

Differential effects of AMP-activated protein kinase in isolated rat atria subjected to simulated ischemia–reperfusion depending on the energetic substrates available

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Abstract AMP-activated protein kinase (AMPK) is a serine–threonine kinase that functions primarily as a metabolic sensor to coordinate anabolic and catabolic processes in the cell, via phosphorylation of multiple proteins involved in metabolic pathways, aimed to re-establish energy homeostasis at a cell-autonomous level. Myocardial ischemia and reperfusion represents a metabolic stress situation for myocytes. Whether AMPK plays a critical role in the metabolic and functional responses involved in these conditions remains uncertain. In this study, in order to gain a deeper insight into the role of endogenous AMPK activation during myocardial ischemia and reperfusion, we explored the effects of the pharmacological inhibition of AMPK on contractile function rat, contractile reserve, tissue lactate production, tissue ATP content, and cellular viability. For this aim, isolated atria subjected to simulated 75 min ischemia–75 min reperfusion (Is-Rs) in the presence or absence of the pharmacological inhibitor of AMPK (compound C) were used. Since in most clinical situations of ischemia–reperfusion the heart is exposed to high levels of fatty acids, the influence of palmitate present in the incubation medium was also investigated. The present results suggest that AMPK activity significantly increases during Is, remaining activated during Rs. The results support that intrinsic activation of AMPK has functional protective effects in the reperused atria when glucose is the only available energetic substrate whereas it is deleterious when palmitate is also

available. Cellular viability was not affected by either of these conditions.

Keywords AMP-activated protein kinase · Compound C · Atria · Simulated ischemia–reperfusion · Metabolism · Palmitate

Introduction

AMP-activated protein kinase (AMPK) is a serine–threonine kinase that functions as a cellular “fuel gauge” enzyme activated when cellular energy is depleted. This enzyme acts as an important regulator of diverse cellular pathways aimed to re-establish energy homeostasis at a cell-autonomous level. It promotes catabolic pathways that generate ATP, including breakdown of energy stores, glucose transport, glycolysis and fatty acid oxidation, and inhibits energy-consuming anabolic processes not essential for short-term cell survival. [25, 86]. It has also been proposed that AMPK promotes autophagy. Activation of this process may be protective by generation of metabolic substrates and removal of malfunctioning mitochondria or it may be harmful by induction of cell death [75, 76].

Mammalian AMPK is a heterotrimeric complex comprising a catalytic α subunit and regulatory β and γ subunits, each subunit being encoded by multiple genes (α_1 , α_2 , β_1 , β_2 , γ_1 , γ_2 , γ_3). The heart predominantly expresses α_2 (contributing 70–80% of the total AMPK catalytic activity), β_2 , and both the γ_1 and γ_2 isoforms [68]. It has been demonstrated that cardiac AMPK is rapidly activated during ischemia [36, 37]. Under this condition, the increase of intracellular AMP/ATP ratio results in allosteric binding of AMP to the AMPK γ

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subunit nucleotide-binding domains [55, 84]. Following this, the conformational change of the AMPK complex facilitates the subsequent phosphorylation of a critical residue at threonine 172 (Thr¹⁷²) in the α subunit by upstream kinases, such as the tumor suppressor kinase LKB1, which appears to be constitutively active [82]. Under normal aerobic conditions, physiologic concentrations of ATP antagonize this effect of AMP and decrease the suitability of AMPK for phosphorylation, keeping AMPK in the inactive state. In some cell types, phosphorylation of Thr¹⁷² could also occur through a different upstream enzyme, such as transforming growth factor- β -activated protein kinase-1 (TAK1) and calmodulin-dependent protein kinase kinase β (CaMKK β) activated by calcium ions, which appears to be particularly important in neurons, endothelial cells and T lymphocytes [50, 83].

Once AMPK is activated, it orchestrates several metabolic pathways to maintain acceptable ATP levels, required for cell survival under metabolic stress conditions. To this respect, AMPK plays an important role in regulating both fatty acid and glucose metabolism. Its activation results in a stimulation of glucose uptake, glycolysis, and fatty acid oxidation. AMPK promotes cellular glucose uptake by translocation of intracellular GLUT4-containing membrane vesicles to the sarcolemma and upregulation of GLUT1. AMPK also phosphorylates the 6-phosphofructo-2-kinase, resulting in the increase of fructose 2,6-bisphosphate, an allosteric activator of 6-phosphofructo-1-kinase, which is an essential regulatory step in the glycolytic pathway [48, 66]. Furthermore, AMPK also suppresses the activity of glycogen synthase, promoting the inhibition of glycogen synthesis, which also contributes to the conservation of ATP [23]. AMPK facilitates both fatty acid uptake and oxidation. This enzyme increases lipoprotein lipase translocation to vascular endothelial surface in the heart, promoting the release of fatty acids from circulating triacylglycerol, which is required for their delivery to cardiomyocytes [2]. In addition, AMPK also facilitates fatty acid uptake into cardiomyocytes through the recruitment of the fatty acid transporter CD36 to the sarcolemma [69]. Activated AMPK is also important for cytosolic fatty acid transport into the mitochondria and, consequently, for its oxidation. In this respect, AMPK phosphorylates and inactivates acetyl-CoA carboxylase, leading to a decrease in the intracellular concentration of malonyl-CoA, a negative regulator of carnitine palmitoyl transferase-I, the rate-limiting enzyme for mitochondrial fatty acid uptake and, therefore, for its oxidation [36].

While the activation of glucose uptake and glycolysis during ischemia has the potential to increase energy supply to the heart, fatty acid oxidation, which suppresses glucose oxidation via the Randle cycle [63], lead to uncoupling of glycolysis and glucose oxidation, proton accumulation, and a more acidic intracellular environment, which has been shown to be associated with a decrease in cardiac function during the critical

period of reperfusion. Additionally, fatty acids require approximately 10–15% more oxygen to generate an equivalent amount of ATP when compared with glucose, making the heart especially vulnerable to damage following ischemia. Whether AMPK plays a critical role in the metabolic and functional responses to myocardial ischemia and reperfusion remains uncertain.

In this study, we determined the effects of endogenous AMPK activation in simulated ischemic-reperfused (Is-Rs) isolated rat atria. Many studies trying to elucidate the role of AMPK in the heart are being carried out employing isolated cardiomyocytes, being the murine atrial cardiomyocyte derived (HL-1) cell line one of the most widely used [16, 52, 65, 69, 71]. Despite the parallel between the behavior of isolated myocytes and intact myocardium, whether the role of AMPK observed in single cells can be reproduced in the intact myocardial syncytium, where cell–cell junctions can modify cellular response as a result of metabolic and electrical communication in the tissue, still remains to be determined. Moreover, it is well known that left atria contribute significantly to total cardiac output with its reservoir, conduit, and pumping function [30], and recent studies suggest that left atrial ischemia might play important roles in the pathophysiology of atrial fibrillation [39, 57], common sustained arrhythmia encountered in clinical which is associated with increased mortality of ischemic-reperfused patients. The use of isolated atria in the present study provides new insights that might help to improve the understanding of the role played by AMPK in the response to ischemia and reperfusion in a more physiologically employable model, while also providing a deeper understanding into the effects of ischemia–reperfusion on atrial function, which are less well established but could provide important information to better understand ischemia–reperfusion injury.

Finally, since in most clinical situations of ischemia–reperfusion the heart muscle is exposed to high levels of fatty acids, the influence of free fatty acids, in the form of 1.2 mM palmitate present in the incubation medium, on AMPK activation and effects was also investigated.

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. 86-23, revised 1996; http://oacu.od.nih.gov/regs/guide/guide_2011.pdf) and Argentine Law No. 14346 concerning animal protection.

Female Sprague-Dawley rats, weighing 220–270 g, fed ad libitum, and maintained on a 12-h dark/light cycle were used in this study. In order to carry out the experiments, rats were

anesthetized by carbon dioxide inhalation, procedure widely used as a safe form of anesthesia for short-term procedure. At concentrations above 70%, carbon dioxide acts as an anesthetic agent that causes rapid loss of consciousness, due to the narcotic effect of the high intake of carbon dioxide on the brain without causing hypoxia, resulting in an effective and humane method for anesthesia and euthanasia of most small animals, with controversial effects on the heart rate but no serious disturbance to cardiac function [5, 14, 15, 22]. Afterwards, hearts were excised quickly and cooled in ice-cold saline until contractions stopped. Left atria were excised and mounted isometrically in 20 ml organ baths at a resting force of 750 mg and paced at 1 Hz with 5–10 V, 0.6 ms square pulses applied through bipolar punctate electrodes during the whole experiment. The bathing medium was a Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl, 120; NaHCO₃, 25; KCl, 4.8; MgSO₄, 1.33; KH₂PO₄, 1.2; CaCl₂, 1.6; and Na₂EDTA, 0.02, continuously bubbled with 95% O₂–5% CO₂, pH 7.4, and kept at 31 °C. As metabolic substrates, the bathing medium contained 10 mM D-glucose or 10 mM D-glucose and 1.2 mM palmitate prebound to 3% bovine serum albumin (BSA). Palmitate concentration was chosen according to plasma fatty acid levels measured within the first 30 min after the appearance of symptoms associated to the development of acute myocardial infarction, which would be due to an increase in catecholamine discharge along with plasma increase of glucocorticoids [38, 46].

