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Influence of fasting on the effects of diazoxide in the ischemic-reperfused rat heart

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This investigation aimed to assess whether the mitochondrial ATP-sensitive potassium channel opener diazoxide could reproduce the protection conferred by ischemic preconditioning and to ascertain whether its effects are associated with changes in glycogen breakdown and glycolytic activity. Hearts of fed and 24-h fasted rats were perfused with 10 mM glucose containing medium and exposed to 25 min no-flow ischemia plus 30 min reperfusion. Diazoxide (10 μM) perfusion was begun 10 min before ischemia and continued throughout the experiment. Fasting accelerated reperfusion recovery of contraction, reduced the post-ischemic contracture and decreased lactate accumulation during ischemia but had no effects on glycogen levels and cellular viability. Diazoxide, did not affect glycogen catabolism but improved reperfusion recovery of contraction. Furthermore, diazoxide reduced ischemic lactate accumulation and contracture amplitude only in the fed group whereas it improved cell viability in the fed and fasted groups. These data indicate that:1) reduced lactate production which may attenuate myocyte acidification might explain, at least in part, the beneficial effects of diazoxide on mechanical function, although data obtained with the fasted rat hearts indicate that other mechanisms must be involved as well; 2) the reduction of lactate production occurring in the fed group, does not seem to be related to glycogenolysis; and 3) since diazoxide improved cell viability in the fasted rat group where it did not reduce glycolytic activity, other mechanisms may be responsible for this cytoprotective effect.

Key words: Diazoxide, Fasting, Glycogen, Glycolysis, Ischemia, Preconditioning.

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Brief intermittent episodes of ischemia protect the heart against subsequent sustained ischemia and reperfusion. This phenomenon, known as ischemic preconditioning (IP), is an inherent capability of the heart to protect itself from ischemic damage (1, 6, 7, 16). Although the molecular basis of this endogenous protective mechanism have yet to be fully understood, several components have been identified. Among those, the mitochondrial ATP-sensitive potassium (K-ATP) channel has been demonstrated to be an important component in perfused heart and cellular models of IP (2, 11, 12, 18). In support of this, the K-ATP channel opener diazoxide (DZ), which in a micromolar range selectively opens the mitochondrial K-ATP channel, was shown to elicit significant cardioprotective effects (8, 11, 12, 18, 29). Furthermore, the mitochondrial K-ATP channel blocker 5-hydroxydecanoic acid, impedes both DZ and IP cardioprotection (5, 6, 14, 18, 29). However, it should be mentioned that some papers question the assumption that the effects to DZ and 5-hydroxydecanoic acid implies the involvement of the K-ATP channel (3, 6).

With regard to the metabolic changes which make myocytes more resistant to ischemic injury, it should be noted that several studies have shown that the protective effect of IP is associated with reduced glycogen breakdown and less glycolysis and protons production during ischemia (7, 27, 30). Inhibition of glycolysis and the consequent production of lactate and protons may indirectly be beneficial by reducing ionic alterations which lead to calcium overload, despite reduced ATP production. On the other hand, similar changes in glucose metabolism may contribute to the beneficial effects of fasting. In this respect, it has been shown that previous fasting, which enhances triglyceride stores and accelerates oxidation of fatty acids derived from endogenous lypolysis (15, 24), reduced lactate production during ischemia, ameliorated the ischemic contracture (15, 26) and shortened the time of return of functional recovery (23, 26). This cardioprotective effect occurred in spite of the ischemic increase of long-chain acyl-CoA levels (26) which are powerful inactivators of mitochondrial K-ATP channels (19). It has also been reported that IP, which did not affect long-chain acyl-CoA levels but lowered lactate production during ischemia in hearts from fed rats, elicited a more thorough functional protection in this nutritional condition with respect to the fasted hearts, and improved cell viability in both groups of hearts (25, 26).

Based on the findings described above and since it is still unclear as to why opening mitochondrial K-ATP channels would be cardioprotective, it seemed interesting to assess whether the mitochondrial K-ATP channel opener DZ, could reproduce the protection conferred by ischemic preconditioning (IP) in hearts from fed and fasted rats. In order to meet this goal, the effects of DZ on functional recovery and cell viability following no-flow global ischemia and reperfusion were assessed in relation to glycogenolysis and lactate production during the ischemic state.

