Effects of 3-methyladenine on isolated left atria subjected to simulated ischaemia–reperfusion

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SUMMARY

Although autophagy is a prominent feature of myocardial ischaemia and reperfusion, its functional significance is unclear and controversial. In order to gain a deeper insight into the role of autophagy in myocardial ischaemia–reperfusion, we explored the effects of the pharmacological inhibitor of autophagy 3-methyladenine (3-MA). Isolated rat atria subjected to simulated 75-min ischaemia/75-min reperfusion (Is-Rs) in the presence or absence of 3-MA were used. The LC3-II/LC3-I ratio, an indicator of autophagosome formation, did not increase after ischaemia either in the presence or absence of 3-MA, but there was significant enhancement during reperfusion, which was prevented by the presence of 3-MA. The autophagy inhibitor also increased p62 protein, one of the specific substrates degraded through the autophagy-lysosomal pathway. Electron micrographs showed double membrane autophagosome-like structures during reperfusion, which were absent in atria subjected to Is-Rs in the presence of 3-MA. These findings suggest that this agent inhibited the autophagic flux under the present experimental conditions. Inhibition of autophagy during Is-Rs was accompanied by a high incidence of tachyarrhythmias during reperfusion, and a decrease in the maximal inotropic response to β-adrenergic and to calcium stimulation at the end of Is-Rs. Deterioration of mitochondrial morphology and function, without affecting cell viability, was observed in atria subjected to Is-Rs in the presence of 3-MA. The present results suggest an association between the inhibition of autophagy and functional alterations of the cells that have undergone sublethal stress, and have been able to recover in this experimental model of ischaemia–reperfusion.

Key words: 3-methyladenine, atria, autophagy, simulated ischaemia–reperfusion.

INTRODUCTION

Macrouautophagy, hereinafter referred to as autophagy, is a cellular catabolic process that occurs constitutively at a low rate in all eukaryotic cells, mainly for house-keeping purposes, such as degradation of protein aggregates too large to be removed by the ubiquitin-proteosomal system and removal of damaged organelles. During autophagy, polyubiquitinated protein aggregates or organelles, such as mitochondria, endoplasmic reticulum and other cytoplasmic constituents, are engulfed into specialized double-membrane structures, the autophagosomes, which then fuse with lysosomes forming the autolysosomes to degrade their cargoes, allowing cells to eliminate damaged or harmful components through catabolism and recycling to regenerate nutrients.1,2 In cardiomyocytes, autophagy occurs at basal levels, and can be further induced by different forms of stress, including ischaemia–reperfusion.3 In particular, myocardial ischaemia-induced autophagy is largely a result of phosphorylation of adenosine monophosphate-activated protein kinase and subsequent inhibition of mammalian target of rapamycin mTOR (mTOR), a powerful negative regulator of autophagy.4 Inhibition of mTOR, in concert with other protein partners, provides the critical step in initiating autophagosome formation. Furthermore, autophagy is induced in the reperfusion phase through a Beclin 1-dependent mechanism.4 Although autophagy is a prominent feature of myocardial ischaemia and reperfusion, its role in the heart is controversial, with findings suggesting both a detrimental role5,6 and a clear protective role.7–9 It has also been reported that ischaemic preconditioning, in which brief periods of ischaemia dramatically protect the heart against damage by subsequent prolonged ischaemia, is associated with increased autophagosome formation in both the in vivo mouse heart and the ex vivo rat heart. Furthermore, the inhibition of autophagy attenuates the protective effects of ischaemic preconditioning.10 In contrast, Dosenko et al. showed that postconditioning, in which brief episodes of ischaemia just at the onset of reperfusion result in highly significant cardioprotection, prevents autophagy in cardiomyocytes subjected to anoxia-reoxygenation.11 Other studies suggest that autophagy could play differing roles during ischaemia...
and reperfusion: autophagy might be beneficial during ischaemia, but harmful during reperfusion.\(^4\) Furthermore, Wagner et al. found that both wortmannin and rapamycin – with opposite effects on the regulation of autophagy – completely blocked the infarct size reduction of postconditioning in open-chest rats subjected to regional myocardial ischaemia–reperfusion.\(^1\)

On the basis of these observations, and in order to gain a deeper insight into the role of autophagy in myocardial ischaemia–reperfusion, we explored the effects of the pharmacological inhibitor of autophagy 3-methyladenine (3-MA) on isolated rat atria subjected to simulated ischaemia–reperfusion (Is-Rs) given that the consequences of ischaemia and reperfusion are less well established at the atrial level. Furthermore, ischaemia has been suggested to contribute to atrial arrhythmias providing the trigger for arrhythmia induction, and coronary artery disease is a frequent risk factor for atrial fibrillation, the most common arrhythmia in clinical practice, contributing to the arrhythmogenic substrate that helps to maintain the arrhythmia. In this regard, the present study provides new insights into the effects of ischaemia–reperfusion on atrial function that might help to better understand the pathophysiology of atrial fibrillation.

