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Effects of wortmannin on cardioprotection exerted by ischemic preconditioning in rat hearts subjected to ischemia-reperfusion

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Abstract Ischemic preconditioning (IPC) is one of the most powerful interventions to reduce ischemia-reperfusion injury. The aim of the present study was to investigate the involvement of the phosphatidylinositol-3-kinases (PI3Ks) family in cardioprotection exerted by IPC and the relationship between preservation of mitochondrial morphology and ATP synthesis capacity. In this regard, macroautophagy (autophagy) is considered a dynamic process involved in the replacement of aged or defective organelles under physiological conditions. IPC consisted of four 5-min cycles of ischemia-reperfusion followed by sustained ischemia. Wortmannin (W), a PI3K family inhibitor, was added to the perfusion medium to study the involvement of autophagy in the beneficial effects of IPC. In the present study, LC3-II/I expression was significantly increased in the IPC group when compared with the control group. The hearts subjected to IPC showed greater degradation of p62 than control groups, establishing the existence of an autophagic flow. Electron microscopy showed that IPC preserves the structural integrity of mitochondria after ischemia and at the end of reperfusion. Moreover, hearts subjected to IPC exhibited increased mitochondrial ATP synthesis. The beneficial effects of IPC were abolished by W in all trials of this study, abolishing the differences between the IPC and control groups. These results suggest that IPC could partly reduce injury by ischemia-reperfusion (I/R) by decreasing mitochondrial damage and promoting autophagy. Since W is a nonspecific inhibitor of the PI3Ks family, further research is required to confirm participation of PI3K in the response to IPC.

Keywords Preconditioning · Ischemia · Heart · Wortmannin · Autophagy · PI3K

Introduction

The interruption of blood flow, essential for the myocardium, is followed by profound functional, biochemical, and morphological consequences, and the myocardium has different endogenous mechanisms to ensure survival when faced with events of ischemia. Single or repetitive brief periods of ischemia followed by intermittent reperfusion render the heart more resistant to a subsequent sustained ischemic period. This is called ischemic preconditioning (IPC). In this regard, acute IPC described for the first time by Murry et al. (1986) has been confirmed in virtually every animal model and in several organ systems [25], although the final effector has not been elucidated yet [13, 21, 22, 29]. The cardioprotective effect of IPC seems to be biphasic, with an early phase occurring within minutes of the initial

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ischemic damage and lasting two to three hours, and a late phase becoming apparent 12 to 24 h later [27]. Despite intensive research into the development of adjuvant therapies to reperfusion interventions aimed at reducing infarct size, clinical trials using IPC mediators that have demonstrated beneficial effects in the laboratory have not yet yielded encouraging results [2, 8].

In this respect, macroautophagy (autophagy) is a dynamic process that under physiological conditions is involved in the replacement of aged or defective organelles and the degradation of potentially harmful molecules; this process begins with the engulfing of cytoplasmic material, forming the autophagosome, which finally fuses with the lysosome to form the autolysosome [7]. The vacuolar content is digested along with the inner membrane and is recycled to provide cells not only with amino acids, fatty acids, and energy but also to allow cells to get rid of damaged mitochondria (mitophagy). Autophagy can be activated by nutritional state in the absence of growth factors and also by a high bioenergetic demand, hypoxia, or cellular remodelling [20, 23]. A basal and necessary level of autophagy is displayed in cardiomyocytes and can be further induced by different forms of stress, including I/R [28]. However, given that publications are few and the results are controversial, the role of autophagy in situations of cardiac I/R remains unclear, with findings suggesting both a detrimental role and a clear protective one [11, 18]. For these reasons, elucidating the role played by autophagy in situations of I/R and its implication for endogenous protective mechanisms available in the myocardium was a fundamental goal of this study.

