J Physiol Sci (2011) 61:303–312
DOI 10.1007/s12576-011-0152-0

ORIGINAL PAPER

Involvement of energetic metabolism in the effects of ischemic postconditioning on the ischemic-reperfused heart of fed and fasted rats

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Received: 26 November 2010 / Accepted: 8 April 2011 / Published online: 6 May 2011

Abstract The effects of ischemic-postconditioning (IPOC) on functional recovery and cell viability of ischemic-reperfused hearts from fed and fasted rats were studied in relation to triacylglycerol and glycogen mobilization, ATP content, glucose-6-phosphate dehydrogenase activity and reduced/oxidized glutathione (GSH/GSSG). Oxidative damage was estimated by measuring thiobarbituric acid reactive substances (TBARS). IPOC improved contractile recovery and cell viability in the fed but attenuated them in the fasted hearts. In both groups ischemia lowered glycogen. IPOC further reduced it. Triacylglycerol remained unchanged during ischemia-reperfusion in both groups, but triacylglycerol mobilization was activated by IPOC in the fasted group. ATP was increased by IPOC in the fed hearts, but lowered in the fasted ones, which appeared to be associated with the rates of ATP synthesis in isolated mitochondria. In the fed hearts IPOC raised glucose-6-phosphate dehydrogenase activity and GSH/GSSG, and lowered TBARS. These results suggest that IPOC effects are associated with changes in the ATP supply, mobilization of energy sources and glutathione antioxidant ratio.

Keywords Postconditioning · Heart · Fasting · Ischemia-reperfusion · Triacylglycerol · Glycogen

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Introduction

After exposure to ischemia, reperfusion of the myocardium is the prerequisite for any potential recovery of contractile function. However, reperfusion by itself can cause additional damage, also known as reperfusion injury [1]. Reperfusion injury could be attenuated by interventions applied either before the onset of ischemia or during reperfusion. Among these procedures, ischemic postconditioning (IPOC), defined as rapid intermittent interruptions of blood flow in the early phase of reperfusion, has been shown to attenuate myocardial injury in many experimental studies as well as in clinical settings [2, 3]. Interestingly, the reduction of infarct size by postconditioning persists for up to 72 h [4], suggesting that IPOC effects the long-term reduction in reperfusion damage and therefore not merely the delay of an inevitable injury. This procedure seems to activate multiple intracellular molecular pathways that attenuate reperfusion injury, but their respective contribution to the amelioration of necrosis, apoptosis, endothelial injury and/or microvascular and macrovascular injury is not yet fully understood [3, 4].

On the other hand, it has been found that previous fasting protects the heart against ischemia-reperfusion injury by improving functional recovery [5, 6], increasing the ratio of reduced to oxidized glutathione (GSH/GSSG) and decreasing oxidative damage in the Langendorff perfused rat hearts submitted to no-flow global ischemia-reperfusion

[6]. These changes appear to be a consequence of the increased hydrolysis of endogenous triacylglycerol (TAG) and the upregulation of glucose-6-phosphate dehydrogenase (G6PDH), the first and rate-limiting enzyme of the pentose phosphate pathway, that occurs in the fasted rat heart perfused under aerobic conditions before ischemia-reperfusion [6]. It is well known that glucose-6-phosphate, which is derived either from glycogenolysis or from glucose taken up by the cell, is metabolized mainly via glycolysis. However, a small amount enters the pentose phosphate pathway in which G6PDH is the first and rate-limiting enzyme. This pathway provides reducing equivalents in the form of NADPH, which is required by the heart in order to maintain tissue levels of GSH that are essential for detoxification of reactive oxygen species. Furthermore, it has been shown that specific adaptive regulation of G6PDH, which is exerted both before and after transcription, is an important component of the integrated response to hormones, nutrients and oxidative stress [7].

Until now no study has examined the modulatory role of the nutritional condition in IPOC-mediated cardioprotection. The aim of the present work was therefore to assess the functional and metabolic response to no-flow ischemia in ischemic-postconditioned hearts from fed and fasted rats and compare this response to non-postconditioned hearts. TAG and glycogen mobilization were measured. Since fasting results in increases in G6PDH activity and the GSH/GSSG ratio, it seemed appropriate to investigate whether the protection afforded by IPOC is associated with changes in G6PDH activity and the GSH/GSSG ratio in the tissue. In order to assess the oxidative damage to the tissue, the concentration of compounds that react with thiobarbituric acid (TBARS) was determined.

Materials and methods

Experimental protocol

This study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996; http://acu.od.nih.gov/regs/guide.pdf) and Argentine Law No. 14346 concerning animal protection.

Female Wistar rats, weighing 250–350 g, maintained on a 12-h dark-light cycle, fed ad libitum or fasted for 24 h, were used in the study. Rats were anaesthetized with diethylether, and then heparin (250 IU) was injected into the jugular vein. Hearts were excised quickly and cooled in ice-cold saline until contractions stopped. Hearts were then mounted on a modified Langendorff apparatus (Hugo Sachs Elektronik, March-Hugstetten, Germany) and perfused at a constant pressure of 70 mmHg with a non-recirculating Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 120; $NaHCO_3$ 25; KCl 4.8; $MgSO_4$ 1.33; KH_2PO_4 1.2; $CaCl_2$ 1.6; Na_2EDTA 0.02; glucose 10. The perfusate was gassed with 95% O_2 and 5% CO_2 (pH 7.4), and kept at a constant temperature of 37°C. In the conventional Langendorff preparation, oxygen is provided by gassing the perfusion solution with a sintered glass bubbling device with high concentrations of oxygen because of the low oxygen carrying capacity of crystalloid buffers. Typically a mixture of 95% oxygen and 5% carbon dioxide is used to ensure adequate O_2 delivery to the cells.