The present results show that inhibition of autophagy during Is-Rs was accompanied by a high incidence of tachyarrhythmias during reperfusion, a decrease in the maximal inotropic response to \(\beta\)-adrenergic and to calcium stimulation at the end of the ischaemic–reperfusion period, and a deterioration of mitochondrial morphology and function.

**RESULTS**

**Western blotting**

During autophagy, the cytosolic form of LC3 (LC3-I) is cleaved and lipidated to form the phosphatidylethanolamine-conjugated form (LC3-II) that is recruited to the autophagosomal membrane.\(^1\) Therefore, increases in this autophagy protein marker can reflect induction of autophagy.\(^14\) In contrast, p62 serves as a link between LC3-II and ubiquitinated substrates, it becomes incorporated into the completed autophagosome and is degraded in autolysosomes, with a general correlation between inhibition of the autophagic flux and increased levels of this protein.\(^1\) It should be noted that induced autophagy leads to increased LC3-II/LC3-I ratio together with decreased levels of p62, while concomitant increases in the LC3-II/LC3-I ratio and p62 reflect inhibition of the autophagosome-lysosome fusion step.\(^1\)

Western blotting analysis showed that Is did not affect the expression of LC3-II either in the presence or absence of 3-MA (Fig. 1a). In contrast, a significant increase in the LC3-II/LC3-I ratio occurred during Rs, which was prevented by the presence of 3-MA (Fig. 1a). Figure 1b shows that the increase in the LC3-II/LC3-I ratio was accompanied by a decrease in p62 signal during Rs, whereas an increase in this protein was observed in the presence of 3-MA, suggesting that the autophagic flux was inhibited by this agent in atria subjected to Is-Rs.

**Electron micrographs**

Electron micrographs obtained after the 60-min stabilization period (Fig. 2a) showed myofibrils tightly placed alongside each other, sarcomeres arranged in a regular pattern surrounded by normal mitochondria with tightly packed cristae, and the absence of wide interstitial space and vacuoles. Ultrastructural analysis of the left atrial myocardium subjected to Is-Rs in the absence of 3-MA (Fig. 2c,e,g) showed mitochondria with disruption and separation of peaks, and clearance of the mitochondrial matrix. Activation of a vacuolar process, and double membrane autophagosome- and autolysosome-like structures were also detected during reperfusion. These vacuoles frequently contained mitochondrial remnants. When electron micrographs were obtained from atria subjected to Is-Rs in the presence of 3-MA (Fig. 2d,f), ultrastructural analysis showed structural disorganization, myofibril disruption, cytosolic oedema, and more severe damaged mitochondria characterized by swelling, loss of cristae and matrix derangement. This was accompanied by absence of a vacuolar process.

**Mitochondrial ATP synthesis and tissue ATP content**

To assess whether the deleterious effects of 3-MA on mitochondrial morphology were accompanied by changes in mitochondrial ATP synthesis capacity, the rate of ATP synthesis by isolated mitochondria at the end of Is-Rs was determined.

Figure 3a shows that simulated ischaemia did not affect mitochondrial ATP synthesis capacity either in the presence or
absence of 3-MA. When atria were exposed to Is-Rs, isolated mitochondria showed a higher rate of ATP synthesis than mitochondria from atria subjected to Is-Rs in the presence of 3-MA.

Changes in ATP content of isolated atria subjected to Is-Rs are shown in Fig. 3b. The ATP level markedly decreased at the end of ischaemia, reaching similar values in 3-MA-treated and non-treated atria; whereas at the end of reperfusion, ATP of 3-MA-treated and non-treated atria recovered to 32% and 51%, respectively.

**Functional parameters**

As shown in Fig. 4, an abrupt decrease in the systolic parameters was observed in the first 10 min of Is. After this early period of Is, the decrease in these parameters occurred gradually, and was not affected by the autophagy inhibitor, showing no significant differences between treated and non-treated ischaemic-reperfused atria. During the 75 min of Rs, contractile parameters gradually recovered and were not affected by 3-MA.

Figure 5 shows that a slightly more pronounced development of contracture was observed during Is, which decreased gradually during subsequent Rs when the experiments were carried out in the presence of 3-MA. Glycolysis is an important source of ATP during ischaemia, and ischaemic contracture is thought to be associated with a decline in glycolytic ATP leading to calcium-independent rigor complex formation. As it has been reported that 3-MA inhibits glycolysis in isolated rat hepatocytes, lactate production during Is was assessed. The present results show that lactate lies beyond the scope of the present investigation.

As expected for normal myocardium, atria subjected to the 60-min aerobic stabilization period was considered 100% viable cells. Cell viability was not affected by either 210 min of aerobic conditions (210 min aerobic 95.7 ± 2.6%) or Is in the presence or absence of 3-MA (Is-Rs 99.0 ± 6.1 vs Is-Rs + 3-MA 101.2 ± 5.3). However, after 75-min reperfusion, the percentage of viable myocytes decreased in both the presence and absence of 3-MA (Is-Rs 76.3 ± 2.2 vs Is-Rs + 3-MA 84.3 ± 4.6, P < 0.05 vs 60-min aerobic stabilization period). Neither ISO nor calcium had any effect on cell viability (data not shown).

