Short-term fasting reduces the extent of myocardial infarction and incidence of reperfusion arrhythmias in rats

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Short title: Cardioprotection conferred by **s**hort-term fasting

Summary

The effect of three-day fasting on cardiac ischemic tolerance was investigated in adult male Wistar rats. Anesthetized open-chest animals (pentobarbitone 60 mg/kg, i.p.) were subjected to 20-min left anterior descending coronary artery occlusion and 3-h reperfusion for infarct size determination. Ventricular arrhythmias were monitored during ischemia and at the beginning (3 min) of reperfusion. Myocardial concentrations of beta-hydroxybutyrate and acetoacetate were measured to assess mitochondrial redox state. Short-term fasting limited the infarct size (48.5 ± 3.3 % of the area at risk) compared to controls (74.3 ± 2.2 %) and reduced the total number of premature ventricular complexes (12.5 ± 5.8) compared to controls (194.9 ± 21.9) as well as the duration of ventricular tachycardia (0.6 ± 0.4 s vs. 18.8 ± 2.5 s) occurring at early reperfusion. Additionally, fasting increased the concentration of beta-hydroxybutyrate and beta-hydroxybutyrate/acetoacetate ratio (87.8 ± 27.0) compared to controls (7.9 ± 1.7), reflecting altered mitochondrial redox state. It is concluded that three-day fasting effectively protected rat hearts against major endpoints of acute I/R injury. Further studies are needed to find out whether these beneficial effects can be linked to altered mitochondrial redox state resulting from increased ketogenesis.

Key words

Myocardial Ischemia/Reperfusion • Arrhythmias • Infarction • Fasting • Ketone Bodies

Introduction

After an acute myocardial infarction, early myocardial reperfusion is the only effective strategy for reducing the extent of infarct size and improving the clinical outcome. However, restoring the blood flow to the ischemic myocardium may induce myocardial reperfusion injury, which reduces the beneficial effects of reperfusion (Yellon and Hausenloy 2007). It is now established that reactive oxygen species (ROS) play a role in the pathogenesis of myocardial injury associated especially with reperfusion. It has been well documented that ROS contribute to early reperfusion arrhythmias and that antioxidants can significantly attenuate their incidence and severity (reviewed in (Li and Jackson 2002; Zweier and Talukder 2006). In contrast to the involvement of ROS in reperfusion arrhythmias, it is still debated whether ROS significantly contribute to other end points of ischemia/reperfusion (I/R) injury. Although numerous experimental studies demonstrated beneficial effects of ROS dampening on ischemic arrhythmias and infarct size, other reports showed only minor (if any) protection (e.g. (Miura *et al.* 1988; Neckar *et al.* 2008; Imani *et al.* 2011). Nevertheless, prevention of production of cytotoxic ROS might be a target for effective cardioprotection, especially during the reperfusion phase.

It has been shown that cell redox state correlates with ROS formation (Stowe and Camara 2009; Wolin 2009; Aon *et al.* 2010). Formation of ROS and subsequent oxidative damage can be reduced by various regimens of long-term dietary restrictions such as reduced energy intake (Gredilla *et al.* 2001; Bevilacqua *et al.* 2004; Colom *et al.* 2007) or intermittent fasting (Wan *et al.* 2003; Johnson *et al.* 2007). Caloric restriction is related to the concept of so called reductive stress. Under normoxic conditions mitochondria operate in a physiological intermediate redox state (Aon *et al.* 2010). The redox couples comprise the relatively oxidized NAD^+/NADH, and relatively reduced couples $NADP^+$/NADPH and GSSG/GSH. The formation of ROS is

supported primarily by reduced pyridine nucleotides, such as NADH or NADPH (Shen *et al.* 2005). Thus, under the conditions of increased concentration of reduced nucleotides, higher rates of ROS production can occur. The increase of reductive power in the mitochondrial compartment is reflected by the increased beta-hydroxybutyrate/acetoacetate ratio (Williamson *et al.* 1967). Beta-hydroxybutyrate (i.e. 3-hydroxybutyrate, BHB) and acetoacetate (AcAc) represent two main ketone bodies (Mitchell *et al.* 1995). The reaction of AcAc with NADH and hydrogen ion, catalyzed by beta-hydroxybutyrate dehydrogenase (Hegardt 1999), results in the formation of BHB and NAD^+. Fasting of laboratory animals increases ketogenesis (Mitchell *et al.* 1995), which can affect cell redox state and ROS formation by consuming reduced nucleotides.