After a 25-min equilibration period, hearts were subjected to 25 min of global ischemia, followed by 30 min of reperfusion (RP). Ischemia was started by shutting off the flow of perfusate. IPOC was induced by six cycles of 10-s reperfusion interspersed by 10-s no-flow ischemia immediately after sustained ischemia.

Only hearts with left ventricular developed pressure (LVDP) >60 mmHg and heart rate (HR) >200 beats/min at the end of the equilibration period were included in the study.

It is worth noting that Langendorff perfused rat hearts subjected to 25 min of total global ischemia followed by 30 min reperfusion have been extensively used for the evaluation of cardioprotective interventions on necrosis, functional recovery and the study of metabolic pathways [6, 8, 9].

Measurement of heart function

The left atrium was removed, and a latex balloon connected to a pressure transducer was inserted into the left ventricle through the mitral valve in order to measure left ventricular pressures. The volume of the balloon was adjusted to obtain an initial left ventricular end diastolic pressure (LVEDP) of 10 mmHg. This allowed continuous measurement of end diastolic and systolic pressure changes during ischemia and reperfusion. Values for LVDP, peak rate of contraction ($+dP/dt$) and peak rate of relaxation ($-dP/dt$) were obtained using a digital data acquisition system. Heart rate was measured by means of a counter that was triggered by the LVDP pulse. Rate-pressure product (RPP) was determined by multiplying HR by LVDP.

Measurement of cell viability

At the end of the RP period the hearts were removed, frozen and cut into six to eight slices of approximately 0.8 up to 1 mm of 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

distinguish clearly stained viable tissue and 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 as percentage of risk area.

TBARS, G6PDH and GSH/GSSG assay

TBARS, G6PDH and GSH/GSSG were measured from parallel experiments in separate hearts treated according to the above protocols. Frozen heart tissue was homogenized in 5 ml of 50 mM cold phosphate buffer (pH 7.4). An aliquot was taken for measurement of TBARS as a marker of lipid peroxidation. The rest of the homogenate was centrifuged at 10,000 rpm for 10 min at 0°C, and the supernatant separated and used for measurement of G6PDH and GSH/GSSG.

G6PDH activity was measured using spectrophotometry [10]. Enzyme activity was expressed as units/g protein. The amount of protein was determined by the method of Lowry et al. [11], with bovine serum albumin (BSA) as the standard.

GSH/GSSG was determined using a commercially available kit (Calbiochem, La Jolla, CA). The technique is based on the enzymatic recycling method described by Tietze [12].

Levels of TBARS were determined using a commercially available kit (Cayman Chemical Co., Ann Arbor, MI) based on the spectrophotometric method described by Yagi [13].

ATP, TAG and glycogen assay

Tissue ATP, glycogen and TAG were measured from parallel experiments in separate hearts treated according to the above protocols. 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.

Tissue levels of ATP were determined by luciferin-luciferase luminometry (Sigma bioluminescent assay kit) in $\sim$200 mg neutralized $HClO_4$ extracts of frozen ventricular tissue according to a standard technique [14].

For measurement of TAG content, $\sim$200 mg frozen heart tissue was homogenized in chloroform:methanol (2:1). Following overnight extraction at 4°C, the homogenate was filtered, mixed with an equal volume of cold saline and centrifuged at 0°C. The lower phase was then transferred to a fresh tube, and the phospholipids were adsorbed by addition of activated silicic and shaking for 90 s using a vortex mixer. The silicic acid was packed by centrifugation at 20°C for 10 min, and an aliquot was transferred to a fresh tube and reduced to dryness. TAG was saponified with ethanolic KOH at 60°C for 15 min. The amount of glycerol was measured by spectrophotometry [15].

Glycogen was determined in $\sim$200 mg samples of frozen ventricular tissue according to the method of Walaas and Walaas [16] with the use of amyloglucosidase.

Measurement of mitochondrial ATP synthesis

At the end of ischemia-reperfusion, hearts were removed from the Langendorff apparatus, and mitochondria were isolated by differential centrifugation after tissue homogenization in ice-cold sucrose buffer solution (300 mM sucrose, 10 mM Tris-Cl, 2 mM EGTA, 5 mg/ml BSA, pH 7.4). The mitochondrial pellet was then washed three times in sucrose isolation buffer solution lacking BSA. Cardiac mitochondria prepared with this procedure have been shown to be metabolically active with respiratory control ratios of 3.5–5.0 with succinate and 8.0–10.0 with glutamate/malate, and corresponding ADP/O ratios of 1.5–1.7 and 2.5–2.7, respectively [17]. Since it is well documented that complex I of the respiratory chain is the most sensitive to reperfusion injury [18], mitochondrial ATP synthesis was measured in the presence of the complex I substrates pyruvate and malate. Mitochondria (1 mg protein/mL) were incubated for 10 min in a 25°C metabolic shaker in a medium containing (mM): KCl 125, Mops 20, Tris 10, EGTA 0.5, KH_2PO_4 2.4, $MgCl_2$ 2.5, malate 2.5 and pyruvate 2.5, pH 7.4. After 2 min, ATP synthesis was initiated by addition of ADP 2.5 mM. ADP 2.5 mM was used because it corresponds to a physiological concentration found in myocytes. Aliquots were taken from the incubation mixture at 3-min intervals for 10 min, and reactions were stopped by adding perchloric acid. The neutralized supernatant was assayed for ATP by luciferin-luciferase luminometry (Sigma bioluminescent assay kit). Mitochondrial protein concentration was determined by the method of Lowry using BSA as a standard, and the rate of mitochondrial ATP synthesis was calculated and expressed as nmol per minute per milligram of mitochondrial protein.