**Cellular viability**

The percentage of viable myocytes was assessed by MTT assay. Atria subjected to the 60-min aerobic stabilization period was considered 100% viable cells. Cell viability was not affected by either 210 min of aerobic conditions (210 min aerobic 95.7 ± 2.6%) or Is in the presence or absence of 3-MA (Is-Rs 99.0 ± 6.1 vs Is-Rs + 3-MA 101.2 ± 5.3). However, after 75-min reperfusion, the percentage of viable myocytes decreased in both the presence and absence of 3-MA (Is-Rs 76.3 ± 2.2 vs Is-Rs + 3-MA 84.3 ± 4.6, P < 0.05 vs 60-min aerobic stabilization period). Neither ISO nor calcium had any effect on cell viability (data not shown).

**DISCUSSION**

The present study investigated the role of autophagy in the response to ischaemia and reperfusion in isolated rat atria. Many studies trying to elucidate the role of autophagy in the heart subjected to ischaemia–reperfusion are being carried out employing isolated cardiomyocytes, with the murine atrial cardiomyocyte-derived (HL-1) cell line being one of the most widely used. Despite the parallel between the behaviour of isolated myocytes and intact myocardium, whether the role of autophagy 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. The use of isolated atria in the present study provides new insights that might help to improve the understanding of the role played by autophagy in the response to ischaemia and reperfusion in a more physiologically employable model, while also providing a deeper understanding into the effects of ischaemia–reperfusion on atrial function, which are less well established.

The present data show that ischaemia did not increase the LC3-II/LC3-I ratio, which is an established indicator of
autophagy, whereas a significant increase in the LC3-II/LC3-I ratio was observed during reperfusion, and this was accompanied by a decrease in the level of the ubiquitin- and LC3-binding protein p62, one of the specific substrates that are degraded through the autophagy-lysosomal pathway. These results suggest that autophagy does not increase during ischaemia and is upregulated during reperfusion. Consistent with the present results, Hamacher-Brady et al. showed that the autophagic flux was null during the ischaemic period and increased at reperfusion in the cardiac HL-1 cells subjected to simulated ischaemia-reperfusion.\(^4\) As the formation of the autophagosome involves several steps that are energy-dependent, it is likely that energy constraints of ischaemia preclude autophagy.\(^22\) However, some studies have described increased autophagy in hearts subjected to ischaemia.\(^4\) The discordance in results might reflect variations in the duration and severity of the ischaemia implemented. The present data also show that in the presence of 3-MA, which is known to inhibit autophagosome formation, an increase of the LC3-II/LC3-I ratio was not observed at reperfusion. Furthermore, administration of 3-MA led to increased levels of p62. These findings suggest that reperfusion-induced autophagy in isolated atria is prevented by 3-MA. Ultrastructural analysis of left atrial myocardium revealed unaltered mitochondrial structure at the end of the 210 min of aerobic conditions. However, myocardium subjected to Is-Rs showed damaged mitochondria characterized by swelling, loss of cristae, and matrix derangement and autophagic vacuole-like structures. These vacuoles frequently contained mitochondrial remnants. 3-MA intensified mitochondrial damage and no autophagosome-like structures were observed in the presence of this inhibitor. It is generally accepted that reactive oxygen species (ROS) induce autophagy,\(^23\) and that autophagy in turn serves to reduce oxidative damage.\(^24\) As mitochondria are the major source of ROS, impairment of autophagy could lead to accumulation of damaged and dysfunctional mitochondria, corresponding to an increase in intracellular ROS levels, with a further increase occurring at reperfusion. Consistent with the ultrastructural findings, mitochondria isolated at the end of Is-Rs in the presence of 3-MA exhibited lower ATP synthesis capacity than those isolated at the end of Is-Rs in absence of the inhibitor. Furthermore, the reduction of ATP synthesis was associated with a significantly lower ATP concentration in isolated atria at the end of reperfusion. The fact that reperfusion led to similar contractile recovery and cell viability preservation in both groups of atria suggests that, in the presence of 3-MA, atria are able to cope with myocardial energy requirements despite increased deterioration of mitochondrial function. Both necrosis and apoptosis could be involved in cellular death after the Is-Rs protocol. In this regard, Vila-Petroff et al. found that the decreased viability of isolated cardiomyocytes was due, in part, to apoptotic cell death after 45-min simulated ischaemia/60-min reoxygenation.\(^25\) The mitochondrion is the primary organelle involved in mediating the intrinsic apoptotic pathway, and damaged mitochondria can be dangerous to the cell by releasing pro-apoptotic factors, such as cytochrome c. In contrast, it has been shown that inhibition of autophagy results in promotion of apoptosis.\(^26\) However, cell death did not increase with treatment with 5 mmol/L 3-MA, and therefore the present study focused on assessing the functionality of still viable cells at the end of Is-Rs in situations of increased energy.