After 60-min stabilization period, atria were subjected to 75-min Is followed by 75-min Rs. The duration of ischemia resulted in ~40% functional recovery and ~20% cell death, allowing demonstration of both beneficial and harmful effects in response to different interventions [28]. For the induction of Is, the medium was bubbled with 95% N₂–5% CO₂ (pH 6.80–7.00) and metabolic substrates were replaced with 10 mM 2-deoxy-D-glucose (Sigma). During Is, the pO₂ of the bathing medium, measured polarimetrically in samples retrieved from the organ bath, was 67 ± 5 mmHg. Rs was initiated by a buffer exchange to normoxic Krebs-Ringer bicarbonate solution. Stability of left atria paced at 1 Hz was maintained for at least 300 min in the Krebs-Ringer bicarbonate solution at 31 °C.

Ten-micromolar compound C, (6-[4-(2-piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine) (Sigma), a cell-permeant pyrazolopyrimidine, the most widely pharmacological inhibitor of AMPK, was used. Compound C was added to the bathing medium at the onset of Is and maintained throughout the experiment. Stock solution was prepared in dimethylsulfoxide (DMSO) at a concentration not to exceed 0.1% DMSO (v/v) in the final solution. The solvent did not show effects by itself in a separate experimental test carried out with isolated left atria (data not shown). Experiments conducted using variable ATP concentrations revealed that compound C is a potent and reversible inhibitor of AMPK, which is competitive with ATP, with Ki = 109 ± 16 nM [88]. It has been

demonstrated that compound C alters the conformation of the AMPK-activation loop and reduces the phosphorylation of Thr¹⁷² by AMPK kinases and/or promote the dephosphorylation of Thr¹⁷² by protein phosphatases [24, 49].

In a subset of experiments, 2 mM oxfenicine (S-4-hydroxyphenylglycine, Sigma), a carnitine palmitoyl transferase-I inhibitor, was added to the bathing medium at the onset of stabilization period and maintained until the end of the experiment.

Western blotting

Atria were homogenized in lysis buffer (18.2 mM HEPES, 1 mM EDTA, 0.28 M sucrose, 2 mM dithiothreitol, pH 7.4, 2 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail from Thermo Scientific), an aliquot was saved to measure proteins by the method of Bradford et al. [8], and the remaining sample was subjected to SDS-PAGE.

Equal amounts of protein were mixed with LAEMMLI sample buffer (BioRad), boiled for 5 min and then resolved on 15% SDS-PAGE gels at 120 V. Next, proteins were transferred to polyvinylidene difluoride membranes at 15 V for 50 min and then blocked for 1 h with 5% non fat powder milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Membranes were then washed with TBS-T and incubated at 4 °C overnight with polyclonal rabbit anti-total AMPKα antibody (Thermo Scientific) at 1:1000, polyclonal rabbit anti-phospho AMPKα Thr¹⁷² antibody (Thermo Scientific) at 1:1000, and polyclonal rabbit anti-β-actin antibody (Thermo Scientific) at 1:1000 dilution. HRP-conjugated donkey anti-rabbit antibody (Thermo Scientific) at 1:250 dilution was then incubated for 2 h at room temperature. After TBS-T washes, bands were detected with Bio-Lumina detection reagent (Kalium Technologies) and exposed to an autoradiography film (Kodak). The films were scanned for band quantification using a Hewlett-Packard scanner and the intensity of the immunoblot bands was analyzed by densitometry using Image J software and normalized to β-actin. Results were expressed in arbitrary units.

Measurement of atrial function

Mechanical variables were recorded with stress transducers coupled to an amplifier and Grass polygraph. Systolic function was assessed by peak developed force (*F*), peak rate of contraction (+ *dF/dt*), and force-time index (FTI), which reflects the mechanical energy of contraction per beat. Peak developed force was calculated as the difference between the peak of developed force curve and the resting force. FTI was obtained from the integration of the area under the systolic portion of the developed force curve. Diastolic function was assessed by developing contracture, measured as the rise in resting force, and the peak rate of relaxation (– *dF/dt*).

F , FTI, and $\pm dF/dt$ were expressed as a percentage of the respective basal values at the end of the 60-min aerobic stabilization period. The rise in resting force was expressed as a percentage of the peak developed force at the end of the 60-min aerobic stabilization period.

Contractile reserve

Contractile reserve was assessed by the evaluation of maximal contractile response to a β -adrenergic agent. For this aim, 2 μ M isoproterenol (ISO, Sigma) was added to the incubation medium at the end of the Is-Rs period. The ISO dose, obtained from a dose-response curve previously performed in left atria subjected to incubation for 210 min in normal aerobic medium, elicited the maximal inotropic response. F , FTI, and $\pm dF/dt$ values were expressed as a percentage of the respective value obtained at the end of the 60-min stabilization period.

Tissue lactate assay

For determination of tissue lactate atria treated following the above described protocols were removed at the end of the 75-min simulated ischemic period and immediately frozen between two blocks of ice at -21 °C. Lactate was extracted from a 40- to 50-mg sample of frozen tissue, added to 6% ice-cold perchloric acid and measured enzymatically [72]. The results were expressed as μ mol of lactate per gram of dry weight.

Tissue ATP content

Samples (40 mg) were homogenized in 500 μ l of 3% (mass/vol) cold perchloric acid for determination of tissue ATP. After removal of the denatured protein by centrifugation at $12,000\times g$ for 5 min, aliquots of supernatant were neutralized with a mixture of 2 M KOH and 0.3 M Mops [7]. Tissue ATP concentrations were determined on the neutralized supernatant by luciferin-luciferase luminometry (Sigma Chemiluminescent Assay kit).

Protein concentrations were determined in the solubilized pellet by the Lowry method [47]. The concentration of ATP was expressed as picomoles of ATP per milligram of tissue protein.

Pyruvate dehydrogenase activity

Mitochondria were isolated from simulated ischemic reperfused atria by differential centrifugation after tissue homogenization in ice-cold sucrose buffer solution (300 mM sucrose, 10 mM Tris-HCl, 2 mM EGTA, 5 mg/ml BSA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) [73]. The mitochondrial pellet was then washed three times in sucrose isolation buffer solution lacking BSA. Cardiac mitochondria prepared with this procedure have shown to be metabolically active, with respiratory control ratios of 3.5–

5.0 with succinate and of 8.0–10.0 with glutamate/malate, and corresponding adenosine diphosphate to oxygen ratios of 1.5–1.7 and 2.5–2.7 [73].

Pyruvate dehydrogenase activity was determined using a coupled enzyme reaction (Pyruvate Dehydrogenase Activity Assay Kit, Sigma). The enzymatic activity was determined by the reduction of NAD^+ to NADH, coupled to the reduction of a reporter dye to yield a colored reaction product. The concentration of this product was monitored by measuring the increase in absorbance at 450 nm, which is proportional to the enzymatic activity present. One unit of pyruvate dehydrogenase is the amount of enzyme that will generate 1 mmol/min of NADH at 37 °C.

Protein concentrations were determined in the solubilized pellet by the Lowry method [62]. PDH activity was expressed as nanomoles per minute (milliunits) per milligram of tissue protein.

Measurement of cellular viability: MTT assay

The reduction of 3-[4,5 dimethylthiazol-2yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma) to blue formazan by mitochondrial dehydrogenases was assessed at the end of each experimental protocol to measure cell viability.

Atria were loaded into a Falcon conical tube and incubated in 2 ml of phosphate-buffer solution (PBS) containing 1 mg/ml MTT for 90 min at 37 °C. Atria were next transferred to 2 ml PBS and shaken for 1 min to remove dye excess. Atria were then homogenized in 2 ml DMSO at 9500 rpm for 1 min and the homogenate was centrifuged at $1000\times g$ for 10 min. The absorbance of the colored supernatant was then measured spectrophotometrically at 520 nm and optical density/mg wet weight of myocardial tissue was calculated [21]. The results were expressed as a percentage of the pre-ischaemic value.

Statistical analysis

All data are presented as mean \pm SEM. Changes in the contractile function were statistically compared using a two-factor ANOVA for repeated measures in one factor followed by Tukey's test. All biochemical parameters were evaluated using one or two-way ANOVA followed by Tukey's test. Statistical analysis was done using GraphPad InStat 4 (GraphPad Software, La Jolla, CA, USA). The probability level of 0.05 or lower was used as a criterion for biological significance.