Statistical analysis

All data are presented as mean ± SEM. Changes in the ventricular contractile function were statistically compared using a three-factor ANOVA for repeated measurements in one factor, followed by Tukey's test. Differences between the same biochemical measurements at different times or between variables in control and postconditioned fed and fasted hearts were assessed using factorial ANOVA followed by Tukey's test. Statistical significance was set at $p < 0.05$.

Results

At the end of the equilibration period, fasting did not exhibit any effect on baseline values of HR, RPP, +d*P*/d*t* and −d*P*/d*t* (data not shown).

The exposure to 25 min of global ischemia led to complete cessation of spontaneous contractions, and during the 30 min of reperfusion the HR gradually returned to pre-ischemic values, which were similar in both fed and fasted control hearts (15-min RP: control fed 190.33 ± 27.35 and control fasted 196.12 ± 16.54; 30-min RP: control fed 222.63 ± 12.69 and control fasted 218.25 ± 8.22; expressed as beats/min.). In addition, there was no significant difference in HR between control and postconditioned hearts during reperfusion, nor between fed and fasted postconditioned hearts (15-min RP: postconditioned fed 207.00 ± 14.21 and postconditioned fasted 191.00 ± 17.9; 30-min RP: postconditioned fed 231.24 ± 5.40 and postconditioned fasted 210.63 ± 5.90 beats/min). Upon reperfusion, hearts from fasted rats exhibited faster recovery of RPP, +d*P*/d*t* and −d*P*/d*t* compared with hearts from fed rats (Fig. 1a–c). The rate of recovery of RPP, +d*P*/d*t* and