![Fig. 2](image-url)  
**Fig. 2** Electron micrographs. (a) Representative electron micrographs obtained from left atria subjected to the 60-min stabilization period show sarcomeres arranged in a regular pattern surrounded by normal mitochondria with tightly packed cristae, myofibrils tightly placed alongside each other and the absence of wide interstitial space and vacuoles. (b) Electron micrographs obtained after 210 min of aerobic conditions show preservation of ultrastructural arrangement. (c-e,g) Electron micrographs obtained after simulated ischaemia-reperfusion (Is-Rs). Ultrastructural analysis of left atrial myocardium subjected to Is-Rs in absence of 5 mmol/L 3-methyladenine (3-MA) shows mitochondria with disruption and separation of peaks, and clearance of the mitochondrial matrix (white arrows) accompanied by activation of a vacuolar process; double membrane autophagosome- and autolysosome-like structures were detected during reperfusion. Black arrows show autophagosome-like structures. Double arrow shows autolysosome-like structures. Head arrow shows an autophagosome with a mitochondrion inside. (d-f) Representative electron micrographs obtained after simulated ischaemia-reperfusion in the presence of 3-MA. Ultrastructural analysis shows structural disorganization with more severely damaged mitochondria characterized by swelling, loss of cristae, and matrix derangement (white arrows), cytosolic edema, myofibrils disruption and absence of vacuolar process.

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The ability of the surviving myocardium to increase its level of contractile function during inotropic stimulation with ISO and the maximal calcium-activated force were decreased vis-à-vis what was observed in the aerobic pre-ischaemic myocardium, and was further diminished when atria were subjected to Is-Rs in the presence of 3-MA. As healthy mitochondria are essential for myocardial function, persistence of a larger amount of dysfunctional mitochondria is likely to contribute to the depressed inotropic response when energy demand rises in the presence of ISO or high calcium concentrations. However, cellular injury at the level of the contractile filaments cannot be excluded.

These findings were accompanied by induction of severe tachyarrhythmias during the reperfusion period in the presence of 3-MA, which were partially prevented by the mBzR ligand PK11195, which, consistently with the lack of effects on the recovery of the tissue ATP level and the mitochondrial ATP synthesis capacity, lacked inotropic effects. In this regard, the mitochondrial inner membrane anion channel has been shown to be activated under moderate oxidative stress and to be responsible for fast mitochondrial membrane depolarization.27

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Fig. 6  Effects of 5 mmol/L 3-methyladenine (3-MA) on the maximal inotropic response to a β-adrenergic agent in isolated left atria subjected to simulated ischaemia–reperfusion. (a) Peak developed force (F), (b) force-time index (FTI), (c) peak rate of contraction (+dF/dt) and (d) peak rate of relaxation (–dF/dt). All values (mean ± SEM, n = 8) were recorded 30 min after addition of 2 μmol/L isoproterenol (ISO) and are expressed as a percentage of the respective basal value at the end of the 60-min stabilization period. End stabilization period: ISO was added to the incubation medium at the end of the 60-min stabilization period. 210 min aerobic: ISO was added to the incubation medium at the end of 210-min incubation in aerobic conditions. Is-Rs: ISO was added at the end of the 75-min simulated ischaemia/75-min reperfusion period. Is-Rs + 3-MA: ISO was added at the end of the 75-min simulated ischaemia/75-minute reperfusion period in the presence of 3-MA. **P < 0.01 versus end stabilization period, 210 min aerobic; *P < 0.05 versus Is-Rs; *P < 0.05 versus end stabilization period, 210 min aerobic.
membrane depolarization through uncoupled mitochondrial ATP consumption and opening of sarcocellular ATP-sensitive potassium channels might create spatial and temporal action potential heterogeneity that can be a substrate for arrhythmias on reperfusion. A confirmatory role for inner membrane anion channel in arrhythmias has been provided by studies showing that the mBzR ligands PK11195 and 4'-chlorodiazepam, prevent mitochondrial depolarization, stabilize the action potential of stressed cardiomyocytes and prevent occurrence of spontaneous arrhythmias on reperfusion in the rabbit heart. Taken together, these findings suggest involvement of mitochondria in the 3-MA-induced reperfusion arrhythmias in isolated atria, providing new insights into the effects of ischaemia–reperfusion on atrial function that might help to better understand the pathophysiology of arrhythmias.