Results

AMPK immunoblotting

We first examined whether Is and/or Rs activates the AMPK pathway in the isolated rat left atria. AMPK α -subunit contains a serine-threonine kinase domain, which has a critical

activating residue at Thr¹⁷² within the catalytic cleft. Phosphorylation status of this amino acid by upstream kinases is essential for AMPK activity and, therefore, it is widely used as an indicator of the activation state of the kinase [26]. In this context, we explored the ratio of phosphorylated α -subunit in Thr¹⁷² (AMPK α -pThr¹⁷²) to total enzyme α -subunit (AMPK α -total), by Western blot analysis at 15-, 40-, and 75-min Is and at 15-, 40-, and 75-min Rs. The results showed a significant increase in the AMPK α -pThr¹⁷²/AMPK α -total ratio during Is, reaching a maximum phosphorylation status at 40-min Is, which was sustained until the end of Is, either in the presence or absence of 1.2 mM palmitate in the bathing medium during the 60-min stabilization period (Fig. 1a). During the 40-min Rs AMPK remained activated, either in the presence or absence of 1.2 mM palmitate. However, at the end of Rs, the results showed a returned to baseline levels in both metabolic conditions (Fig. 1b). On the other hand, AMPK pharmacological inhibitor, compound C, prevented phosphorylation in Thr¹⁷² during the whole experiment, either in the presence and absence of palmitate.

Functional parameters

As shown in Fig. 2, an abrupt decrease in the systolic parameters was observed in the first 10 min of Is. Following this early period, the decrease in these parameters occurred gradually. After 75-min Is, peak developed force and force-time index showed a slightly more pronounced decrease in the absence of compound C, either in the presence and in the absence of 1.2 mM palmitate in the bathing medium during the 60-min stabilization period. During the 75 min of Rs, contractile parameters gradually recovered. Atria subjected to Is-Rs in the presence of 1.2 mM palmitate in the bathing medium during the 60-min stabilization period and 75-min Rs showed a decrease in the recovery of contractile function compared with atria incubated in the presence of glucose as sole exogenous substrate. AMPK inhibitor did not affect systolic function recovery in either metabolic condition.

Figure 3 shows that maximum ischemic contracture developed earlier in the atria stabilized with glucose and palmitate, compared with atria stabilized in the presence of glucose as sole exogenous substrate (20- vs 40-min Is, $p < 0.05$). Compound C did not affect the development of ischemic contracture when the atria were stabilized with glucose only. However, in the experiments stabilized with glucose and palmitate as exogenous substrates, the addition of compound C increased maximum ischemic contracture, without affecting the time when this peak appeared (Fig. 3b). On the other hand, during simulated ischemia compound C generated a less pronounced decrease in the relaxation rate, either in the presence of glucose as sole exogenous substrate or glucose and palmitate during 60-min aerobic stabilization period. After 75-min Rs, atria subjected to Is-Rs in the presence of 1.2 mM

palmitate in the bathing medium during the 60-min stabilization period and 75-min Rs showed a decrease in the recovery of peak rate of relaxation compared with atria incubated in the presence of glucose as sole exogenous substrate. Compound C reversed the harmful effect of palmitate over this functional parameter.

In order to investigate the ability of the surviving myocardium to increase its level of contractile function in situations of increased energy demand, inotropic stimulation with ISO was assessed. As expected for normal myocardium, atria subjected to the 60-min stabilization period or to 210 min of aerobic conditions developed a similar increase in contractile parameters in response to β -adrenergic stimulation. This increase in cardiac contractility was attenuated in atria subjected to Is-Rs. This effect was even more pronounced in the experiments performed in the presence of glucose and palmitate as exogenous substrates. Compound C reversed the harmful effects in myocardial contractility as a response to β -adrenergic stimulation in the atria subjected to ischemia–reperfusion in the presence of palmitate. However, in the presence of glucose as sole exogenous substrate, the AMPK inhibitor attenuated the response to ISO even more (Fig. 4).

To assess the influence of palmitate oxidation in the present contractile results, oxfenicine (2 mM), a carnitine palmitoyl transferase-I inhibitor, was used for the limitation of mitochondrial fatty acid uptake rate and therefore its oxidation. The results showed that oxfenicine reversed the harmful effects of palmitate on functional recovery, while in the atria subjected to Is-Rs in the presence of glucose as sole exogenous substrate the contractile reserve was reduced by oxfenicine similarly to that observed in the presence of compound C (Table 1).

Lactate tissue content

It has been reported that AMPK stimulates glucose transport and glycolysis during ischemia, which are particularly important for metabolic adaptation under this condition. However, during ischemia, glycolysis becomes uncoupled from glucose oxidation, resulting in the accumulation of deleterious by-products of glycolysis within the cardiac cells. This can lead to the redirection of ATP away from myocardial contraction and toward the clearance of glycolytic by-products, leading to a decrease in both cardiac function and efficiency [17, 41, 42]. To assess if these metabolic pathways are enhanced by AMPK during ischemia, tissue lactate content, the final product of glycolysis under ischemic condition, was measured at the end of this period. Results showed that after aerobic incubation in the presence of 1.2 mM palmitate lactate accumulation was reduced (Fig. 5). Atria subjected to Is in the presence of compound C, performed either in the presence of glucose as sole exogenous substrate or glucose and palmitate during 60-min aerobic stabilization period, showed an even more

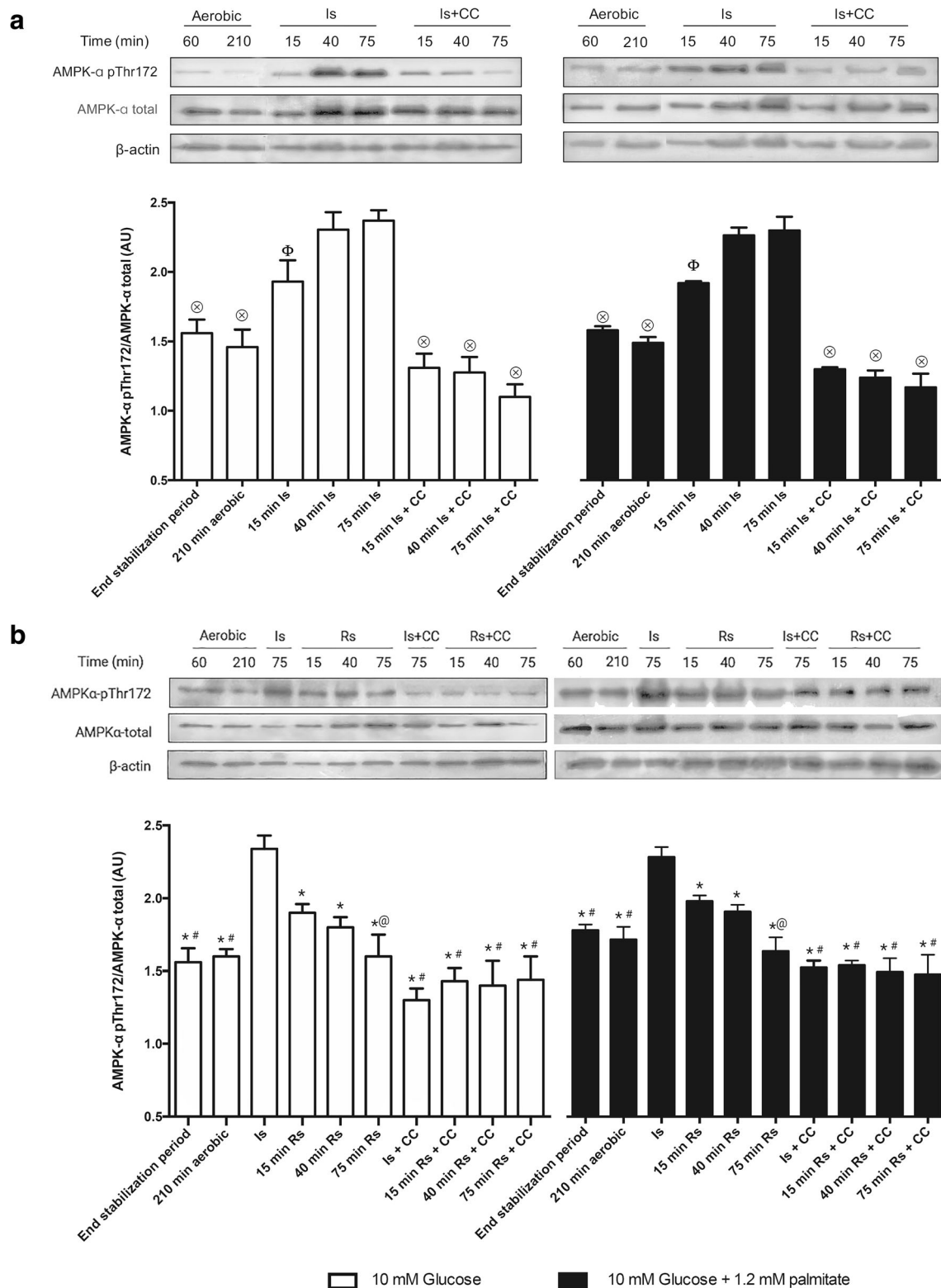


Fig. 1 Western blot Immunoblot composite images, showing phosphorylated α -subunit in Thr¹⁷² (AMPK α -pThr¹⁷²) and total enzyme α -subunit (AMPK α -total) expression in rat atria **a** subjected to 15-, 40-, and 75-min simulated ischemia and **b** subjected to 15-, 40-, and 75-min simulated reperfusion. Immunoblot bands were normalized to corresponding β -actin. The values for the AMPK α -pThr¹⁷² and AMPK- α

total ratio are mean \pm SEM ($n = 5$ /each condition). CC, 10 μ M compound C. $\otimes p < 0.05$ vs 15, 40, and 75 min Is in both metabolic conditions; $\Phi p < 0.05$ vs 40 and 75 min Is in both metabolic conditions; $*p < 0.05$ vs Is in both metabolic conditions; $\#p < 0.05$ vs 15 and 40 min Rs in both metabolic conditions; $\oplus p < 0.05$ vs 15 min Rs in both metabolic conditions