Phosphatidylinositol-3-kinase (PI3K) belongs to a family of enzymes that phosphorylates the 39-hydroxyl group on the inositol ring of phosphoinositides. Different studies indicate that PI3K is essential in the regulation of many membrane-flow-dependent processes [9, 17]. Wortmannin (W) is a potent inhibitor of mammalian PI3Ks that covalently binds to the enzyme, which is then irreversibly inhibited [1].

On the basis of these observations and in order to gain a deeper insight into the mechanisms involved in the protective effects of IPC, the effects of the pharmacological inhibitor W on isolated Langendorff perfused rat hearts subjected to I/R were explored.

Materials and methods

Experimental protocol

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and Argentine Law No. 14346 concerning animal protection. Fifty-eight female Wistar rats fed ad libitum, weighing 250–350 g and maintained on a 12-h dark/light cycle, were used. Rats were anesthetized with diethylether and heparin (250 IU) was injected into the jugular vein. The hearts were quickly excised and cooled in ice-cold saline until contractions stopped. The hearts were subsequently mounted on a modified Langendorff apparatus (Hugo Sachs Elektronik) and isovolumically perfused at a constant pressure of 70 mmHg with a non-recirculating Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 120, NaHCO3 25, KCl 4.8, $MgSO_4$ 1.33, KPO_4H_2 1.2, $CaCl_2$ 1.6, Na_2EDTA 0.02, and glucose 10. The perfusate was gassed with 95 % O_2/5 % CO_2 (pH 7.4) and kept at a constant temperature of 37 °C. After instrumentation, hearts were randomly assigned to one of the following groups ($n = 16$ /group): Control (C): after a 60-min equilibration period hearts were subjected to 25 min of global ischemia and 60 min of reperfusion (RP) (ischemia was started by shutting off the perfusate flow); ischemic preconditioning (IPC): four 5-min cycles of ischemia followed by 5 min of RP before sustained ischemia; C + W: wortmannin (100 nM, Sigma, St Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO) was added to the perfusion medium 45 min before sustained ischemia and maintained until the end of ischemia; IPC + W: wortmannin was added to the perfusion medium 5 min before application of IPC and maintained until the end of ischemia; DMSO: 0.01 % dimethyl sulfoxide was added to the perfusion medium 45 min before sustained ischemia and maintained until the end of ischemia.

Measurement of heart function

For measurement of left ventricular pressure, the left atrium was removed and a latex balloon connected to a pressure transducer was inserted into the left ventricle through the mitral valve. The volume of the balloon was adjusted to obtain left ventricular end-diastolic pressure (LVEDP) of 10 mmHg. Values for left ventricular

developed pressure (LVDP), peak rate of contraction (+dP/dt), and peak rate of relaxation (−dP/dt) were obtained using a digital data acquisition system (Unkel Scope Configuration Program for the PCLabCard Data Acquisition Boards from Advantec, USA; this program was adapted and modified by the technical assistant). Heart rate (HR) was measured with a counter triggered by the LVDP pulse. Rate-pressure product (RPP) was determined by multiplying HR by LVDP.

Only hearts with LVDP > 60 mmHg and HR > 200 bpm at the end of the equilibration period were included in the study.

Creatine kinase release

Spectrophotometric measurement of creatine kinase (CK) released (UI/g wet weight) in the coronary effluent during the first 10 min of RP was used as an indicator of cardiac injury. Since measure of CK in the effluent is subject to changes in coronary flow, it is worth noting that there were no differences between groups in the effluent volume during the first 10 min of RP.

Western blotting

Hearts 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, 1X protease inhibitor cocktail from Thermo Scientific), an aliquot was saved to measure proteins by the method of Bradford et al. [3], 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. Proteins were afterwards transferred to polyvinylidene difluoride membranes at 15 V for 50 min and the membranes were then blocked for 1 h with 5 % non-fat powdered milk in Tris-buffered saline containing 0.1 % Tween 20 (TBS-T). Membranes were next washed with TBS-T and incubated overnight at 4 °C 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. Then HRP conjugated donkey anti-rabbit antibody (Thermo Scientific) at 1:2500 dilution was incubated 1 h at room temperature. Following TBS-T washes, bands were detected with Bio-Lumina detection reagent (Kalium Technologies) and exposed to an autoradiography film (Roche). For band quantification, the film was scanned 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.