Fig. 7 Effects of 5 mmol/L 3-methyladenine (3-MA) on calcium response of isolated left atria subjected to simulated ischaemia-reperfusion. (▲) Increasing concentrations of calcium were added to the incubation medium at the end of the 60-min stabilization period; (●) increasing concentrations of calcium were added to the incubation medium at the end of 210 min of aerobic conditions; (■) increasing concentrations of calcium were added at the end of 75-minute simulated ischemia/75-minute reperfusion (Is-Rs); (○) increasing concentrations of calcium were added at the end of Is-Rs in the presence of 3-MA. Values (mean ± SEM, n = 6) are expressed as a percentage of the respective basal value at the end of the 60 min aerobic stabilization period. **P < 0.01 versus 60 min stabilization period, 210 min aerobic conditions; *P < 0.05 versus Is-Rs; **P < 0.05 versus 60 min stabilization period, 210 min aerobic conditions.

Fig. 8 Representative mechanically recorded contractions and tachyarhythmias. (a) Recordings of left atria subjected to simulated ischaemia-reperfusion in absence of 3-methyladenine (3-MA). Regular morphology and rate contractions (60 contractions/min) can be observed. (b) Recordings of representative tachyarrhythmias developed in left atria subjected to simulated ischaemia-reperfusion in the presence of 5 mmol/L 3-MA. Sustained tachyarrhythmia of regular morphology and rate contractions can be observed, with frequencies ranging between 270 and 300 contractions/min.

Table 1 Incidence of tachyarrhythmias during reperfusion in isolated atria subjected to simulated ischaemia-reperfusion

| Experiments (n) | Atria showing tachyarrhythmia |
|----------------|-------------------------------|
| Is-Rs          | 11                            | 0 | 0 |
| Is-Rs + 3-MA   | 15                            | 7 | 47** |
| Is-Rs + 3-MA + PK11195 | 7          | 2 | 29*** |

Results are expressed as the percentage of atria that showed tachyarrhythmia during the reperfusion period. *P < 0.05 versus left atria subjected to 75 min simulated ischaemia and 75 min reperfusion (Is-Rs + 3-MA), **P < 0.01 versus left atria subjected to 75 min simulated ischaemia and 75 min reperfusion (Is-Rs). PK11195 was added at the onset of reperfusion until the end of the experiment. Is-Rs + 3-MA + PK11195, left atria subjected to 75 min simulated ischaemia and 75 min reperfusion in the presence of 3-MA.

As in addition to its action as an inhibitor of autophagy 3-MA also exerts other effects on cellular functions, it is not possible to posit that the detrimental effects of this agent on the ischaemic reperfused atria are caused solely by the inhibition of autophagy. 3-MA inhibits all phosphatidylinositol-3 kinases (PI3K) when used at mmol/L concentrations. Class I and class III PI3K play integral and reportedly opposing roles in the autophagic process: class III PI3K forms a complex together with Beclin 1 and other proteins to facilitate assembly and elongation of the autophagosomal membrane, whereas Class I PI3-kinase inhibits the induction of autophagy through the PI3K–Akt–mTOR signalling pathway. In this regard Wu et al. found that 5 mmol/L 3-MA has a dual role in modulation of autophagy. Consistent with the present results, these authors found that 3-MA was capable of suppressing autophagy under nutrient-rich conditions for a relatively short period of time (4 h), but prolonged treatment (up to 9 h) under nutrient-rich conditions induced autophagy. They also concluded that induction of autophagy by 3-MA was a result of its persistent inhibition on class I PI3K, whereas class III PI3K suppression was transient, suggesting that great caution needs to be exercised when 3-MA is used over extended periods of treatment, in contrast with the present conditions. Furthermore, PI3K–Akt–mTOR are also components of the reperfusion injury salvage kinase or the RISK pathway, which, when activated, has been shown to attenuate ischaemia–reperfusion injury. Whether the noxious effects of 3-MA treatment are associated with both inhibition of class III PI3K and the Akt signalling pathway remain to be tested in further studies. However, the ability of 3-MA to suppress the formation of autophagosomes visible by electron microscopy – undoubtedly the gold standard for the detection and assessment of autophagy –, to decrease the expression of the autophagy-related protein LC3-II and to increase p62, accompanied by post-ischaemic myocardial dysfunction and a greater deterioration of mitochondrial structure and function without affecting cell viability, suggests an association between the inhibition of autophagy and functional alterations of the cells that have undergone sublethal stress and been able to recover in this experimental model of moderate ischaemia.
METHODS
Experimental protocol
The present study conformed to the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication No. 85-23, revised 1996; http://oacu.od.nih.gov/regs/guide/guide_2011.pdf) and Argentine Law No. 14346 regarding 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 the study. Rats were anesthetized with carbon dioxide, and 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 (mmol/L): NaCl 120; NaHCO3 25; KCl 4.8; MgSO4 1.33; KH2PO4 1.2; CaCl2 1.6; Na2EDTA 0.02; D-glucose 10, pH 7.4, continuously bubbled with 95% O2–5% CO2. The temperature was kept at 31.5°C. After a 60-min stabilization period, atria were subjected to 75-min Is followed by 75-min Rs.