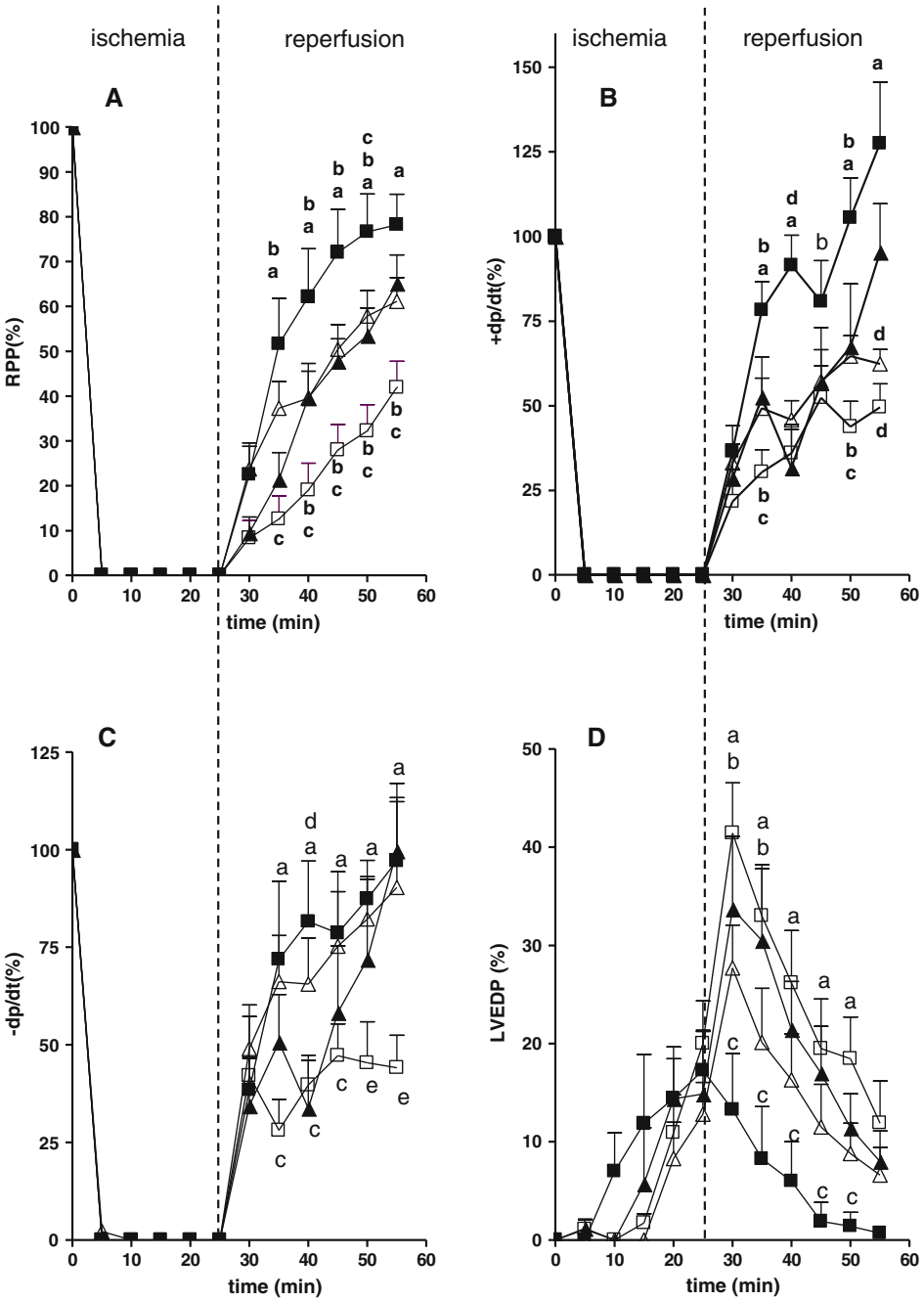

Fig. 1 Changes in: **A** rate-pressure product (RPP); **B** peak rate of contraction (+d*P*/d*t*); **C** peak rate of relaxation (−d*P*/d*t*) and **D** left ventricular end-diastolic pressure (LVEDP) due to ischemia-reperfusion in control and postconditioned hearts from fed and fasted rats. Values are expressed as percentages of the respective basal values at the end of the 25-min equilibration period. *Open symbols* fed hearts. *Filled symbols* fasted hearts. *Squares* control hearts. *Triangles* postconditioned hearts. Values are mean ± SEM ($n = 8$) **A**, **B**, **C**: *a*, $p < 0.01$ versus control fed, *b*, $p < 0.05$ versus postconditioned fasted, *c*, $p < 0.05$ versus postconditioned fed, *d*, $p < 0.01$ versus postconditioned fasted, *e*, $p < 0.01$ versus postconditioned fed. **D** *a*, $p < 0.05$ versus control fasted, *b*, $p < 0.05$ versus postconditioned fed., *c*, $p < 0.05$ versus postconditioned fasted

$-dP/dt$ in fed rat hearts was improved by IPOC, but markedly reduced in hearts from fasted rats. During reperfusion and concurrently with the faster rate of recovery of contractile function, hearts from fasted rats exhibited a smaller amplitude of LVEDP than hearts from fed animals (Fig. 1d). The amplitude of LVEDP was significantly reduced by IPOC in the fed hearts, but increased in the fasted hearts when compared with the control groups. Hence, the difference between hearts from fed and fasted rats disappeared in postconditioned hearts.

In accordance with previous data [6] the percentage of viable myocytes was similar in both nutritional conditions (fed 41.2 ± 1.1% and fasted 40.1 ± 0.5%; $n = 8$). Compared with hearts from control animals, cell viability was increased in the postconditioned fed hearts (61.0 ± 0.7%, $p < 0.05$ vs. control fed; $n = 8$), whereas it was decreased in the postconditioned fasted hearts (33.5 ± 1.2%, $p < 0.05$ vs. control fasted; $n = 8$).

Glycogen content measured in vivo ($n = 8$) was higher in the fasted group than in the fed group (362.15 ± 8.12 vs. 229.21 ± 6.11 μg/100 mg dry weight; $p < 0.01$) and decreased only in the fasted hearts during the pre-ischemic stabilization period (fasted: 321.35 ± 7.23 μg/100 mg dry weight, $p < 0.05$ vs. in vivo; fed: 247 ± 4.11 μg/100 mg dry weight, n.s. vs. in vivo). In spite of this, the fasted group exhibited a higher glycogen content before the onset of ischemia compared with the fed group (Fig. 2). During ischemia, glycogen content fell to similar values in both nutritional groups, and no further decrease occurred during reperfusion. These changes in glycogen content resulted in a marked difference in glycogen breakdown, which was about 25% higher in the fasted than in the fed group. Interestingly, IPOC reduced glycogen content close to exhaustion during reperfusion in both nutritional conditions (Fig. 2).

TAG content measured immediately after excision of the hearts (in vivo, $n = 8$), was higher in the fasted group than in the fed group (19.52 ± 1.77 vs. 8.66 ± 1.88 μmol/g dry weight; $p < 0.01$). No change in TAG content was seen throughout the experiment in the hearts from fed rats (Fig. 3). In contrast, during the pre-ischemic period, the TAG content of the hearts from the fasted group decreased compared with that in hearts immediately after their removal from rats to 16.29 ± 1.24 μmol/g dry weight ($p < 0.05$). This indicates that the consumption of TAG increased in fasted rat hearts. However, at the end of the ischemic-RP period, there was no further change in TAG levels in fasted rats, indicating that, in this group, the utilization of endogenous TAG did not persist during the post-ischemic period (Fig. 3). TAG content at the end of the 30-min reperfusion period was not affected by IPOC in the fed group, but was markedly lowered in the fasted group (Fig. 3). Oxfenicine (2 mM), a carnitine palmitoyltransferase I inhibitor, added