Electron micrographs

After 60 min of RP in the presence or absence of W, heart tissue samples (~1 mm thick) were fixed with 2.5 % glutaraldehyde in phosphate buffer (pH 7.4) for 4 h and then postfixed in 1 % osmium tetroxide for 1 h. After this, bloc staining with 2 % uranyl acetate, dehydration in a graded series of ethanol, and imbibition in Durcupan resin were performed. 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).

Measurement of mitochondrial ATP synthesis

At the end of ischemia-reperfusion, hearts were removed from the Langendorff apparatus and mitochondria were isolated by differential centrifugation after tissue homogenization in ice-cold sucrose buffer solution (300 mM sucrose, 10 mM Tris-Cl, 2 mM EGTA, 5 mg/ml BSA, pH 7.4). The mitochondrial pellet was then washed three times in sucrose isolation buffer solution lacking BSA. Cardiac mitochondria prepared with this procedure have been shown to be metabolically active with respiratory control ratios of 3.5–5.0 with succinate and of 8.0–10.0 with glutamate/malate and corresponding ADP/O ratios of 1.5–1.7 and 2.5–2.7 [32]. Since it is well documented that complex I of the respiratory chain is the most sensitive to reperfusion injury [31], mitochondrial ATP synthesis was measured in the presence of the complex I substrates pyruvate and malate. Mitochondria (1 mg protein/mL) were incubated for 5 min in a metabolic shaker at 30 °C, in a medium containing (mM): KCl 125, Mops 20, Tris 10, EGTA 0.5, KH_2PO_4 2.4, $MgCl_2$ 2.5, malate 2.5, and pyruvate 2.5, pH 7.4. ATP synthesis was then initiated by addition of 2.5 mM ADP, which is the amount of ADP that corresponds to the physiological concentration found in myocytes. Aliquots of 100 uL were taken from the

incubation mixture for the assay and ATP synthesis was measured by luciferin-luciferase luminometry (Sigma bioluminescent assay kit) every 30 s over a 4-min period. Mitochondrial protein concentration was determined by the method of Lowry using BSA as a standard, and the rate of mitochondrial ATP synthesis was calculated and expressed as mol per microgram of mitochondrial protein [19].

Statistical analysis

Values are mean ± SEM. Changes in ventricular contractile function were compared statistically using a three-factor ANOVA for repeated measures on one factor followed by Tukey's test. All other experimental data were evaluated statistically using two-way ANOVA followed by Tukey's test. Significance was set at $p < 0.05$.

Results

Measurement of heart function

At the end of the equilibration period, neither IPC nor C nor W rats exhibited any effect on the baseline values of HR, RPP, +dP/dt, and −dP/dt (data not shown).