The duration of ischaemia resulted in ~40% functional recovery and ~20% cell death, allowing demonstration of both beneficial and harmful effects in response to different interventions. For the induction of Is, the medium was bubbled with 95% N2–5% CO2 (pH 6.80–7.00), and D-glucose was replaced with 10 mmol/L 2-deoxy-D-glucose (Sigma, St. Louis, MO, USA). During Is, the pO2 of the bathing medium, measured polarimetrically in samples retrieved from the organ bath, was 67 ± 5 mm Hg. 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 31.5°C.

Then, 5 mmol/L 3-MA (Sigma) was added to the bathing medium at the onset of Is and maintained throughout the experiment. 3-MA is an inhibitor of class III phosphatidylinositol 3-kinase, which is involved in the formation of autophagosomes and initiation of autophagy. As this inhibitor is 3-kinase, which is involved in the formation of autophagosome. 3-MA is an inhibitor of class III phosphatidylinositol 3-kinase, which is involved in the formation of autophagosomes and initiation of autophagy.

In a subset of experiments, 50 μmol/L PK11195 (Sigma), a peripheral benzodiazepine receptor ligand, was added to the bathing medium at the onset of Rs and maintained until the end of 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).

Western blotting
Atria were homogenized in lysis buffer (18.2 mmol/L HEPES, 1 mmol/L EDTA, 0.28 mol/L sucrose, 2 mmol/L dithiothreitol, pH 7.4, 2 mmol/L phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail from Thermo Scientific, Rockford, IL, USA), an aliquot was saved to measure proteins by the method of Bradford et al., and the remaining sample was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Equal amounts of protein were mixed with LAEMMLI sample buffer (BioRad, Hercules, CA, USA), boiled for 5 min and then resolved on 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis 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. Membranes were then washed with Tris-buffered saline containing 0.1% Tween 20 and incubated at 4°C overnight with polyclonal rabbit anti-LC3B antibody (Thermo Scientific) at 1:3000 dilution, polyclonal rabbit anti-p62 antibody (Thermo Scientific) at 1:1500 dilution, and polyclonal rabbit anti-β-actin antibody (Thermo Scientific) at 1:2000 dilution. Horseradish peroxidase conjugated donkey anti-rabbit antibody (Thermo Scientific) at 1:2500 dilution was then incubated for 1 h at room temperature. After Tris-buffered saline containing 0.1% Tween 20 washes, bands were detected with Bio-Lumina detection reagent (Kalium Technologies, Buenos Aires, Argentina) and exposed to an autoradiography film (Roche Life Science, Mannheim, Germany). The film was scanned for band quantification using a Hewlett-Packard scanner and the intensity of the immunoblot bands was analysed by densitometry using Image J software (NIH, Bethesda, MD, USA) and normalized to β-actin. Results were expressed in arbitrary units.

Electron micrographs
Atria (~1-mm thick) were fixed for 4 h with 2.5% glutaraldehyde in phosphate-buffer (pH 7.4) and then postfixed for 1 h in 1% osmium tetroxide. After this, staining en bloc with 2% uranyl acetate, dehydration in a graded series of ethanol, and imbibition in Durcupan resin were carried out. Thin sections were prepared with a diamond knife and stained with lead citrate. Grids were examined under a Zeiss 109 electron microscope (Laboratorio Nacional de Investigación y Servicios en Microscopía Electrónica, University of Buenos Aires, Buenos Aires, Argentina).

Measurement of mitochondrial ATP synthesis
Mitochondria were isolated from simulated ischaemic reperfused atria by differential centrifugation after tissue homogenization in ice-cold sucrose buffer solution (300 mmol/L sucrose, 10 mmol/L Tris-HCl, 2 mmol/L EGTA, 5 mg/mL bovine serum albumin (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 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. As it is well documented that complex I
of the respiratory chain is most sensitive to reperfusion injury, mitochondrial ATP synthesis was measured in the presence of the complex I substrates pyruvate and malate. Mitochondria (750 μg protein/mL) were incubated for 10 min in a medium containing (mmol/L): KCl 125, Mops 20, Tris 10, EGTA 0.5, KH$_2$PO$_4$ 2.4, MgCl$_2$ 2.5, malate 2.5, pyruvate 2.5 and pH 7.4, in a metabolic shaker at 30°C. ATP synthesis was then initiated by the addition of 2.5 mmol/L adenosine diphosphate, which corresponds to the physiological concentration found in myocytes. Aliquots were taken from the incubation mixture at 3-min intervals for 9 min, and the reaction was stopped with perchloric acid. The neutralized supernatant was assayed for ATP by luciferin-luciferase luminometry (Sigma chemiluminescent assay kit). Mitochondrial protein concentration was determined by the method of Lowry et al. using BSA as a standard. The rate of mitochondrial ATP synthesis was calculated and expressed as nmol/min/mg of mitochondrial protein.