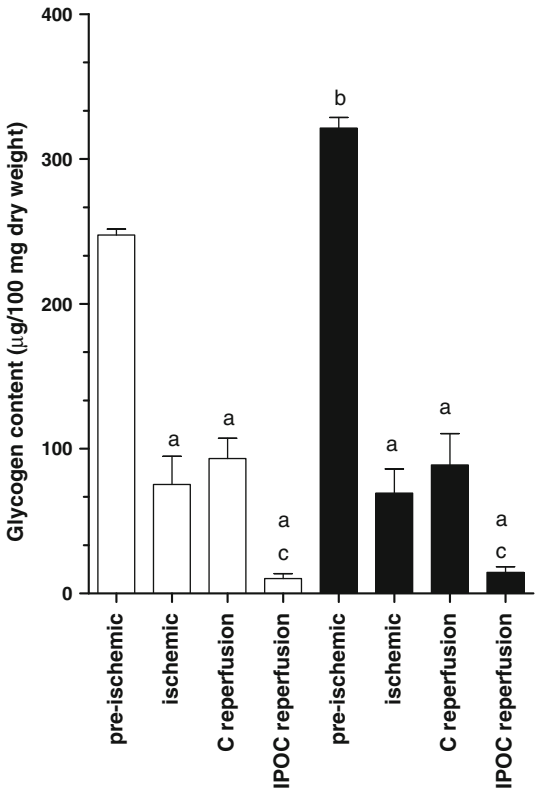

Fig. 2 Glycogen content of hearts subjected to ischemia-reperfusion. Values are mean ± SEM ($n = 8$) and are expressed as μg glycogen/100 mg dry weight. *Open bars* fed hearts, *filled bars* fasted hearts. Pre-ischemic refers to the end of the 25-min pre-ischemic period. Ischemic refers to the end of the 25-min ischemic period. Reperfusion refers to the end of the 30-min reperfusion period. *C* control hearts. *IPOC* postconditioned hearts. *a*, $p < 0.01$ versus pre-ischemic in the same nutritional condition; *b*, $p < 0.01$ versus fed pre-ischemic; *c*, $p < 0.01$ vs. C reperfusion in the same nutritional condition

during the 30-min RP period abolished TAG breakdown in the postconditioned fasted hearts (postconditioned fasted plus oxfenicine: 12.90 ± 1.31 vs. postconditioned fasted: 7.77 ± 1.21 μmol/g dry weight; $p < 0{,}01$) and the noxious effects of IPOC on functional recovery in the fasted hearts (Table 1), but did not affect the recovery in the postconditioned fed ones.

Immediately before the beginning of ischemia, the myocardial ATP level was similar in both nutritional groups (Fig. 4). At the end of ischemia, ATP levels declined to the same extent in both groups, but at the end of reperfusion they were higher in the fasted group (Fig. 4). Accordingly, the rate of ATP synthesis in mitochondria isolated from hearts exposed to ischemia-reperfusion was higher in the fasted than in the fed group (Fig. 5). At the end of reperfusion, IPOC markedly raised myocardial ATP content in the fed condition, whereas this intervention decreased myocardial ATP content in the hearts from fasted rats (Fig. 4) compared with the control ones. Furthermore, the

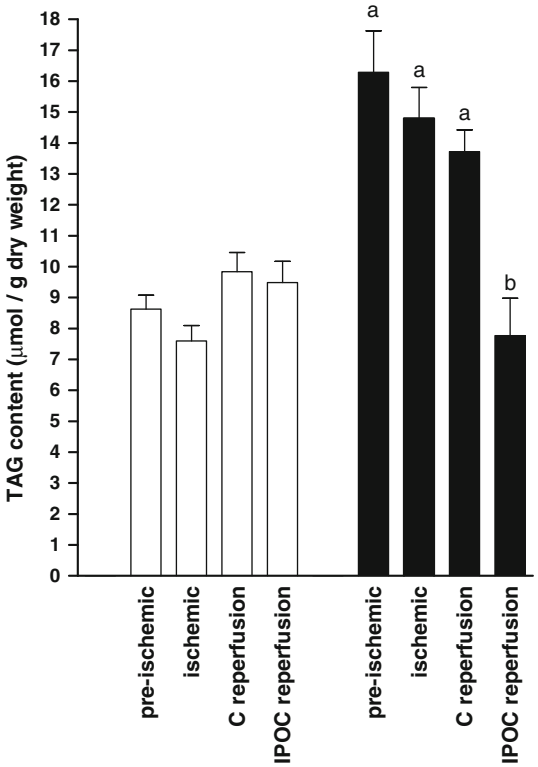

Fig. 3 TAG content of hearts subjected to ischemia-reperfusion. Values are mean ± SEM ($n = 8$) and are expressed as µmol/g dry weight. *Open bars* fed hearts, *filled bars* fasted hearts. Pre-ischemic refers to the end of the 25-min pre-ischemic period. Ischemic refers to the end of the 25-min ischemic period. Reperfusion refers to the end of the 30-min reperfusion period. *C* control hearts. *IPOC* postconditioned hearts. *a*, $p < 0.01$ versus fed hearts, same treatment; *b*, $p < 0.01$ versus IPOC reperfusion fasted heart and versus pre-ischemic fasted hearts

Table 1 Effects of oxfenicine on contractile recovery of postconditioned fasted hearts

	C	IPOC	IPOC + OX
RPP	62 ± 10	40 ± 7*	67 ± 9
+d*P*/d*t*	78 ± 8	53 ± 11**	78 ± 14
−d*P*/d*t*	82 ± 15	34 ± 11**	98 ± 9