While cardioprotective effects of various regimens of long-term dietary restrictions have been well documented (e.g. (Chandrasekar *et al.* 2001; Ahmet *et al.* 2005; Varady *et al.* 2009), the impact of short-term fasting (days before the ischemic insult) on cardiac tolerance to acute I/R injury has been rarely examined. In the present study, we therefore evaluated infarct size and the incidence of ventricular arrhythmias induced by regional I/R in rats subjected to fasting for 3 days. Redox state of myocardial mitochondria was assessed by measuring the BHB/AcAc ratio.

Methods

Adult male Wistar rats (AnLab, Czech Republic) were divided into two groups. Animals in the experimental group did not have access to food for 3 days but had free access to common drinking water from supply system. The duration of fasting was determined arbitrarily. Control group had free access to water and a standard laboratory diet. All experiments were performed in accordance with the European Community and NIH guidelines for use of experimental animals and approved by the animal studies committee at our institution.

Surgical procedure

Animals were anesthetized by sodium pentobarbitone (Sanofi, France; 60 mg/kg body weight, i.p.). Heparinized cannula was placed in the left carotid artery for blood pressure monitoring with a pressure transducer (Gould P23Gb, USA). Tracheotomy was performed, the rats were intubated with a cannula connected to a rodent ventilator (Ugo Basile, Italy) and ventilated with room air at 65-70 breaths/min (tidal volume of 1.2 ml/100 g body weight). A single-lead electrocardiogram (ECG) was continually recorded. Both blood pressure and ECG signals were subsequently analyzed by our custom-designed software. The rectal temperature was maintained within 36.5 and 37.5 °C by a heated table throughout the experiment. Left thoracotomy was performed and left anterior descending (LAD) coronary artery was occluded for 20 minutes about 1-2 mm distal to the origin. Characteristic changes in the configuration of the ECG and a transient decrease in blood pressure verified the coronary artery occlusion. After releasing the occlusion reperfusion was indicated by changes of the ECG, transient decrease of blood pressure and appearance of reperfusion arrhythmias.

Analysis of arrhythmias

The incidence and severity of ventricular arrhythmias during ischemic insult and the first 3 min of reperfusion were assessed according to the Lambeth Convention (Walker *et al.* 1988). Premature ventricular complexes (PVCs) occurring as singles, salvos or tachycardia (a run of 4 or more consecutive PVCs) were counted separately. The incidence of ventricular tachycardia (VT) and fibrillation (VF) was also evaluated. VF lasting more than two minutes was considered as sustained (VFs).

Measurement of infarct size

After 3 hours of reperfusion, the hearts were excised and washed with 20 ml saline through the cannulated aorta. The area at risk and the infarct size were determined as described elsewhere (Neckar *et al*. 2002) by staining with potassium permanganate (Lachema, Czech Republic) and 2,3,5-triphenyltetrazolium chloride (Sigma, USA) dissolved in 0.1 mol/l phosphate buffer (pH 7.4). The hearts were cut perpendicularly to the long axis of the left ventricle into slices 1 mm thick and stored overnight in 10 % neutral formaldehyde solution. The day after the heart staining, the right ventricular free wall was separated and both sides of slices were photographed. The infarct size (IS), the size of the area at risk (AR) and the size of the left ventricle (LV) were determined by a computerized planimetric method. The IS was normalized to the AR (IS/AR) and the size of AR was normalized to the LV (AR/LV).

Redox state assessment

In separate groups of anesthetized open-chest animals, the hearts were excised either before or after 20 min of myocardial ischemia, the atria and right ventricle were removed and the left ventricle was frozen in liquid nitrogen. About 1.5 g of pre-weighted, minced, frozen tissue was added to the same volume of perchloric acid and after 10 min of proteins precipitation the mixture was centrifuged (6,000 RPM, 30 min). Then the supernatant was separated and neutralized by potassium carbonate. After 20 min, it was centrifuged again for 15 min and the homogenized supernatant was divided into three aliquots (each about 0.5 g) used for measurements. Concentrations of BHB and AcAc were assessed according to Mintz and Robin (Mintz and Robin 1971). Briefly, BHB was determined by adding beta-hydroxybutyrate dehydrogenase (Sigma, USA), NAD+ and tris buffer (pH 8.5) to the final supernatant; NADH was measured using spectrophotometer Helios (Unicam, United Kingdom). In a similar manner,

concentration of AcAc was measured by adding NADH, tris buffer (pH 7.0) and the appropriate enzyme to the final supernatant, and by measuring the decrease in fluorescence as the NADH was oxidized to NAD+.

Statistical analysis

All results are expressed as means ± S.E.M. The statistical significance of differences between groups was determined by one-way ANOVA and subsequent Fisher's PLSD test and the Games/Howell post hoc test, as appropriate. Differences were considered as statistically significant when $p < 0.05$.