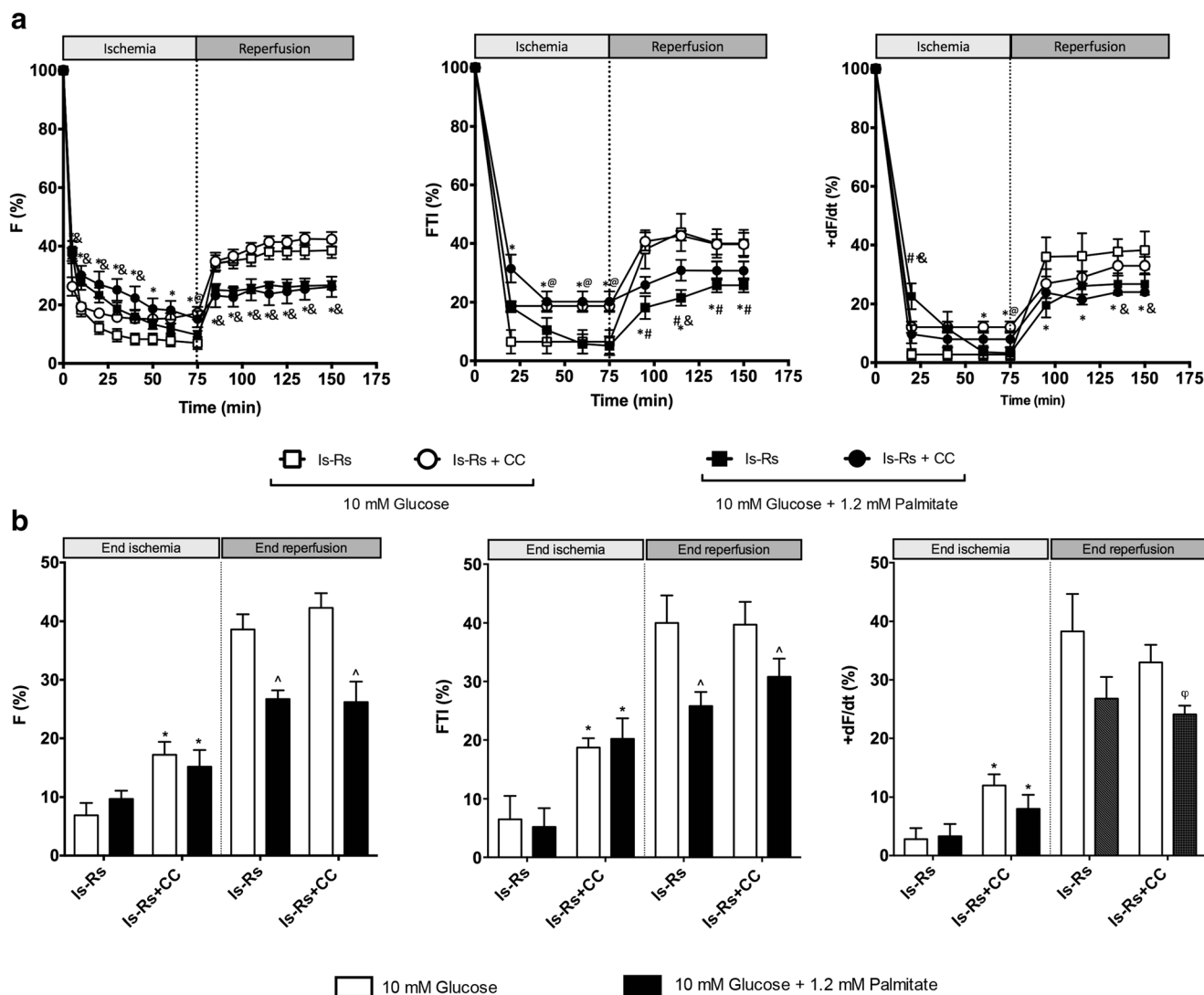


Fig. 2 Atrial systolic function. Effects of 10 μ M compound C (CC) on peak developed force (F), peak rate of contraction ($+dF/dt$) and force-time index (FTI) in left isolated rat atria subjected to simulated ischemia reperfusion in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. Values (mean \pm SEM, $n = 8$ /each condition) are expressed as a percentage of the respective basal values at the end of the 60-min stabilization period. **a** Effects of compound C on atrial contractile function during 75 min of simulated ischemia and 75 min reperfusion. * $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate; # $p < 0.05$ vs Is-Rs + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates; & $p < 0.05$

vs Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate; @ $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates. **b** Effects of compound C on atrial contractile function at the end of simulated ischemia and reperfusion. * $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs in the presence of CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates; ^ $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrates; $\varphi p < 0.05$ vs all end reperfusion groups

pronounced reduction in lactate accumulation during the ischemic period, showing no significant differences between both groups (Fig. 5).

Tissue ATP content

To assess whether the differences observed between groups in peak ischemic contracture development were accompanied with differences in ATP levels, tissue ATP content was determined at 20-min Is. Figure 6 shows that ATP levels fell to

lower values when atria were incubated with 10 mM glucose and 1.2 mM palmitate. Moreover, when the intrinsic AMPK activation was inhibited, the atria stabilized in the presence of glucose and palmitate contained substantially less ATP than all other groups. However, in atria stabilized with glucose as sole exogenous substrate, inhibition of AMPK did not affect ATP content, which was similar to control atria. At the end of 75-min Is, tissue ATP levels fell to even lower values, being this reduction more pronounced by the presence of palmitate as exogenous substrate. Compound C reduced ATP content