Exposure to 25 min of global ischemia led to complete cessation of spontaneous contractions and, over the 60 min of RP, HR gradually returned to pre-ischemic values (pre-ischemic: C 233 ± 14, IPC 222 ± 12; 60-min RP: C 240 ± 13, IPC 232 ± 12 beats/min). W did not exert any effect on HR in either C or IPC hearts (pre-ischemic: C + W 228 ± 12, IPC + W 223 ± 15; 60-min RP: C + W 228 ± 14, IPC + W 237 ± 17 beats/min). In agreement with previous studies on IPC performed in our laboratory [33, 34], recovery of RPP, +dP/dt, and −dP/dt was improved by IPC (Fig. 1a–c), and amplitude of LVEDP during the earliest phase (min) of RP was significantly reduced (5-min RP LVEDP (%): C 33.43 ± 5.39, IPC 10.23 ± 3.25, $p < 0.05$; 10-min RP: C 19.50 ± 5.88, IPC 7.22 ± 2.65, $p < 0.05$) (Fig. 1d). These beneficial effects were abolished by W (5-min RP LVEDP (%): C + W 30.80 ± 7.0, IPC + W 28.00 ± 5.10; 10-min RP: C + W 26.50 ± 7.00, IPC + W 28.00 ± 8.20) (Fig. 1a–d). However, W did not change RPP, +dP/dt, −dP/dt, or LVEDP in C hearts (Fig. 1a–d). DMSO did not show effects by itself in a separate experimental test (data not shown).
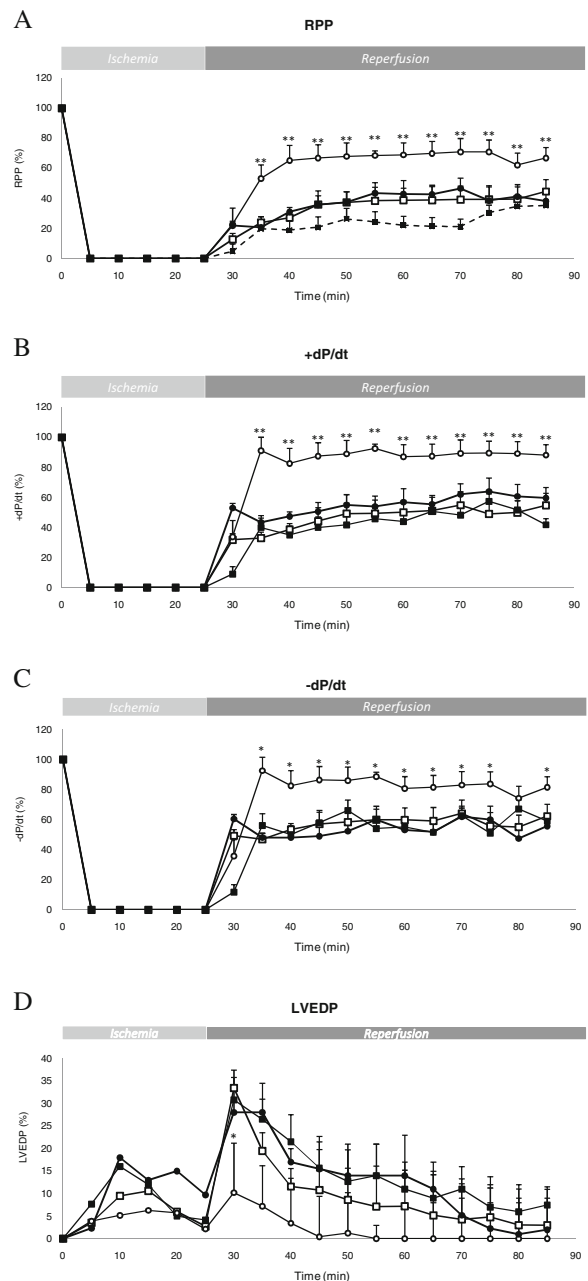
Fig. 1 Effects of wortmannin (W) on: **a** rate-pressure product (RPP); **b** peak rate of contraction (+dP/dt); **c** peak rate of relaxation (−dP/dt); and **d** left ventricular end-diastolic pressure (LVEDP) due to ischemia-reperfusion in C and IPC hearts. Values are expressed as a percentage of the respective basal value at the end of the 60-min equilibration period. *Squares* C hearts, *circles* IPC hearts, *filled symbols* hearts perfused with W added to the perfusion medium 45 min before sustained ischemia and maintained until the end of ischemia. Ischemic preconditioning was induced by four 5-min cycles of ischemia followed by 5 min of reperfusion (RP) before sustained ischemia. Values are mean ± SEM ($n = 8$). *$p < 0.05$ vs. C, C + W, IPC + W; **$p < 0.01$ vs. C, C + W, IPC + W

Creatine kinase release

As shown in Fig. 2, concomitant with the improvement in contractile function, IPC attenuated the ischemia-induced CK release during the first 10 min of reperfusion (CK UI/gww: C 38.6 ± 2.8 vs. IPC 22.6 ± 5, $p < 0.05$). This effect was abolished by W (CK UI/gww: IPC+W: 34.0 ± 4.6), and no effects were observed in C hearts (CK UI/gww: C+W 38.4 ± 5).