Results

Ischemia/reperfusion arrhythmias and infarct size

Three-day fasting tended to reduce the incidence of ischemic ventricular arrhythmias as documented by total number of PVCs (Fig. 1A) and duration of ventricular tachycardia (Fig. 1B) by about 40 – 50% but the differences between fasted and control groups did not reach statistical significance. However, fasting resulted in a significant suppression of arrhythmias occurring at early reperfusion as indicated by marked decreases of both parameters (Fig. 2A,B).

The area at risk normalized to the left ventricle did not significantly differ between the fasted and control groups (38.5 ± 3.1% and 42.3 ± 4.8%, respectively). The infarct size in the control group reached 74.3 ± 2.2 % of the area at risk and it was significantly decreased by fasting to 48.5 ± 3.3 % (Fig. 3).

Concentrations of ketone bodies

Myocardial concentrations of ketone bodies are shown in Table 1. Fasting significantly increased myocardial concentration of BHB to 15.58 ± 2.56 mM/g compared with control animals (1.22 ± 0.21 mM/g) but the concentration of AcAc did not differ between the groups. Although the ratio of BHB/AcAc increased six-fold in the fasted group, the difference did not reach statistical significance due to high variation in this group (Fig. 4). Ischemia tended to further increase the BHB concentration in the fasted group compared with controls while the concentration of AcAc remained unchanged in both groups. Consequently, the BHB/AcAc ratio after ischemia was significantly higher in the fasted group (Fig. 4).

Discussion

The present study demonstrated that short-term water-only fasting of rats limited the extent of acute myocardial infarction induced by regional I/R and markedly reduced the total number of PVCs and duration of ventricular tachycardia occurring at early reperfusion period. Additionally, fasting increased the myocardial BHB/AcAc ratio reflecting altered mitochondrial redox state. Consistent with our observation, fasting of rats for only 24 h improved the postischemic recovery of contractile function and reduced the lactate dehydrogenase release in isolated hearts subjected to global I/R (Marina Prendes *et al.* 2005). Hearts of overnight (16 – 20 h) fasted rats also exhibited better recovery of contractile performance and preservation of the structural integrity of mitochondria following I/R than hearts of fed animals (Doenst *et al.* 1996). Moreover, 3 days of water-only fasting conferred protection against renal I/R injury in mice, similar in magnitude to 2 – 4 weeks of 30 % dietary restriction. Interestingly, the functional protection of kidneys by this short-term fast was rapidly lost within hours of refeeding (Mitchell

et al. 2010) suggesting that the underlying mechanism(s) may differ from that of the long-term dietary restriction.

Although the cardioprotective mechanism of short-term fasting remains poorly understood, it seems likely that the decreased ROS formation and reduced tissue oxidative damage may play at least partial role. It was shown that hearts of rats fasted for 24 h exhibited higher GSH/GSSG ratio and lower levels of lipid peroxidation markers after I/R than hearts of fed animals (Marina Prendes *et al.* 2009). There is ample evidence that longer durations of various dietary restriction regimens also limited formation of ROS. For example, Bevilacqua *et al.* showed decreased skeletal muscle mitochondrial production of hydrogen peroxide in rats subjected to 40 % caloric restriction for 2 weeks to 6 months (Bevilacqua *et al.* 2004). Similar regimen (lasting 6 weeks) lowered hydrogen peroxide production in rat liver mitochondria (Gredilla *et al.* 2001). Judge *et al.* also reported lower myocardial mitochondrial hydrogen peroxide production in the calorie restricted rats (10 – 40 % restriction during 2 months) compared to fed animals (Judge *et al.* 2004). Furthermore, 3 months of 40 % caloric restriction in rats resulted in lower content and improved efficiency of myocardial mitochondria that generated less hydrogen peroxide than fed animals, consistent with attenuated tissue oxidative damage (Colom *et al.* 2007). As recently demonstrated, 8 weeks of intermittent fasting in overweight humans markedly decreased markers of oxidative stress such as 8-isoprostane, nitrotyrosine and protein carbonyls (Johnson *et al.* 2007) supporting clinical relevance of the above-mentioned animal data.