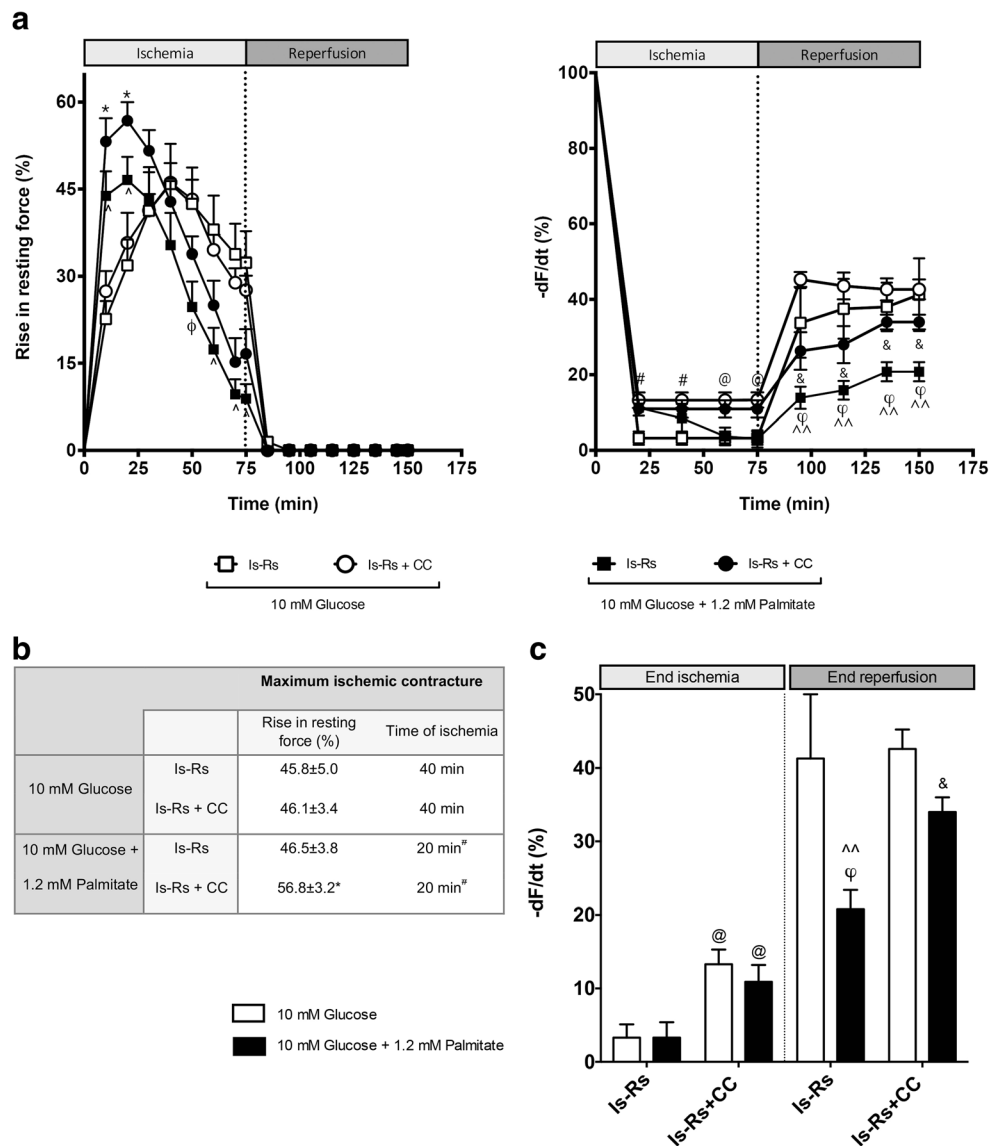


Fig. 3 Atrial diastolic function. **a** Effects of 10 μ M compound C (CC) on resting force and peak rate of relaxation ($-dF/dt$) in isolated left atria subjected to 75 min simulated ischemia–75 min reperfusion (Is-Rs) in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. All values are expressed as mean \pm SEM ($n = 8$ /each condition). Rise in resting force is expressed as a percentage of the peak force developed at the end of the stabilization period and $-dF/dt$ as a percentage of the respective basal value at the end of the stabilization period. ^{*} $p < 0.05$ vs Is-Rs in both metabolic conditions and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate; [#] $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate; [@] $p < 0.05$ vs Is-Rs in both metabolic conditions; [^] $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate; [^] $p < 0.01$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate; [&] $p < 0.05$ vs Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate; ^φ $p < 0.05$ vs Is-Rs + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates; ^φ $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs + CC in both metabolic conditions. **b** Effects of compound C on

maximum ischemic contracture. Data shows rise in resting force, expressed as a percentage of the peak force developed at the end of the stabilization period, when maximum ischemic contracture was developed in isolated left atria subjected to simulated ischemia in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. The time of ischemia maximum ischemic contracture was developed is also shown. All values (mean \pm SEM, $n = 8$ /each condition). ^{*} $p < 0.05$ vs Is-Rs in both metabolic conditions and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate; [#] $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate. **c** Effects of compound C on atrial maximum peak of relaxation at the end of simulated ischemia and reperfusion. All values are expressed as mean \pm SEM ($n = 8$ /each condition). $-dF/dt$ is expressed as a percentage of the respective basal value at the end of the stabilization period; [@] $p < 0.05$ vs Is-Rs in both metabolic conditions; [^] $p < 0.01$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate and Is-Rs + CC in the presence of 10 mM glucose as sole exogenous substrate; [&] $p < 0.05$ vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate; ^φ $p < 0.05$ vs Is-Rs + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates

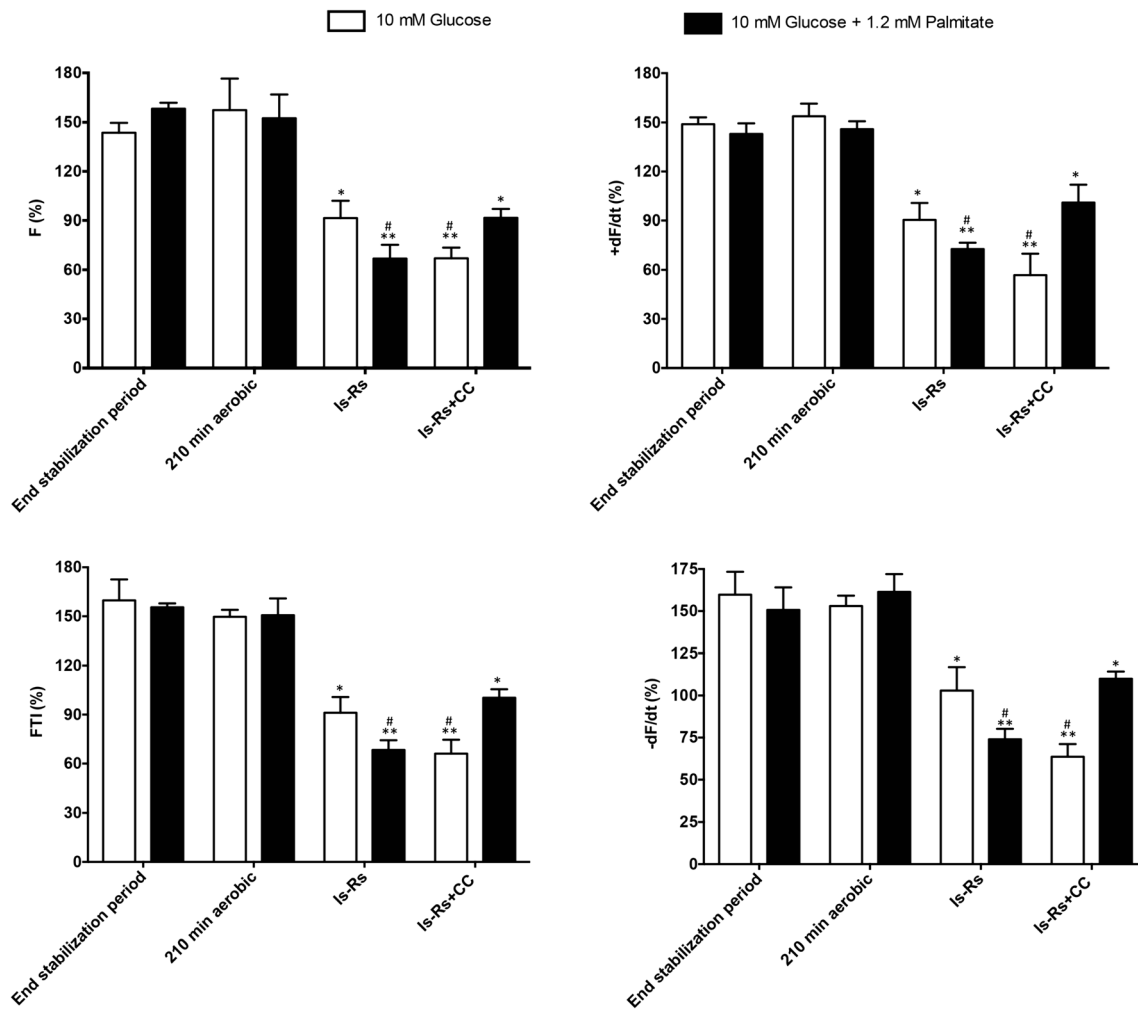


Fig. 4 Contractile reserve. Effects of 10 μ M compound C (CC) on the maximal inotropic response to a β -adrenergic agent (2 μ M isoproterenol) in isolated left atria subjected to simulated ischemia–reperfusion in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. For this aim peak developed force (F), force-time index (FTI), peak rate of contraction (+ dF/dt), and peak rate of relaxation ($-dF/dt$) was assessed. All values (mean \pm SEM, $n = 8$ /each condition)

were recorded 30 min after the addition of 2 μ M isoproterenol (ISO), at this time atria of all groups reached maximal inotropic response and are expressed as a percentage of the respective basal value at the end of the 60-min stabilization period. ** $p < 0.01$ vs end stabilization period and 210 min aerobic in both metabolic conditions; * $p < 0.05$ vs end stabilization period and 210 min aerobic in both metabolic conditions; # $p < 0.05$ vs Is-Rs in both metabolic conditions

even more when atria were incubated with glucose as sole exogenous substrate; however, it did not affect tissue ATP levels in the presence of palmitate.

Figure 6 also shows that changes in contractile recovery were accompanied by similar changes in tissue ATP content. The results also demonstrate that the effects observed in the presence of oxfenicine were accompanied by similar levels of tissue ATP content observed in the presence of compound C in each metabolic condition.

Pyruvate dehydrogenase activity

The mitochondrial oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 is catalyzed by the multienzyme pyruvate dehydrogenase (PDH) complex. The enzyme complex

plays an important role in the regulation of glucose oxidation, determining whether the mitochondria metabolize glucose or lipid fuels for ATP generation. In this study, the PDH activity was assessed to determine if the effects observed in the presence of palmitate were accompanied by PDH inhibition through Randle cycle effect.

