **131:** 172–178.
30. Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 1990; **66:** 913–931.
31. Kolocassides KG, Seymour AM, Galinanes M, Hearse DJ. Paradoxical effect of ischemic preconditioning on ischemic contracture? NMR studies of energy metabolism and intracellular pH in the rat heart. *J Mol Cell Cardiol* 1996; **28:** 1045–1057.
32. Wolfe CL, Sievers RE, Visseren FL, Donnelly TJ. Loss of myocardial protection after preconditioning correlates with the time course of glycogen recovery within the preconditioned segment. *Circulation* 1993; **87:** 881–892.
33. Docherty JC, Gunter HE, Kuzio B, Shoemaker L, Yang L, Deslauriers R. Effects of cromakalim and glibenclamide on myocardial high energy phosphates and intracellular pH during ischemia-reperfusion: ^{31}P NMR studies. *J Mol Cell Cardiol* 1997; **29:** 1665–1673.
34. Dzeja PP, Bast P, Ozcan C, Valverde A, Holmuhamedov EL, Van Wylen DG, et al. Targeting nucleotide-requiring enzymes: Implications for diazoxide-induced cardioprotection. *Am J Physiol Heart Circ Physiol* 2003; **284:** H1048–H1056.
35. Rouslin W, Broge CW, Grupp IL. ATP depletion and mitochondrial functional loss during ischemia in slow and fast heart-rate hearts. *Am J Physiol* 1990; **259:** H1759–H1766.
36. Askenasy N. Glycolysis protects sarcolemmal membrane integrity during total ischemia in the rat heart. *Basic Res Cardiol* 2001; **96:** 612–622.
37. Fukuda H, Luo CS, Gu X, Guo L, Digerness SB, Li J, et al. The effect of KATP channel activation on myocardial cationic and energetic status during ischemia and reperfusion: Role in cardioprotection. *J Mol Cell Cardiol* 2001; **33:** 545–560.
38. Zimmer G, Freisleben HJ, Fuchs J. Influence of pH on sulfhydryl groups and fluidity of the mitochondrial membrane. *Arch Biochem Biophys* 1990; **282:** 307–317.
39. Rouslin W. Mitochondrial complexes I, II, III, IV, and V in myocardial ischemia and autolysis. *Am J Physiol* 1983; **244:** H743–H748.
40. Seki S, Taniguchi M, Takeda H, Nagai M, Taniguchi I, Mochizuki S. Inhibition by KB-R7943 of the reverse mode of the Na^+/Ca^{2+} exchanger reduces Ca^{2+} overload in ischemic-reperfused rat hearts. *Circ J* 2002; **66:** 390–396.
41. Halestrap AP, Kerr PM, Javadov S, Woodfield KY. Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim Biophys Acta* 1998; **1366:** 79–94.
42. King LM, Opie LH. Does preconditioning act by glycogen depletion in the isolated rat heart? *J Mol Cell Cardiol* 1996; **28:** 2305–2321.
43. Opie LH, Sack MN. Metabolic plasticity and the promotion of cardiac protection in ischemia and ischemic preconditioning. *J Mol Cell Cardiol* 2002; **34:** 1077–1089.
44. Sargent CA, Dzwonczyk S, Sleph P, Wilde M, Grover GJ. Pyruvate increases threshold for preconditioning in globally ischemic rat hearts. *Am J Physiol* 1994; **267:** H1403–H1409.