**Tissue ATP content**

Samples (40 mg) were homogenized in 500 μL of 3% (mass/vol.) cold perchloric acid for determination of tissue ATP. After removal of the denatured protein by centrifugation at 12 000 g for 5 min, aliquots of supernatant were neutralized with a mixture of 2 mol/L KOH and 0.3 mol/L Mops. 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. The concentration of ATP was expressed as nanomoles of ATP per mg of tissue protein.

**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 ±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.

**Inotropic response to a β-adrenergic agent**

Contractile response to a β-adrenergic agent was evaluated at the end of the Is-Rs period by addition of 2 μmol/L isoproterenol (ISO; Sigma) to the incubation medium. This ISO dose, obtained from a dose–response curve previously carried out in left atria subjected to incubation for 210 min in normal aerobic medium, elicited the maximal inotropic response. F, FTI and ±dF/dt values were expressed as a percentage of the respective value obtained at the end of the 60-min stabilization period.

**Peak developed force in response to calcium**

Peak force generation was measured at a range of calcium concentrations (0–10.2 mmol/L) at the end of the Is-Rs period. Values were expressed as a percentage of the value obtained at the end of the 60-min stabilization period.

**Measurement of cellular viability: MTT Assay**

The reduction of 3-(4,5 dimethylthiazol-2-yl)-2.5 diphenyltetrazo- lium 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 MTT (1 mg/mL) for 90 min at 37°C. Atria were then 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 g for 10 min. The absorbance of the coloured supernatant was then measured spectrophotometrically at 520 nm, and optical density/mg wet weight of myocardial tissue was calculated. The results were expressed as a percentage of the pre-ischaemic value.

**Tissue lactate assay**

Atria treated following the aforementioned protocols were removed at the end of the 75-min simulated ischaemic period and immediately frozen between two blocks of ice at −21°C for determination of tissue lactate. Lactate was extracted from a 40-50-mg sample of frozen tissue, added to 6% ice-cold perchloric acid and measured enzymatically. The results were expressed as μmol of lactate per gram of dry weight.

**Statistical analysis**

All data are presented as mean ± 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 carried out using GraphPad Instat 4 (GraphPad Software, La Jolla, CA, USA). Incidences of arrhythmias were compared using the χ$^2$-test. The probability level of 0.05 or lower was used as a criterion for biological significance.