Results showed that PDH activity after 75-min simulated ischemia increased 37% in the atria incubated in the presence of 10 mM glucose as sole exogenous substrate. The activity of the enzyme remained elevated after 75-min reperfusion. However, in the atria subjected to Is-Rs in the presence of glucose and palmitate, Fig. 7 shows that PDH activity was significantly reduced. On the other hand, when intrinsic AMPK activation was inhibited by compound C, the effects of palmitate on PDH activity were partially reverted,

Table 1 Effects of oxfenicine on contractile reserve

		Exogenous substrates present in the experiments performed					
		10 mM Glucose			10 mM glucose + 1.2 mM palmitate		
		Is-Rs	Is-Rs + CC	Is-Rs + Oxf	Is-Rs	Is-Rs + CC	Is-Rs + Oxf
Maximal inotropic response (2 pM ISO)	<i>F</i>	91.4 ± 10.56	67.0 ± 6.6 [#]	69.1 ± 6.1 [#]	66.9 ± 8.4 [#]	91.6 ± 5.4	94.3 ± 10.3
	+ <i>dF/dt</i>	90.4 ± 10.3	56.8 ± 13.1 [#]	67.9 ± 1.1 [#]	72.7 ± 3.9 [#]	102.0 ± 11.0	89.2 ± 10.7
	FTI	91.1 ± 9.6	66.1 ± 8.5 [#]	63.2 ± 7.5 [#]	68.3 ± 6.0 [#]	100.3 ± 5.3	90.3 ± 6.5
	− <i>dF/dt</i>	103.0 ± 13.9	63.6 ± 7.6 [#]	60.8 ± 7.6 [#]	74.0 ± 6.2 [#]	110.0 ± 4.3	100.1 ± 7.7

Effects of oxfenicine on the maximal inotropic response to 2 μM isoproterenol (ISO) in isolated left atria subjected to simulated ischemia–reperfusion in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. For this aim, peak developed force (*F*), force-time index (FTI), peak rate of contraction (+ *dF/dt*), and peak rate of relaxation (− *dF/dt*) was assessed. All values (mean ± SEM, *n* = 6–8/each condition) were recorded 30 min after addition of ISO and are expressed as a percentage of the respective basal value at the end of the 60-min stabilization period

CC, 10 μM compound C

[#] *p* < 0.05 vs Is-Rs in the presence of 10 mM glucose as sole exogenous substrate, Is-Rs + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrate, and Is-Rs + Oxf + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrate

increasing the enzyme activity 18% compared with aerobic control. Conversely, when atria were incubated in the presence of glucose as sole exogenous substrate, compound C reduced PDH activity (Fig. 7).

Cellular viability

Atria subjected to a 60-min aerobic stabilization period were considered 100% viable cells. Cell viability was not affected by 210-min of aerobic conditions or Is, either in the presence of glucose as sole exogenous substrate or glucose and palmitate (Fig. 8). After 75-min reperfusion, the percentage

of viable myocytes was reduced reaching similar values in both metabolic conditions. Compound C did not affect cellular viability percentage after reperfusion period, showing no significant differences between treated and non-treated groups (Fig. 8).

Discussion

In the present study, we investigated the role of intrinsic AMPK activation in the response to simulated ischemia–reperfusion in isolated rat left atria. For this purpose, the well-studied inhibitor of AMPK, compound C, was added to the bathing medium at the onset of simulated ischemia and maintained throughout the experiment [6, 31]. Since in most clinical situations of ischemia–reperfusion the heart muscle is exposed to high levels of fatty acids within the first 30 min from the appearance of symptoms associated with the development of acute myocardial infarction, which would be due to an increase in catecholamine discharge along with the plasma increase of glucocorticoids, the influence of palmitate present in the incubation medium was also investigated [12, 38, 46, 53, 56]. For this aim, atria subjected to Is-Rs were incubated in two different metabolic conditions, containing the bathing medium 10 mM D-glucose as sole exogenous substrate or 10 mM D-glucose and 1.2 mM palmitate pre-bound to 3% bovine serum albumin. The results of this study showed that AMPK is activated during Is, remaining activated during Rs. During Is, peak contracture developed earlier in the atria stabilized with palmitate. In this condition, AMPK inhibition also increased maximum contracture developed. These results were consistent with parallel changes in tissue ATP content. At the end of Rs contractile function recovery was reduced by palmitate but not by AMPK inhibition. However, AMPK

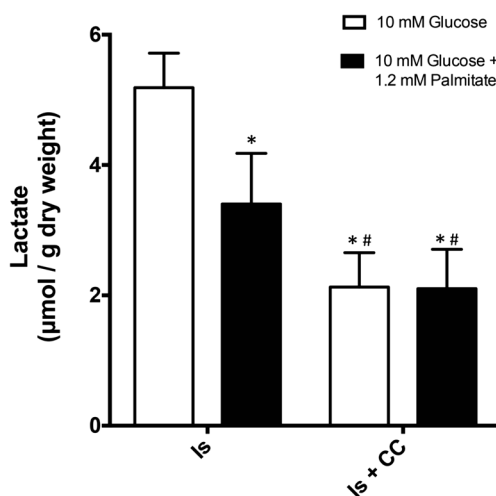


Fig. 5 Lactate tissue content. Effects of 10 μM compound C (CC) on lactate tissue content in isolated left atria subjected to simulated ischemia in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates during 60-min aerobic stabilization period. Values are mean ± SEM (*n* = 8/each condition) and are expressed as μmol/g dry weight. **p* < 0.05 vs Is in the presence of 10 mM glucose as sole exogenous substrate; [#]*p* < 0.05 vs Is in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates

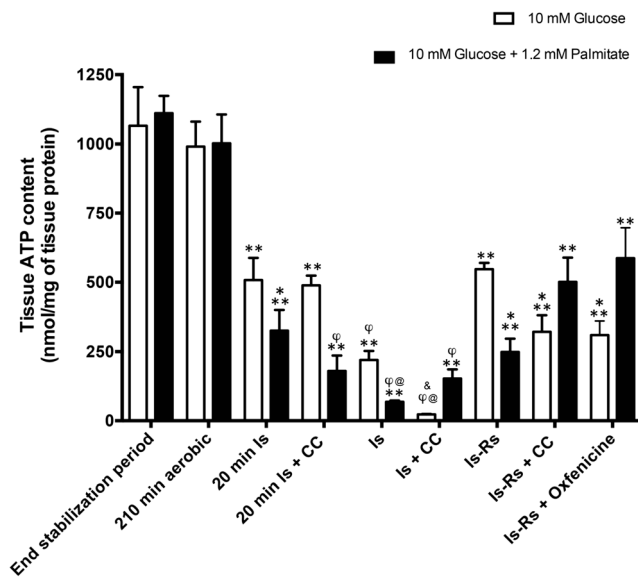


Fig. 6 Tissue ATP content. Effects of 10 μ M compound C (CC) on tissue ATP content in isolated left atria subjected to simulated ischemia in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. Values are mean \pm SEM ($n = 8$ /each condition) and are expressed as pmol/mg tissue protein $**p < 0.05$ vs end stabilization period and 210 min aerobic in both metabolic conditions; $*p < 0.05$ vs 20 min Is in the presence of 10 mM glucose as sole exogenous substrate and 20 min Is + CC in the presence of 10 mM glucose as sole exogenous substrate, Is-Rs in the presence of 10 mM glucose as sole exogenous substrate, and Is-Rs + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates; $^{\psi}p < 0.05$ vs 20 min Is in the presence of 10 mM glucose as sole exogenous substrate, 20 min Is + CC in the presence of 10 mM glucose as sole exogenous substrate, and 20 min Is in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates, Is-Rs in the presence of 10 mM glucose as sole exogenous substrate, and Is-Rs + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates; $^{\circ}p < 0.05$ vs 20 min Is + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates, Is in the presence of 10 mM glucose as sole exogenous substrate, and Is + CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates; $^{\&}p < 0.05$ vs all other groups

inhibition showed dual effect on contractile reserve, it increased this functional parameter attenuation in the presence of glucose but reversed the harmful effect of palmitate. These results were accompanied by parallel changes in tissue ATP content, although the rate of mitochondrial ATP synthesis was not affected. However, AMPK inhibition showed dual effect on PDH activity, decreasing its activity in the atria incubated with glucose, but partially reverting the decrease in PDH activity induced by palmitate. Cellular viability was not affected by palmitate or AMPK inhibition. These results support that intrinsic activation of AMPK has functional protective effects in the reperfused atria when glucose is the only available energetic substrate whereas it is deleterious when palmitate is also available.