Although neither ROS formation nor oxidative stress markers were measured in the present study, we found significant effect of three-day fasting on mitochondrial redox state revealed by assessing the BHB/AcAc ratio. The increased production of ketone bodies and the BHB/AcAc was detected also after prolonged fasting (Laffel 1999). These data are in line with

the concept of reductive stress which can increase ROS formation by several mechanisms including (1) mitochondrial leak due to nonenzymatic reactions, (2) increased activity of NAD(P)H oxidase, (3) increased activity of xanthine oxidase, (4) increased cyclo-oxygenase activity, and (5) reduction of ferric ions in ferritin to the ferrous state (Williamson *et al.* 1993; Tilton *et al.* 1997). However, this issue remains controversial (Ghyczy and Boros 2001; Lipinski 2002; Zhang *et al.* 2010) and further studies are needed to elucidate the role of ketone bodies in limiting ROS formation and oxidative injury. Nevertheless, our finding of altered mitochondrial redox state points to mitochondria as a key structure in the acquisition of cardiac tolerance to I/R injury. In particular, mitochondrial ATP-sensitive potassium channels (mK_{ATP}) and permeability transition pore (mPTP) are important ROS-regulated targets of various forms of cardioprotection. Limited evidence suggests that the protective mechanism of short-term fasting against I/R-induced myocardial contractile dysfunction involves both the activation of mK_{ATP} (Marina Prendes *et al.* 2005) and prevention of mPTP opening (Marina Prendes *et al.* 2009). The potential role of these components in fasting-induced cardioprotection against other endpoints of I/R injury is unknown. Recent studies in isolated heart model have tested activators of AMP-activated protein kinase (AMPK), a stress signaling enzyme involved in regulation of energy-generating and -consuming pathways, to protect the heart against ischemia. It was suggested that possible cardioprotective effect could be mediated through the inhibition of mPTP opening (Kim *et al.* 2011; Paiva *et al.* 2011).

It has been shown that administration of exogenous BHB reduced myocardial infarct size and apoptosis induced by I/R in rats (Zou *et al.* 2002). Considering the role of ketone bodies in cardioprotection induced by fasting, the comparison with early stages of diabetes mellitus might be of interest. Chen *et al.* examining effects of experimental streptozotocin-induced diabetes in rats on myocardial metabolism demonstrated significant hyperketonemia as early as 2 days after

the streptozotocin injection (Chen *et al.* 1984). Interestingly, smaller infarct size compared to that of non-diabetic controls was found in rats subjected to myocardial I/R one week after the induction of diabetes (Ravingerova *et al.* 2003). Thus, it cannot be excluded that short-term fasting and early stages of experimental diabetes may utilize, at least in part, similar mechanisms to improve cardiac ischemic tolerance, possibly involving ketone bodies.

Short-term water-only fasting of animals is likely associated with stress which can precondition their hearts against subsequent stresses including I/R. In fact, cardiac protection induced by fasting was shown to be as effective as ischemic preconditioning (Doenst *et al.* 1996). In view of the complex molecular mechanism of preconditioning, it can be assumed that protective effects of fasting may also involve a variety of cell survival pathways. For example, it can be speculated that ischemia-tolerant phenotype of fasted animals is attributed to increased sympathetic stimulation and protective signaling triggered by catecholamines. The ability of catecholamines to precondition the heart against I/R injury has been well documented (Ravingerova *et al.* 1995; Lochner *et al.* 1999).

In conclusion, our results demonstrated that three-day fasting effectively protected rat hearts against two major endpoints of acute I/R injury: myocardial infarction and reperfusion ventricular arrhythmias. These beneficial effects can be linked to altered mitochondrial redox state resulting from increased ketogenesis but further studies are needed to test this possibility.

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Table 1. Concentrations of beta-hydroxybutyrate (BHB) and acetoacetate (AcAc) in left ventricular myocardium of control and fasted rats before and after 20-min ischemia.

Group	n	BHB (mM/g)	AcAc (mM/g)
Control	9	1.22 ± 0.21	0.29 ± 0.07
Fasted	9	15.58 ± 2.56*	0.70 ± 0.20
Control + ischemia	9	2.24 ± 0.35	0.34 ± 0.06
Fasted + ischemia	11	18.71 ± 3.15*	0.38 ± 0.08

Values are means ± S.E.M. * $P < 0.05$ vs. controls.

Figure legends

Fig. 1

(A) Total number of premature ventricular complexes (PVCs) and (B) total duration of ventricular tachycardia (VTD) over 20-min ischemic period in control and fasted rats. Values are means ± S.E.M. from 12 fasted rats and 11 control animals.

Fig. 2

(A) Total number of premature ventricular complexes (PVCs) and (B) total duration of ventricular tachycardia (VTD) during the first 3 min of reperfusion in control and fasted rats. Values are means ± S.E.M. from 12 fasted rats and 11 control animals. * $P < 0.05$.

Fig. 3

Myocardial infarct size expressed as a percentage of the area at risk (IS/AR) in control and fasted rats. Values are means ± S.E.M. from 6 fasted rats and 5 control animals. * $P < 0.05$.