Materials and Methods

Experimental protocol.– The investigation was conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23,revised 1985) and the Argentine Republic Law N° 14346 concerning animal protection. Fifty two Wistar rats of either sex weighing 250-350 g, maintained

on a 12-h dark-light cycle, either fed *ad libitum* or fasted 24 h, were anesthetized with diethylether and heparin (250 UI) was injected into the jugular vein. The hearts were rapidly isolated and cooled in ice-cold saline until contractions stopped. Afterwards the hearts were mounted on a modified Langendorff apparatus and isovolumically perfused at a constant pressure of 70 mm Hg with a nonrecirculating Krebs-Ringer bicarbonate solution composed of the following (mM): NaCl 120, $NaHCO_3$ 25, KCl 4.8, $MgSO_4$ 1.33, KPO_4H_2 1.2, $CaCl_2$ 1.6, Na_2EDTA 0.02 and glucose 10. The perfusate was gassed with 95% O_2-5% CO_2 (pH 7.4) and kept at a constant temperature of 37 °C. Throughout the entire experiment the heart was placed in a water-jacketed organ bath in order to maintain the temperature constant during the ischemic period.

After a 25 min equilibration period, the hearts were treated with vehicle: 0.01 % dimethyl sulfoxide or 10 μM DZ (Sigma Chem.Co.). The respective drugs were included in the perfusate and kept throughout the experiment. Ten min later, the hearts were subjected to 25 min global ischemia and 30 min reperfusion (RP). Ischemia was initiated by completely shutting off perfusate flow. Consistent with GARLID *et al.* (9) observation, the dose of DZ used in this study did not affect coronary flow (data not shown). Only hearts with LVDP > 60 mmHg and HR > 200 beats/min at the end of baseline perfusion period were included.

Measurement of heart function.– To measure left ventricular pressures, the left atrium was removed and a latex balloon connected to a pressure transducer was inserted through the mitral valve into the left ventricle. The volume of the balloon was adjusted to obtain left ventricular end diastolic pressure (LVEDP) of 10 mm Hg. Values for the left ventricular developed pressure (LVDP), peak rate of contraction (+dP/dt) and peak rate of relaxation (–dP/dt) were obtained using a digital data acquisition system. Heart rate (HR) was measured by means of a HR counter triggered by the LVDP pulse. Rate-pressure product (RPP) was determined by multiplying HR and LVDP.

Measurement of cell viability.– At the end of the RP period and after removal of connective tissue, the hearts were frozen and cut into 6-8 slices of approximately 0.8 mm up to 1.0 mm in thickness. Following defrosting, the slices were incubated at room temperature with 1% triphenyltetrazolium chloride in phosphate buffer (100 mM, pH 7.4) for 90 min and fixed in 10 % formaldehyde solution to clearly distinguish stained viable tissue from unstained necrotic tissue. The areas of viable tissue were determined by computer morphometry (Scion Image B 4). The risk area was the sum of total ventricular area minus cavities. The cellular viability was calculated and presented as percentage of risk area. It is worth noting that in this model, necrosis evolution is incomplete after 30 min RP and it is possible that our results would vary using a longer RP duration leading to the ultimate extent of necrosis (13).

Biochemical assays.– Additional hearts treated according to above protocols were removed and immediately frozen between two blocks of ice at –21 °C just before the onset of sustained ischemia and at the end of the 25 min ischemia for determination of tissue glycogen and lactate. A sample of approximately 60 mg of wet tissue was used to determine the dry-to-wet ratio and to calculate the total dry weight (g) of the heart.

Glycogen, in ~200 mg of frozen ventricular tissue, was determined by the method of WALAAS and WALAAS with the use of amyloglucosidase (28). Glycogen values were expressed as µg per 100 mg dry weight .Lactate was extracted from ~100 mg of frozen ventricular tissue into 6% ice-cold perchloric acid and measured enzymatically (22). Lactate values were expressed as µmol per gram dry weight.