The present data shows that AMPK phosphorylation at Thr¹⁷² increased during simulated ischemia, demonstrating that AMPK activation occurs during myocardial ischemia and persists at least during 40-min reperfusion. On the other

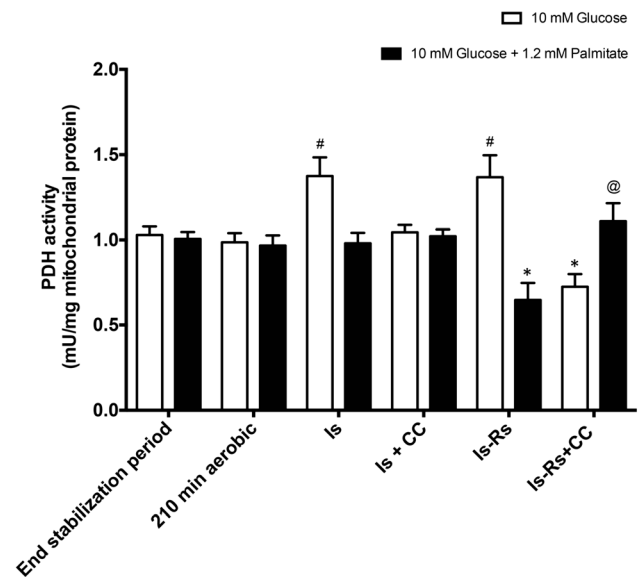


Fig. 7 Pyruvate dehydrogenase activity. Effects of 10 μ M compound C (CC) on PDH activity in isolated left atria subjected to simulated ischemia in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. Values are mean \pm SEM ($n = 8$ /each condition) and are expressed as milliunits per microgram of mitochondrial protein. $*p < 0.05$ vs all groups; $^{\#}p < 0.05$ vs end stabilization period aerobic in both metabolic conditions, 210 min aerobic in both metabolic conditions, Is in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates, and Is + CC in both metabolic conditions, Is-Rs+CC in the presence of 10 mM glucose and 1.2 mM palmitate as exogenous substrates; $^{\circ}p < 0.05$ vs Is in the presence of 10 mM glucose, Is-Rs in both metabolic conditions, Is-Rs+CC in the presence of glucose 10 mM

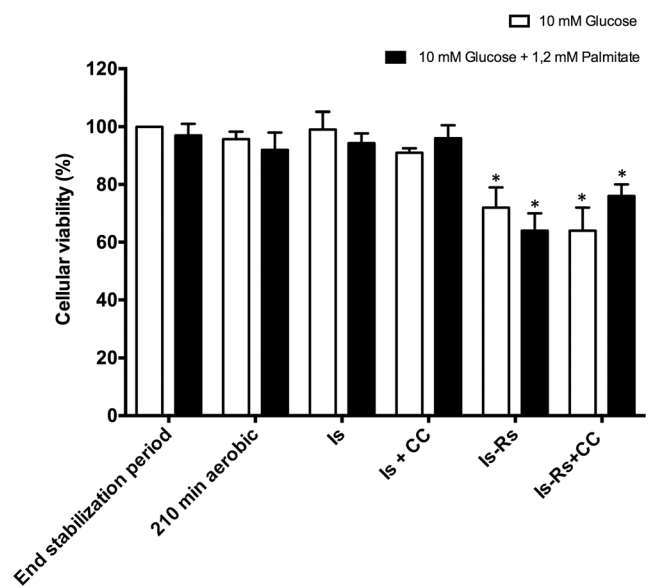


Fig. 8 Cellular viability. Effects of 10 μ M compound C (CC) on cellular viability, in isolated left atria subjected to simulated ischemia in the presence of 10 mM glucose or 10 mM glucose and 1.2 mM palmitate exogenous substrates. Atria subjected to the 60-min aerobic stabilization period were considered 100% viable cells. Values are mean \pm SEM ($n = 8$ /each condition) and are expressed as percentage of the pre-ischaemic value. $*p < 0.05$ vs end stabilization period, 210 min aerobic, Is, and Is + CC, all in both metabolic conditions

hand, compound C was able to inhibit AMPK activation. These results are consistent with the findings reported by other authors, who demonstrated that in Langendorff perfused rat hearts subjected to 30-min period of global no-flow ischemia and a subsequent 60-min period of aerobic reperfusion, AMPK activity was significantly elevated in both the ischemic and reperfused ischemic hearts, compared with those aerobically perfused [4, 36]. Kudo et al. proposed that the activation of AMPK during the ischemic period occurs secondary to the increase in 5'-AMP, either due to a direct activation of AMPK or secondary to phosphorylation of AMPK by AMPK upstream kinase. The authors also state that during reperfusion the activity of AMPK remains elevated, even though 5'-AMP levels return to pre-ischemic values [21].

The lack of oxygen during myocardial ischemia determines an immediate blocking of oxidative phosphorylation, with failure of aerobic ATP synthesis and accumulation of oxidative phosphorylation substrates. However, in this condition anaerobic glycolysis remains active. In this process, glucose is formed by endogenous glycogen stores and catabolized to pyruvate, which is in turn converted to lactate by lactate dehydrogenase, yielding 2 mol of ATP/mol of glucose [32, 79]. On the other hand, it is well known that extracellular levels of fatty acids are an important determinant of myocardial substrate use; in 1960s, Randle et al. discovered that high levels of fatty acids decrease glucose utilization under well oxygenated conditions (Randle effect) [19]. Considering that in most clinical situations of ischemia–reperfusion the myocardium is exposed to high levels of fatty acids, the present results suggest that after aerobic incubation in the presence of 1.2 mM palmitate, the Randle effect continues to function during simulated ischemia, since the anaerobic glycolysis, estimated from lactate accumulation, was reduced. Moreover, peak ischemic contracture occurred much more rapidly, shifting from 40- to 20-min reperfusion. To this respect, it has been shown by Hearse et al. that ischemic contracture is closely related to tissue ATP content [27]. It has been shown that the gradual decrease in the concentration of tissue ATP, below a critical concentration, contributes to the development of ischemic contracture [1, 54]. In this regard, the main cause related to the development of contracture, although not the only one, would be the formation of stable junctions or “rigor” actin-myosin junctions, which require ATP to dissociate [61]. This type of contracture is reversible, usually moderate and, although it does not generate great structural damage, produces alterations in the cytoskeleton that make myocytes more susceptible to mechanical damage [61]. At the time peak ischemic contracture occurred in atria incubated with glucose and palmitate, ATP levels fell to lower values than in atria incubated with glucose as sole exogenous substrate. To this respect, it has been reported that during ischemia AMPK has a dual role, increasing both glycolysis and fatty acid oxidation to increase overall energy production in response to high

energy demand [17, 42]. Our results show that when the intrinsic AMPK activation was inhibited, the atria stabilized in the presence of palmitate exhibited enhanced peak ischemic contracture and contained substantially less ATP than all other groups. However, in atria stabilized with glucose as sole exogenous substrate, inhibition of AMPK did not affect contracture and ATP content was similar to control atria after 20-min ischemia. Present data suggests that the quantity of ATP depletion may be responsible for peak contracture and time to peak contracture [33], probably producing rigor bonds, although other variables involving cytosolic calcium levels and intracellular pH may contribute to contracture in this experimental model. Lactate accumulation during ischemia suggests that anaerobic glycolysis, major source of ATP during ischemia, is partly stimulated by AMPK; however, it is not possible to associate changes in contracture with lactate accumulation. In the study by Xing et al. [85], isolated ischemic-reperfused hearts from transgenic mice with cardiac-specific overexpression of a dominant negative mutant of the AMPK α_2 catalytic subunit exhibited exacerbated ATP depletion during ischemia and enhanced left ventricular end-diastolic pressure that could not be attributed to impaired glycolytic ATP synthesis. Since it is well known that fatty acid utilization is stimulated by AMPK, utilization of glucose may be affected by more complex ways in the presence of compound C than in its absence.

On the other hand, the presence of high levels of palmitate resulted in a smaller post-ischemic functional recovery and contractile reserve, which was accompanied by a lower recovery of tissue ATP content, without affecting cell viability. To this respect, reperfusion of ischemic hearts with high levels of fatty acids, which is seen in most clinically relevant conditions of ischemia, results in a rapid recovery of fatty acid oxidation [43]. It has been demonstrated that the increase in the mitochondrial acetyl CoA/CoA and NADH/NAD⁺ ratios, caused by excessive rates of fatty acid oxidation during reperfusion, lead to the inhibition of PDH, due to the activation of PDH kinase (PDK) which phosphorylates and inactivates PDH, resulting in the reduction of pyruvate decarboxylation to acetyl-CoA and CO₂ [59]. Consistent with this, in the present study data shows that when the atria were incubated with glucose and high levels of palmitate, PDH activity was significantly reduced during the reperfusion period. The resulting decrease in glucose oxidation, caused by an impaired coupling of glycolysis and glucose oxidation, might lead to the reduction of energy efficiency in terms of ATP produced/mol of oxygen consumed [74]. To this respect, it is well known that fatty acid oxidation requires a greater rate of myocardial oxygen consumption for a given rate of ATP synthesis than do carbohydrates, causing a reduction of energy efficiency for basal metabolism and for generating contractile power [10, 35]. In addition to this, it has also been proposed that fatty acids waste ATP through the extrusion of long-chain fatty

acids out of the mitochondria via uncoupling protein 3-mediated futile cycle, possibly contributing to reduce myocardial ATP content [29, 70].