Data are the mean ± SEM ($n = 8$)

Values are expressed as percentages of the respective basal values at the end of the 25-min equilibration period

RPP rate-pressure product at 15 min of reperfusion, *+dP/dt* peak rate of contraction at 15 min of reperfusion, *−dP/dt* peak rate of relaxation at 15 min of reperfusion, *OX* oxfenicine 2 mM added during the 30 min reperfusion period, *C* control hearts, *IPOC* postconditioned hearts, *IPOC + OX* postconditioned hearts in the presence of OX

* $p < 0.05$ versus C and IPOC + OX

** $p < 0.01$ versus C and IPOC + OX

rate of ATP synthesis in the isolated mitochondria of fed rat hearts was increased by IPOC, but reduced in the fasted group (Fig. 5). It is worth noting that the effects of IPOC on

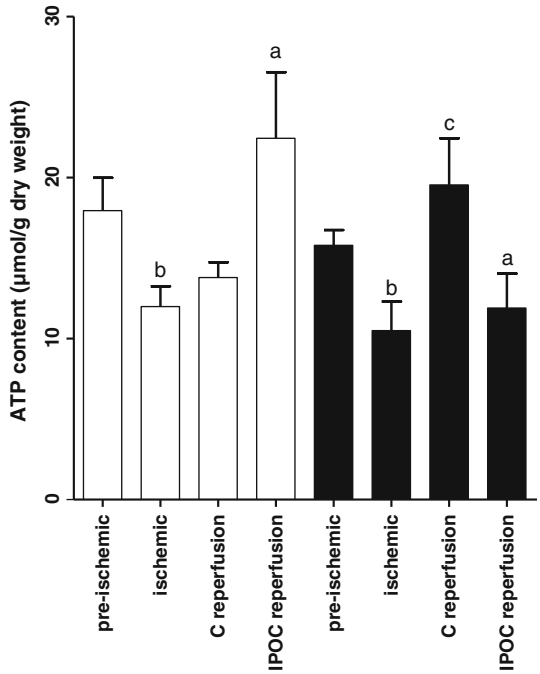

Fig. 4 ATP content of hearts subjected to ischemia-repefusion. Values are mean ± SEM ($n = 8$) and are expressed as µmol/g dry weight. *Open bars* fed hearts; *filled bars* fasted hearts. *Pre-ischemic* refers to the end of the 25-min pre-ischemic period. Reperfusion refers to the end of the 30-min reperfusion period. *C* control hearts. *IPOC* postconditioned hearts. *a*, $p < 0.05$ versus C reperfusion in the same nutritional condition. *b*, $p < 0.05$ versus pre-ischemic in the same nutritional condition. *c*, $p < 0.05$ versus C reperfusion fed and versus ischemic fasted

functional recovery and the extent of cell viability in the fed and fasted hearts appeared to be associated with the changes in the in vitro rates of ATP production, suggesting that the mitochondrial ATP synthesis capacity observed in vitro could also occur in situ.

As indicated in Table 2, fasting increased G6PDH activity 1.44 times, increased the GSH/GSSG ratio by a factor of 3.63 and decreased TBARS levels by 44% in the pre-ischemic hearts. Ischemia-reperfusion did not affect G6PDH activity, but decreased the GSH/GSSG ratio by approximately 50% in both the fed and fasted groups. Thus, fasted hearts at the end of reperfusion exhibited a markedly higher GSH/GSSG ratio than fed ones. Along with the changes in glutathione status, tissue levels of TBARS were increased during ischemia-reperfusion in both nutritional conditions (Table 2). Despite these increases, TBARS levels remained lower in the fasted group compared with the fed one (Table 2). In the reperfused fed hearts, IPOC significantly raised G6PDH activity and the GSH/GSSG ratio, and reduced TBARS content (Table 2). However, in the fasted group IPOC did not cause changes in any of these variables. Accordingly, the differences between the fed and fasted rat hearts were abolished in the IPOC-treated hearts.

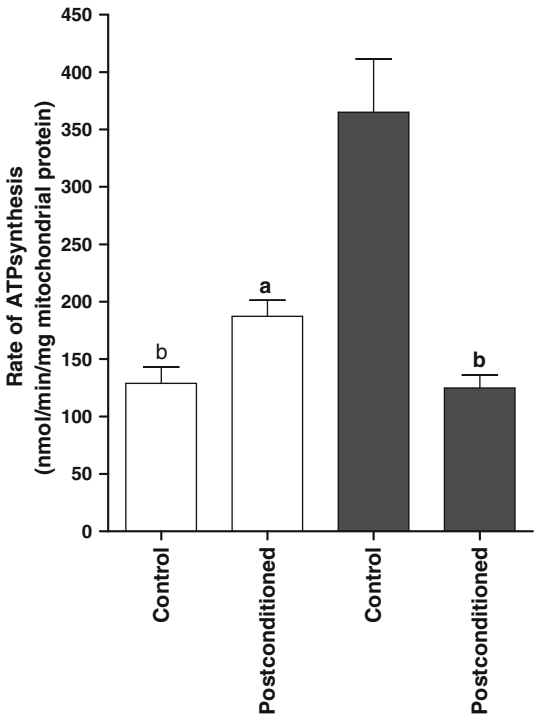

Fig. 5 ADP-stimulated ATP production in isolated mitochondria from hearts subjected to ischemia-reperfusion. *Open bars* fed hearts; *filled bars* fasted hearts. Values are mean ± SEM ($n = 10$) and are expressed as nmol/min/mg mitochondrial protein. *a*, $p < 0.05$ versus control fed and versus postconditioned fasted; *b*, $p < 0.01$ versus control fasted