Statistical analysis.– Values represent the mean ± SEM. Changes of the ventricular contractile functions were statistically compared using a three factors ANOVA for repeated measures in one factor followed by Tukey's test. Lactate and glycogen were statistically evaluated using a three factors ANOVA followed by Tukey's test. Significance was set at the $p<0.05$ level.

Results

Effects of fasting and diazoxide (DZ) on heart functions.– In both the fed and fasted rat hearts the exposure to 25 min no-flow global ischemia led to a complete cessation of the spontaneous contractions, and during the 30 min reperfusion the heart rate returned progressively to preischemic values . DZ in both groups had no effect during ischemia or RP (data not shown).

As indicated by the RPP, the peak +dP/dt and the -dP/dt (Fig. 1) upon RP, the left ventricle contractile function recovered faster in the fasted than in the fed rat hearts although in both groups the recovery attained similar values toward the end of the experiment. DZ improved the recovery of all contractile parameters which reached similar values in both nutritional groups.

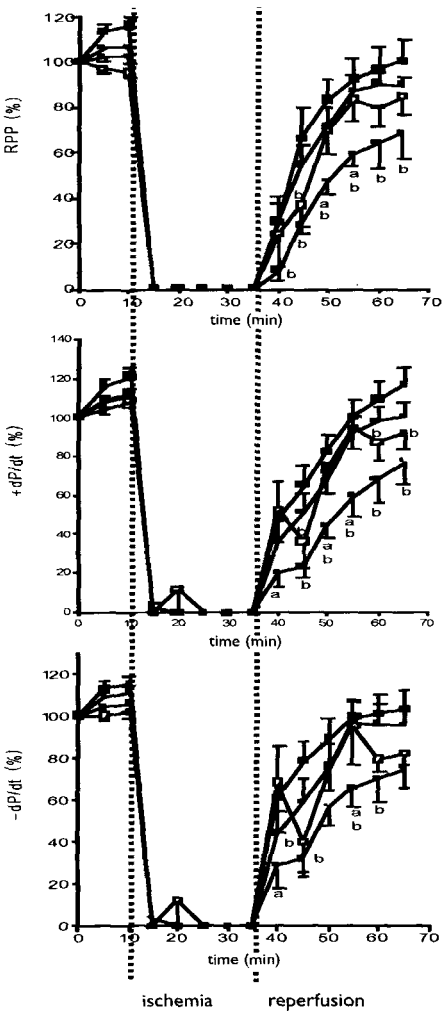

Fig 1. *Changes in rate-pressure product (RPP), peak rate of contraction (+dP/dt), and peak rate of relaxation (-dP/dt) due to ischemia-reperfusion in control and diazoxide treated hearts from fed and fasted rats.* Values are expressed as percentages of respective basal values at the end of the 25 min equilibration period. Squares: fed rats. Circles: 24-h fasted rats. Open symbols: hearts perfused with control medium containing 0.01% dimethylsulfoxide. Closed symbols: hearts perfused with medium containing 10 µM diazoxide plus dimethylsulfoxide. Drugs were added to the perfusion medium 10 min before the onset of 25 min ischemia-30 min reperfusion. Values are the average of 8 hearts ± SEM in each group. a: $p < 0.01$ *vs.* control fasted, b: $p<0.01$ *vs.* diazoxide treated hearts in the same nutritional state.

Figure 2 depicts the effect of ischemia-RP on LVEDP. During ischemia, hearts developed pronounced myocardial contracture, which was comparable between hearts from fasted and fed rats. However, during the first 15 min of RP, and concurrent with the faster recovery of contractile function, hearts from fasted animals exhibited lower diastolic pressures than hearts from fed rats. DZ significantly reduced LVEDP at the end of the ischemic period and during RP in fed rat hearts, so that the difference between hearts of fed and fasted rats disappeared in DZ treated hearts.

Effects of fasting and DZ on cell viability.– After the 25 min global ischemia and 30 min RP periods, a substantial amount of the ventricular muscle was irreversibly damaged in control fed and fasted hearts as indicated by the low percentage of viable tissue, although there was no significant difference between these two nutritional conditions (21.2±6.6 and 18.2±7.9 5 % in hearts from fed and fasted rats, respectively). DZ significantly increased the percentage of viable tissue to a similar extent in the hearts from fed and fasted rats (56.3±6.9 and 57.9±4.2 % in hearts from fed and fasted rats, respectively. $p<0.01$ *vs.* control hearts in the same nutritional state, n=5).