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REFERENCES

1. Abounit K, Scarabelli TM, McAuley RB. Autophagy in mammalian cells. *World J. Biol. Chem.* 2012; 3: 1–6.
2. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. *Mol. Cell* 2010; 40: 280–93.
3. Przyklenk K, Dong Y, Undyala VV, Whittaker P. Autophagy as a therapeutic target for ischaemia/reperfusion injury? Concepts, controversies, and challenges. *Cardiovasc. Res.* 2012; 94: 197–205.
4. Matsu T, Takagi H, Qu X et al. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ. Res.* 2007; 100: 914–22.
5. Osipov RM, Robich MP, Feng J et al. Effect of hydrogen sulfide in a porcine model of myocardial ischemia-reperfusion: comparison of different administration regimens and characterization of the cellular mechanisms of protection. *J. Cardiovasc. Pharmacol.* 2009; 54: 287–97.
6. Valentim L, Lawrence KM, Townsend PA et al. Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischaemia/reperfusion injury. *J. Mol. Cell. Biol.* 2006; 40: 846–52.
7. Gurusamy N, Lekli I, Gorbunov NV, Gherghiceanu M, Popescu M, Das DK. Cardioprotection by adaptation to ischaemia augments autophagy in association with BAG-1 protein. *J. Cell. Mol. Med.* 2009; 13: 373–87.
8. Yan L, Sadoshima J, Vatner DE, Vatner SF. Autophagy in ischemic preconditioning and hibernating myocardium. *Autophagy* 2009; 5: 709–12.
9. Yitzhaki S, Huang C, Liu W et al. Autophagy is required for preconditioning by the adenosine A1 receptor-selective agonist CCPA. *Basic Res. Cardiol.* 2009; 104: 157–67.
10. Huang C, Yitzhaki S, Perry CN et al. Autophagy induced by ischemic preconditioning is essential for cardioprotection. *J. Cardiovasc. Transl. Res.* 2010; 3: 365–73.
11. Dosenko VE, Nagibin VS, Tumanovskaya LV, Moibenko AA, Vajage J. Postconditioning prevents apoptotic necrotic and autophagic cardiomyocyte cell death in culture. *Fiziol. Zh.* 2005; 51: 12–7.
12. Wagner C, Tillack D, Simonis G, Strasser RH, Weinbrenner C. Ischemic post-conditioning reduces infarct size of the in vivo rat infarct: role of PI3-K, mTOR, GSK-3beta, and apoptosis. *Mol. Cell. Biochem.* 2010; 339: 135–47.
13. Mizushima N, Yamamoto A, Matsu M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *J. Biol. Chem.* 2004; 279: 10915–22.
14. Klionsky DJ, Cuervo AM, Seglen PO. Methods for monitoring autophagy from yeast to human. *Autophagy* 2007; 3: 181–206.
15. Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. *Biochem. Biophys. Res. Commun.* 2004; 313: 453–8.
16. Ichimura Y, Kumanomidou T, Sou Y et al. Protein synthesis, post-translational modification, and degradation: structural basis for sorting mechanism of p62 in selective autophagy. *J. Biol. Chem.* 2008; 283: 22847–57.
17. Bricknell OL, Daries OP, Opie LH. A relationship between adenine triphosphate, glycolysis and ischaemic contracture in the isolated rat heart. *J. Mol. Cell. Cardiol.* 1981; 13: 941–5. PMID: 7310897
18. Caro LHP, Ploomp PJAM, Wolvegtan EJ, Kerkhof C, Meijer AJ. 3-Methyladename, an inhibitor of autophagy, has multiple effects on metabolism. *Eur. J. Biochem.* 1988; 17: 325–9.
19. Chauvine AH, Hajjar RJ. AKT signalling in the failing heart. *Eur. J. Heart Fail.* 2011; 13: 825–9.
20. Brown DA, O’Rourke B. Cardiac mitochondria and arrhythmias. *Cardiovasc. Res.* 2010; 88: 241–9.
21. Hamacher-Brady A, Brady NR, Gottlieb RA. Cardiac myocytes against ischemia/reperfusion injury in enhancing macroautophagy protects. *J. Biol. Chem.* 2006; 281: 29776–87.
22. Ichimura Y, Kirisako T, Takao T et al. A ubiquitin-like system mediates protein lipidation. *Nature* 2000; 408: 488–92.
23. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem. Sci.* 2011; 36: 30–38.
24. Moore MN. Autophagy as a second level protective process in conferring resistance to environmentally-induced oxidative stress. *Autophagy* 2008; 4: 254–6.
25. Vila-Petroff M, Salas MA, Said M et al. CaMKII inhibition protects against necrosis and apoptosis in irreversible ischemia-reperfusion injury. *Cardiovasc. Res.* 2007; 73: 689–98.
26. Boya P, Gonzalez-Polo RA, Casares N et al. Inhibition of macro-autophagy triggers apoptosis. *Mol. Cell. Biol.* 2005; 25: 1025–40.
27. O’Rourke B. Mitochondrial ion channels. *Annu. Rev. Physiol.* 2007; 69: 19–49.
28. Aon MA, Cortassa S, Akar FG, Brown DA, Zhou L, O’Rourke B. From mitochondrial dynamics to arrhythmias. *Int. J. Biochem. Cell Biol.* 2009; 41: 1940–8.
29. Akar FG, Aon MA, Tomaselli GF, O’Rourke B. The mitochondrial origin of postischemic arrhythmias. *J. Clin. Invest.* 2005; 115: 3527–35.
30. Brown DA, Aon MA, Akar FG, Liu T, Sorarrain N, O’Rourke B. Effects of 4-chlorodiazepam on cellular excitation-contraction coupling and ischaemia-reperfusion injury in rabbit heart. *Cardiovasc. Res.* 2008; 79: 141–9.
31. Miller S, Tavshanjian B, Oleksy A et al. Shaping development of autophagy inhibitors with the structure of the lipid kinase Vps34. *Science* 2010; 327: 1638–42.
32. Wu Y-T, Tan H-L, Shui G et al. Dual role of 3-methyladename in modulation of autophagy by different temporal patterns of inhibition on Class I and III Phosphoinositide 3-Kinase. *J. Biol. Chem.* 2010; 285: 10850–61.
33. Klionsky DJ, Abeliovich H, Agostinis P et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 2008; 4: 151–75. PMCID: PMC2654259.
34. Petti A, Ogier-Denis E, Blommaart EF, Meijer AJ, Codogno P. Distinct classes of phosphatidylinositol 3-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J. Biol. Chem.* 2000; 275: 992–8.
35. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976; 72: 248–54.
36. Solem L, Wallace K. Selective activation of the sodium-independent, cyclosporin A sensitive calcium pore of cardiac mitochondria by doxorubicin. *Toxicol. Appl. Pharmacol.* 1993; 121: 150–7. PMID: 7687798.
37. Solani G, Harris DA. Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion. *Biochem. J.* 2005; 390: 377–94.
38. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951; 193: 265–75.
39. Blommaart EF, Krause U, Schellens JP, Vreeling-Sindel MA, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur. J. Biochem.* 1997; 243(1–2): 240–6.
40. Golomb E, Schneider A, Houminer E et al. Occult cardiotoxicity: subtoxic dosage of Bis (2-chloroethoxy) methane impairs cardiac response to simulated ischemic injury. *Toxicol. Pathol.* 2007; 35: 383–7. PMID: 17455086.
41. Shimoj N, Fujino K, Kitahashi S, Nakao M, Naka K, Okuda K. Lactate analyzer with continuous blood sampling for monitoring blood lactate during physical exercise. *Clin. Chem.* 1991; 37: 1978–80. PMID: 1934473.