To confirm that myocardial energy substrate utilization is an important determinant of the ability of cardiac muscle to recover after an ischemic episode, oxfenicine, an inhibitor of carnitine palmitoyl transferase-I, the rate-limiting enzyme for mitochondrial fatty acid uptake, was used in this study. Present results show that oxfenicine was able to reverse the harmful effects of fatty acids on functional recovery, suggesting that the deleterious effects of palmitate on the still viable myocardium may be the result of its oxidation. In these conditions, oxfenicine reverted partially the recovery of tissue ATP, reaching similar values to that observed when atria were incubated with glucose as sole exogenous substrate, supporting the existence of some relation between mitochondrial palmitate oxidation and the reduction of energy production, being this consistent with a reduction of contractile function recovery.

To investigate the role of intrinsic AMPK activation on post-ischemic recovery, the enzyme was pharmacologically inhibited by the well-studied inhibitor compound C, which was added to the bathing medium at the onset of simulated ischemia and maintained throughout the experiment. When AMPK was inhibited in the absence of palmitate, a decrease in the recovery of contractile reserve at the end of the reperfusion period was observed. These results were accompanied by a lower tissue ATP content recovery, suggesting that intrinsic AMPK activation contributes to myocardial cellular energy production and contractile function recovery when glucose is provided as sole exogenous substrate. To this regard, the beneficial role of AMPK activation observed in this study is consistent with the results obtained by other authors, in various ischemic-reperfused experimental models in the absence of high levels of fatty acids [11, 58, 66, 87]. The major previous study that correlated AMPK activity to reperfusion contractile function was carried out by Russel et al. The study demonstrated that hearts from transgenic mice expressing a kinase dead mutation of the α_2 catalytic subunit of AMPK, perfused with glucose and physiological concentration of oleate, showed a reduced recovery of contractile function, which was associated with the inability to increase glucose uptake and utilization [66]. The fact that compound C led to similar contractile recovery during reperfusion when glucose was present as only exogenous substrate suggests that in the present experimental conditions, atria are able to cope with myocardial energy requirements despite decreased of ATP content. However, when myocardial energy demand rises in the presence of ISO, reduced ATP content recovery in the presence of compound C is likely to contribute to the depressed inotropic response.

In the present study, the atria subjected to Is-Rs in the presence of glucose as sole exogenous substrate showed that

PDH activity was enhanced when AMPK was activated, but it was reduced in the presence of AMPK inhibitor. These results suggest that an increase in mitochondrial oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 could be taking place when AMPK is activated. Since AMPK increases cardiac glucose utilization, by the promotion of glucose uptake and glycolysis, pyruvate production might be enhanced. In this regard, it has been demonstrated that PDH activity is regulated by pyruvate [59]. High levels of pyruvate increase PDH activity, due in part to an inhibition of PDK, which as part of the PDH complex phosphorylates and inactivates PDH [59, 64]. These conditions might contribute to increase pyruvate oxidation, resulting in higher ATP content restoration during reperfusion and therefore, contributing to contractile reserve recovery. Supporting these results, Bunger et al. reported that reperfusion of previously ischemic hearts with high concentrations of pyruvate improves the recovery of mechanical function, which was related to an increased oxidative flux because of PDH activation [9]. On the other hand, when glucose was used as sole exogenous substrate, oxfenicine reduced contractile reserve showing similar results to that observed in the experiments carried out in the presence of compound C, suggesting that the beneficial effects of endogenous AMPK activation may also be, at least in part, due to enhancement of endogenous myocardial fatty acid oxidation. In the present experimental conditions, oxidation of endogenous fatty acid may not have the detrimental effects of high exogenous levels of fatty acids, contributing to mitochondrial energy production. To this regard, it has been suggested that intracellular fatty acids and triacylglycerol are important energy sources during reperfusion [18]. In the absence of exogenous fatty acids, it has been demonstrated that intracellular triacylglycerol oxidation increases during reperfusion, being this associated with improved functional recovery, suggesting that fatty acids derived from endogenous triacylglycerol do not contribute to reperfusion injury, as has been suggested for exogenous fatty acids [67].

Paradoxically, when atria were incubated with 1.2 mM palmitate, the inhibition of AMPK by compound C reverted the harmful effects exerted by this fatty acid, suggesting that intrinsic AMPK activation might be harmful for post-ischemic recovery when glucose and high levels of fatty acids are available as energy substrate. Supporting this, the limitation of palmitate oxidation through the addition of the pharmacological inhibitor of carnitine palmitoyl transferase-I to the bathing medium showed that contractile reserve and tissue ATP content recovery at the end of reperfusion improved, reaching similar values to those observed in the presence of compound C. These results support the fact that harmful effects of AMPK during reperfusion when glucose and high levels of palmitate are available as exogenous substrates, may be due to enhancement of fatty acid oxidation rate. To this respect, during reperfusion, AMPK is thought to increase

not only glucose utilization but also fatty acid oxidation. It has been proposed that AMPK regulates acetyl-CoA carboxylase activity by phosphorylation, which inhibits the enzyme and thus decreases the production of malonyl-CoA, an inhibitor of carnitine palmitoyl transferase-I [80, 81]. In the presence of increased fatty acid oxidation rates during reperfusion, several studies have shown a reduction in glucose oxidation, at least in part, by the inhibition of the PDH complex due to an activation of PDK by increased intramitochondrial acetyl-CoA and NADH/NAD⁺ ratios, being this metabolic condition associated with poor functional recovery and a decrease in cardiac efficiency [40, 44, 45]. In this study, we demonstrated that AMPK inhibition reverted partially the effects of high levels of palmitate over PDH activity, suggesting that increased oxidation of fatty acids might be involved, at least in part, in the decreased PDH activity. Moreover, as glucose uptake and glycolysis are limited when endogenous AMPK activation is inhibited, the enhancement of PDH activity developed in the presence of glucose as sole exogenous substrate is only partially observed. The significance of these results is that in most clinically relevant conditions of reperfusion after ischemia, a high level of fatty acid is immediately detected. In this situation, intrinsic AMPK activation increases glucose uptake and glycolysis as well as fatty acid oxidation, but this condition might uncouple glycolysis to glucose oxidation. This situation leads to a reduction in cellular energetic production and functional recovery of myocardium subjected to ischemia–reperfusion, being AMPK activation detrimental for myocardium recovery after an ischemic period, when high levels of fatty acids are present as exogenous substrates. As AMPK effects are less well studied when high levels of fatty acids are provided as exogenous substrates, more studies are required to characterize the role of the enzyme in the ischemic-reperfused myocardium.

Limitation of the study

Pharmacologic inhibitors are useful reagents to probe the function of protein kinases in both cells and intact animals. Unfortunately, like many kinase inhibitors, several studies have reported that the widely used AMPK inhibitor compound C, may also inhibit other protein kinases, such as extracellular signal-regulated kinase 8 (ERK8), Src kinase and mitogen-activated protein kinase-interacting serine/threonine kinase 1 (MNK1). To this respect, although several observations has suggested a potential role for ERK8 in the regulation of DNA damage [34], its activation in ischemic-reperfused myocardium has not been reported. On the other hand, Src family of tyrosine kinase have been shown to mediate cellular responses to stress. However, the effect of myocardial ischemia on Src tyrosine kinases is unknown. It has been reported that Src tyrosine kinase inhibition might block the cardioprotection provided by ischemic preconditioning. To

this respect, it has been suggested that Src might play an important role in the genesis of late preconditioning by serving as downstream elements of protein kinase C (PKC)-mediated signal transduction. Considerable evidence indicates that activation of PKC plays an important role in the development of ischemic preconditioning and that ξ , the isoform of PKC is specifically involved as an upstream signaling element. Nevertheless, the downstream effectors of the signaling transduction pathway in which PKC operates remain poorly defined [3, 60]. Finally, it has been shown that MNK1 is the only isoform that can be activated by the p38-MAPK pathway in vivo. MNK1 has low basal activity in cells and is responsive to both stress and mitogen-stimulated pathways. MNK1 was found to be recruited to the eIF4F complex, the cap-initiation complex that functions to bridge mRNA, the ribosome and the initiation translation machinery [20], making it a likely candidate kinase that mediates eIF4E phosphorylation. Although the biological significance of eIF4E phosphorylation is not completely understood [13, 51, 77], a slight decrease in eIF4E phosphorylation has been reported during global cerebral ischemia.

Although involvement of other mechanisms in the effects observed in the presence of compound C may not be discarded, various reports suggest that in specific circumstances compound C inhibits AMPK with expected results [78].

Conclusion

In conclusion, these results demonstrate that AMPK has a critical role in mediating the metabolic and functional responses of the heart to simulated ischemia and reperfusion. Taken together, these results support that intrinsic activation of AMPK has cardioprotective effects in the reperfused atria when glucose is the only available energetic substrate, but it results deleterious when palmitate is also available. Consequently, it appears that the role of AMPK during ischemia and reperfusion is dependent on the substrate availability and on the balance of the effects of AMPK on glucose and fatty acid metabolism.

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