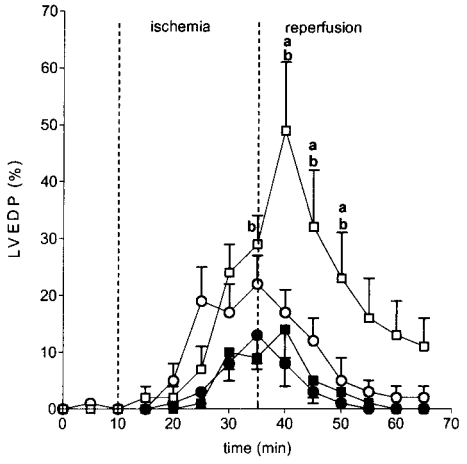

Fig 2. *Changes in left ventricular end diastolic pressure (LVEDP) due to ischemia-reperfusion in control and diazoxide treated hearts from fed and fasted rats.*
Values and symbols as in Fig. 1.

Effects of fasting and DZ on glycogen and lactate tissue levels.– Table I shows that the lactate content of samples from fed rats was higher than in hearts from fasted animals, at the end of the ischemic period. DZ reduced lactate accumulation in the fed but not in the fasted rat hearts. Hence, the difference of lactate content at the end of the 25 min ischemic period between the hearts of fed and fasted rats disappeared in the DZ treated hearts.

Preischemic glycogen content was similar in both fed and fasted hearts and decreased similarly during ischemia in both nutritional groups. DZ did not exert any effect on the heart content of glycogen during the aerobic nor ischemic conditions in neither the fed nor the fasted groups (Table I).

Discussion

The present study confirms the previously reported protective effect of fasting on myocardial performance in the ischemic-reperfused hearts (4, 10, 15, 20, 21, 26). Coinciding with earlier findings (15, 21), either during the equilibration and ischemic periods, glycogen stores were depleted to a comparable extent in hearts from fasted and fed rats. Thus, preservation of myocardial glycogen cannot explain the observed improvement of contractile function upon RP in the fasted hearts. However, consistent with previous observations (10, 26), even though the

Table I: *Changes in ventricular tissue levels of glycogen (µg/100 mg dry weight) and lactate (pmol/g dry weight) in fed and fasted rats.*
Values are the mean ± SEM, n = 8. Preischemic refers to the end of the 35 min preischemic period. Ischemic refers to the end of the 25 min ischemic period. DZ: diazoxide treated hearts.

	Glycogen		Lactate	
	Fed	Fasted	Fed	Fasted
Control				
Preischemic	254.9 ± 33.6	326.5 ± 27.5	15.6 ± 1.9	10.9 ± 2.7
Ischemic	78.9 ± 21.0*	69.2 ±16.6*	153.0 ± 14.1*+	112.1 ± 15.3*[a]
DZ-treated				
Preischemic	233.0 ± 48.6	243.1 ± 34.3	3.0 ± 0.3	2.7 ± 0.3
Ischemic	58.19 ± 10.0*	69.6 ± 13.7*	104.8 ± 12.4*	106.3 ± 10.6*

*$p < 0.01$ *vs.* preischemic hearts in the same nutritional state. +$p < 0.01$, *vs.* ischemic DZ-treated fed rat hearts. [a]$p < 0.01$, *vs.* ischemic hearts of control rats.

preischemic glycogen content tended to be higher in the hearts from fasted rats, they exhibited a lower lactate production. Since glycolytic flux elicits an acidification of the ischemic heart fibers which has been reported to compromise the contractile function, it seems likely that the reduced glycolytic flux observed in the fasted rat hearts could contribute to the protective effects of fasting. On the other hand, the beneficial effects of fasting on the heart function cannot be explained by an improvement in cell survival. This finding is in agreement with previously reported data which showed that post-ischemic creatine kinase leakage (26) as well as infarct size (31) were similar in the fed and fasted rat hearts. These data seem to indicate that preservation of the functional properties of the remaining left ventricular viable myocardium might have occurred in the fasted group.