Fig. 4

Beta-hydroxybutyrate/acetoacetate (BHB/AcAc) ratio in left ventricular myocardium of control and fasted rats before and after 20-min ischemia. Values are means ± S.E.M. from 9 - 11 hearts in each group. * $P < 0.05$ vs. controls.

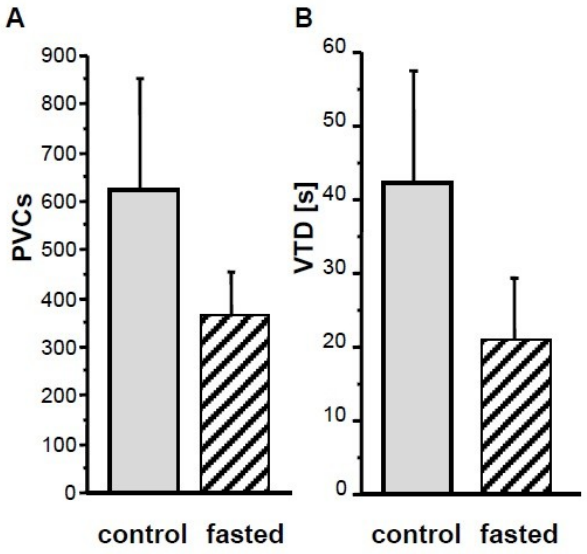
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Fig. 1

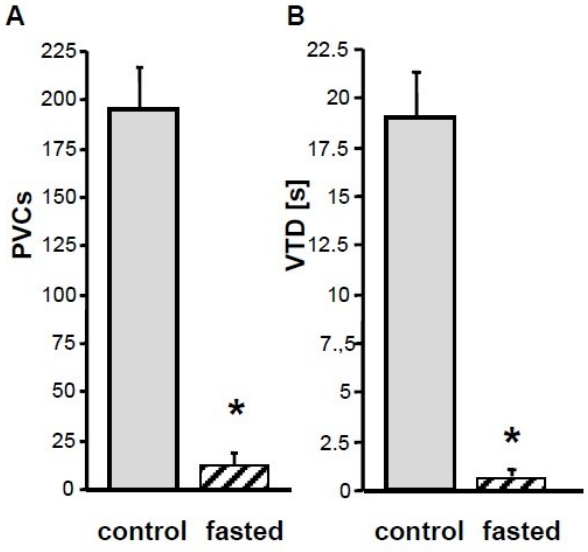
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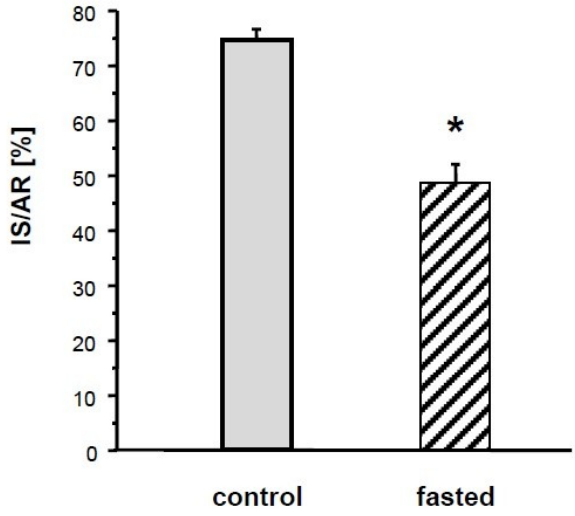
Fig. 2



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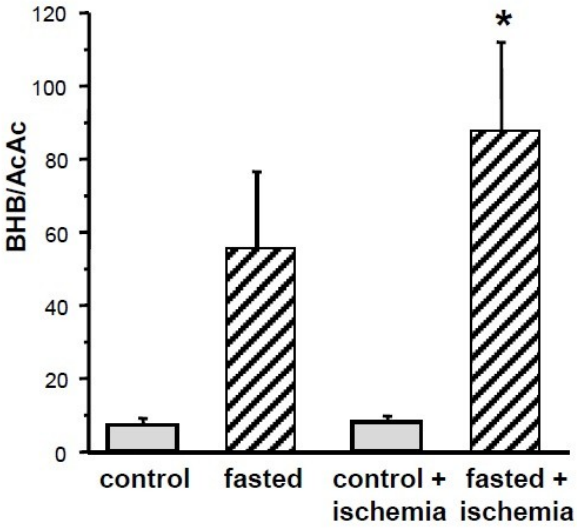
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0
BHB/AcAc
*
control
fasted
control + ischemia
fasted + ischemia

Fig. 4