Discussion

In agreement with previous reports, present data indicate that IPOC accelerated functional recovery of isolated hearts from fed rats [2–4]. Fasting status per se exerted protective effects on the contractile function [5, 6], which were attenuated by IPOC, suggesting that the latter might interfere with some of the "beneficial" biochemical pathways activated during fasting. These findings of greater or lesser rates of functional recovery—specially during early reperfusion—in the fed and fasted postconditioned hearts were supported by concordant measurement of cell viability. Thus, the internal consistency of these data strongly supports the protective or deleterious effects of this intervention.

Consistent with earlier observations, the hearts from fasted rats had higher in vivo TAG and glycogen levels than the fed hearts [6, 19]. Fasting is a condition in which serum fatty acids and myocardial fatty acids uptake are elevated, and a shift in substrate preference from glucose to fatty acids occurs. The enhanced oxidation of fatty acids increases glycogen content through the inhibition of the glycolytic pathway. In addition, in this state, myocardial TAG also increases because fatty acids and acyl-CoA that are not used for β-oxidation are sequestered into TAG. In the absence of exogenous fatty acids, TAG levels decreased during the preischemic period only in the fasted hearts, but remained higher with regard to the fed hearts. It seems reasonable to

Table 2 Glucose-6-phosphate dehydrogenase (G6PDH) activity, reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio and thiobarbituric acid-reactive substances (TBARS) levels in control and postconditioned hearts from fed and fasted rats

	Fed				Fasted			
	Preischemic	Ischemic	Reperfusion		Preischemic	Ischemic	Reperfusion	
			C	IPOC			C	IPOC
G6PDH (units/g protein)	1.45 ± 0.24	1.28 ± 0.17	1.01 ± 0.35	2.11 ± 0.32*$^{+}$	2.09 ± 0.51†	2.31 ± 0.64†	2.25 ± 0.36†	2.27 ± 0.46
GSH/GSSG	14.58 ± 3.49	12.76 ± 1.77	7.92 ± 1.70*	17.69 ± 4.64^{+}	52.96 ± 9.84†	44.66 ± 8.45†	24.38 ± 6.14*†	16.89 ± 2.98*
TBARS (nmol/gww)	7.70 ± 1.09	9.72 ± 1.23	20.73 ± 2.10*	12.12 ± 2.51*$^{+}$	3.42 ± 0.42†	5.91 ± 0.87†	14.38 ± 1.76*†	15.93 ± 2.51*

Data are the mean ± SEM ($n = 8$)

Pre-ischemic, at the end of the 25 min equilibration period; ischemic, at the end of the 25 min ischemic period; reperfusion, at the end of the 30 min reperfusion period

C control hearts, *IPOC* postconditioned hearts, *G6PDH* glucose-6-phosphate-dehydrogenase, *GSH/GSSG* reduced glutathione/oxidized glutathione ratio, *TBARS* thiobarbituric acid-reactive substances

* $p < 0.05$ compared with pre-ischemic and ischemic hearts in the same nutritional state

† $p < 0.05$ compared with hearts from fed rats in the same period

$^{+}$ $p < 0.05$ versus C reperfusion in the same nutritional state

suggest that oxidation of endogenous fatty acids is responsible for TAG breakdown. On the other hand, the breakdown of glycogen during the pre-ischemic period, observed only in hearts from fasted rats, could be due to oxidation of glucose through the pentose phosphate pathway.

As has been previously shown [6], ischemia evoked a significant decrease in glycogen content in both nutritional groups, but since the pre-ischemic glycogen level was higher in the fasted group, the extent of glycogen breakdown was higher in the fasted condition. However, although pre-ischemic TAG content was higher in the fasted group, no lipolysis occurred after ischemia-reperfusion in either group. Therefore, during ischemia, the energy supply was greater in the fasted than in the fed condition. However, since IPOC evoked a further decrease in glycogen content in both nutritional conditions, it evidently improved the glucose supply during reperfusion. Furthermore, IPOC increased lipolytic activity during reperfusion, but only in the fasted group, which suggests that it might have evoked a catabolic shift towards lipid oxidation through the increase in the supply of endogenous fatty acids. Thus, it is plausible to speculate that the differences between the two groups might be related to the kind of endogenous substrates supplied during reperfusion.

On the basis of these findings, it is possible that glycogen breakdown may give rise to a pyruvate pool that is more tightly coupled with oxidation than pyruvate derived from extracellular glucose [20]. Therefore, increased glycogen breakdown elicited by IPOC in the fed rat hearts may contribute to the increased rate of mitochondrial ATP production and ATP content, consequently accelerating functional recovery. In very broad terms, the present findings agree with those of Correa et al. [21], who have reported that glucose uptake and consumption were significantly increased during the early RP phase in postconditioned rat hearts, suggesting that IPOC enhances the contribution of glucose catabolism to the total energy expenditure during reperfusion.