In accordance with studies from other laboratories (8, 11, 29), DZ improved the post-ischemic mechanical function. This effect was similar in absolute values in both nutritional states although proportionally greater in the fed rat hearts. The elevated LVEDP upon RP observed in the fed hearts reflects a diastolic functional impairment that is generally ascribed to Ca^{2+} overload which occurs via sequential H^+/Na^+ and Na^+/Ca^{2+} exchange (17, 23). DZ markedly attenuated the increase of LVEDP and interestingly this effect attained in the fed rat group, coincided with the lowering of lactate accumulation during ischemia. Consistent with this finding, FORBES *et al.* (8) have found that DZ significantly reduced intracellular acidification during ischemia. This ability of DZ is proposed by these authors to be secondary to glycogen depletion at the onset of ischemia. However, present data demonstrate that in DZ treated hearts, preischemic glycogen levels are similar to controls. Furthermore, since DZ did not exhibit any effect on glycogen breakdown during ischemia, the decreased lactate production in the fed hearts cannot be ascribed to a lowered availability of glycolytic substrate from glycogen, but rather to the inhibition of the Embden-Meyerhoff pathway. These findings, combined with the results showing that the functional protection afforded by previous fasting is associated with a reduction of lactate production, reemphasize the

possibility of a link between inhibition of glycolytic activity during ischemia and the improvement of post-ischemic cardiac function. However, since in the fasted rats the protective effect of DZ, although slight, could be observed in the absence of any change in lactate production, the effects of DZ most likely depend on some other mechanisms as well that cannot be clarified from the present experiments.

On the other hand, DZ preserved cell viability in fed as well as in fasted hearts and interestingly, this cytoprotection was equivalent to that of IP previously reported (25). In this respect, it should be noted that the present findings combined with those obtained by IP (25, 26) indicate that it is possible to observe cytoprotective effects in the absence of any modification in anaerobic glycolysis, just as it occurred in DZ-treated and ischemic-preconditioned fasted hearts (25). Furthermore, the present results suggest that different mechanisms might be involved in the improved RP contractile recovery and in the anti-necrotic cardioprotection. Finally, whatever mechanisms are implicated, the beneficial effects of DZ show a fairly good similarity to the previously reported effects of IP (25, 26). The present data further support the notion that mitochondrial K-ATP channel might be involved in the beneficial effects of IP.

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Se estudia en este trabajo si el diazóxido, que activa los canales mitocondriales de potasio sensibles al ATP, reproduce los efectos del precondicionamiento isquémico y si tales efectos se asocian con cambios en la glucogenolisis y la actividad glicolítica. Corazones perfundidos de ratas alimentadas y tras 24 h de ayuno se exponían a 25 min de isquemia con reperfusión de 30 min. La perfusión con diazóxido (10 µM) comenzaba 10 min antes de la isquemia y continuaba durante todo el experimento. El ayuno acelera la recuperación de la contracción al reperfundir, reduce la contractura post-isquémica y disminuye la acumulación isquémica de lactato, pero no tiene efectos sobre los niveles de glucógeno y la viabilidad celular. El diazóxido no afecta a la glucogenolisis, pero mejora la recuperación post-isquémica de la contracción. Además, el diazóxido reduce la acumulación isquémica de lactato y la amplitud de la contractura sólo en el grupo alimentado, pero mejora la viabilidad celular en ambos grupos. Los resultados indican que: 1) la reducción de la producción de lactato, que atenuaría la acidificación del miocito, explicaría, al menos en parte, los efectos beneficiosos del diazóxido sobre la función mecánica, aunque los resultados de las ratas en ayunas indican la implicación de otros mecanismos; 2) la reducción de la producción de lactato obtenida en el grupo alimentado no parece estar relacionada con la glucogenolisis; y 3) como el diazóxido mejora la viabilidad celular en los corazones de ratas en ayunas en los cuales no reduce la glicólisis, otros mecanismos serían responsables del efecto citoprotector

Palabras clave: Diazóxido, Ayuno, Glucógeno, Isquemia, Precondicionamiento.

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