The protective effects of fasting have been ascribed to metabolic changes occurring during ischemia, such as reduced glycolysis [6]. The latter would provide protection, at least in part, by decreasing the exchange of Na^+ for H^+ which in turn reduces calcium overload [6, 22]. These changes probably arise as a consequence of the increased catabolism of fatty acids derived from endogenous TAG that occurs in hearts from fasted rats during the aerobic pre-ischemic period [6]. Interestingly, according to present and previous data, TAG mobilization does not persist during reperfusion [6]. It has been reported that reperfusion of ischemic hearts results in a rapid recovery of the ability to oxidize fatty acids [23, 24], and an over-reliance on fatty acid oxidation has a detrimental effect on functional recovery [25]. Since endogenous TAG, through lipolysis, is a potential source of fatty acids for oxidative metabolism [5, 15, 26], it is plausible to infer that IPOC may accelerate fatty acid β-oxidation in fasted rat hearts. Hence, the noxious effects of IPOC in this nutritional condition might be the consequence of increased lipolysis during reperfusion. Supporting this hypothesis, oxfenicine, a carnitine palmitoyltransferase I inhibitor, added immediately upon RP abolished TAG breakdown and the noxious effects of IPOC on functional recovery in the fasted hearts, but did not affect the recovery in the postconditioned fed ones. Therefore, while endogenous pre-ischemic TAG breakdown is beneficial to the ischemic-reperfused heart, it becomes harmful when it occurs during reperfusion. In regard to the latter finding, it is worthwhile to recall that an increased rate of fatty acid β-oxidation inhibits glucose utilization, with glucose oxidation being inhibited to the largest extent, followed by a smaller effect on glycolysis and glucose uptake [27–29]. Since glycogenolysis is stimulated by IPOC, the concomitant stimulation of TAG breakdown may lead to an increase in H^+ production during the critical period in which the heart is attempting to recover from the acidosis that occurred during ischemia [24, 30, 31]. Consequently, calcium overload might be enhanced [32], leading to contracture [33] and having adverse effects on mitochondrial function and contractile recovery [34]. Calcium overload, especially when this is accompanied by oxidative stress and adenine nucleotide depletion, conditions that are exactly those that the heart experiences during post-ischemic reperfusion, provides a powerful stimulus to mitochondrial permeability transition pore (MPTP) opening. When it opens, the MPTP allows free passage of any molecule <1,500 Da across the mitochondrial inner membrane, including protons. Under such conditions the mitochondria are unable to synthesize ATP by oxidative phosphorylation; indeed by reversing the proton-translocating ATPase such uncoupled mitochondria actively hydrolyse ATP synthesized by glycolysis and remaining healthy mitochondria [26, 34].

AMP-activated protein kinase (AMPK), which plays an important role in regulating both fatty acid and glucose metabolism by switching on catabolic pathways that generate ATP [31, 35, 36], has been shown to be upregulated in the setting of IPOC [36]. The present data show that IPOC caused an increase in TAG breakdown in the fasted heart, where TAG was elevated, increasing the chances that AMPK mediates that process. As a consequence, it is possible that these changes lead to a glucose-sparing effect concomitant with an "over-dependence" on endogenous fatty acids as the energy source for the reperfused fasted rat hearts, which may be linked to the effects of IPOC on functional performance, ATP synthesis and tissue damage. However, assessment of the role of AMPK in IPOC was beyond the scope of this study.

This study also demonstrates that protection induced by IPOC in the fed hearts is associated with an increase in G6PDH activity and a resultant increase in the glutathione antioxidant ratio, which in turn reduces oxidative damage. It has been traditionally thought that G6PDH, the rate limiting enzyme of the pentose phosphate pathway, is a typical "housekeeping" enzyme that provides the cytosolic NADPH required to regenerate reduced glutathione, essential for detoxification of reactive oxygen species [37, 38]. The present findings support the hypothesis that G6PDH activity is associated with the preservation of the GSH/GSSG ratio and subsequent protection against oxidative damage [39]. Furthermore, IPOC increased the ATP content and mitochondrial rate of ATP synthesis in the fed group. It should also be noted that IPOC did not, however, affect the fasting-induced upregulation of G6PDH activity, the GSH/GSSG ratio or TBARS levels, yet it decreased cell viability and delayed functional recovery. Since IPOC decreased ATP synthesis and ATP content in the fasted group, it is likely that these noxious effects of IPOC are mainly linked to the decrease in ATP supply and not to the oxidative status.

In summary, the present results are consistent with previously reported protective effects of IPOC and provide evidence that this protection occurs together with stimulation of glycogen breakdown and the pentose phosphate pathway, an increase in ATP content and synthesis and an increase in the GSH/GSSG ratio in fed rat hearts. On the other hand, IPOC partially attenuates the protection afforded by previous fasting, although in this nutritional condition it increases both glycogen and TAG breakdown without changing either the oxidative status of glutathione or the pentose pathway activity.

Acknowledgments The authors thank Norma Gladys Infante for technical assistance and Ariel Jaitovich for proofreading, grammar review and stylistic improvement. This research was supported, in part, by grants from the Universidad de Buenos Aires, PIP-CONICET 5976, ANPCyT BID contract 1728/OC-AR project 26026 and IQ-UIMEFA-CONICET.

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