Western blotting

During autophagy, the cytosolic form of LC3 (LC3-I) is cleaved and lipidated to form the phosphatidylethanolamine-conjugated form LC3-II, which is recruited to the autophagosomal membrane. Consequently, increases in the LC3-II/LC3-I ratio are interpreted as induction of autophagy [20]. p62 was also studied in order to establish an autophagic flux since this protein serves as a link between LC3-II and ubiquitinated substrates. p62 is incorporated into the autophagosome and then degraded in autolysosomes. Thus, it allows to correlate the formation of autophagosomes with active autophagy through its clearance in autolysosomes [14]. Western blotting analysis showed a significant increase in the LC3-II/LC3-I ratio for IPC hearts in both periods of time, which was prevented by W (Fig. 3a). IPC generated greater disappearance of p62 with significant differences between end times of reperfusion. In the presence of W, which interferes with the signalling cascade that induces autophagy, differences in the LC3-II/ LC3-I ratio were
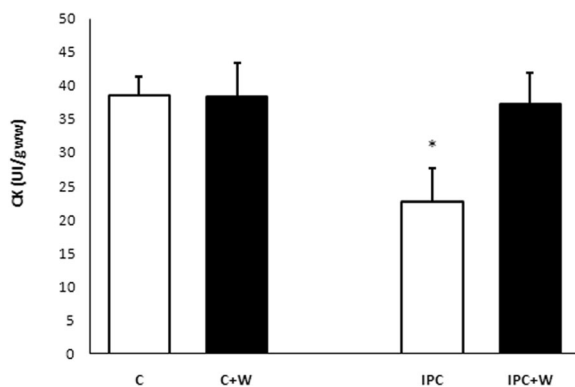
abolished, along with disappearance of p62 at different times tested, suggesting that autophagy was inhibited by this agent in all groups (Fig. 3b). Figure 3 shows that the increase in the LC3-II/LC3-I ratio was accompanied by an increase in p62 degradation, suggesting that IPC may modulate autophagy.

Electron micrographs

As illustrated in Fig. 4, electron micrographs showed higher mitochondrial conservation in IPC hearts, while other groups showed a greater separation between peaks and clearance from the mitochondrial matrix. IPC preserved mitochondrial ultrastructure at the end of RP. IPC mitochondria had fewer cristae disruptions and reduced amounts of amorphous density formation.

Measurement of mitochondrial ATP synthesis

To determine whether the preservation of mitochondrial structure generated by IPC was accompanied by preservation of ATP synthesis capacity, the rate of ATP synthesis by isolated mitochondria at the end of RP was assessed.

Mitochondria isolated from IPC hearts showed greater capacity for ATP synthesis compared with those from groups C, C+W and IPC+W (Fig. 5).

Discussion

Present data confirm previous observations that rat hearts subjected to IPC show an improvement in functional recovery during reperfusion and reduced release of CK [21, 35]. Our results showed, at least in part, that cardioprotection elicited by ischemic preconditioning was associated with PI3K since W reversed the beneficial effects of IPC on post-ischemic functional recovery in the isolated rat heart model. W is a cell-permeable, fungal metabolite that acts as a potent and irreversible inhibitor of the PI3K family and is considered an invaluable tool for elucidating the roles of PI3K in signal transduction pathways. These findings are consistent with recent and accumulating reports indicating that PI3K could be involved in the protection afforded by IPC [12, 15]. In this respect, different studies have shown that these kinases are required for many processes involving membrane traffic, not only translocation of GLUT1 and GLUT4 to the plasma membrane but also
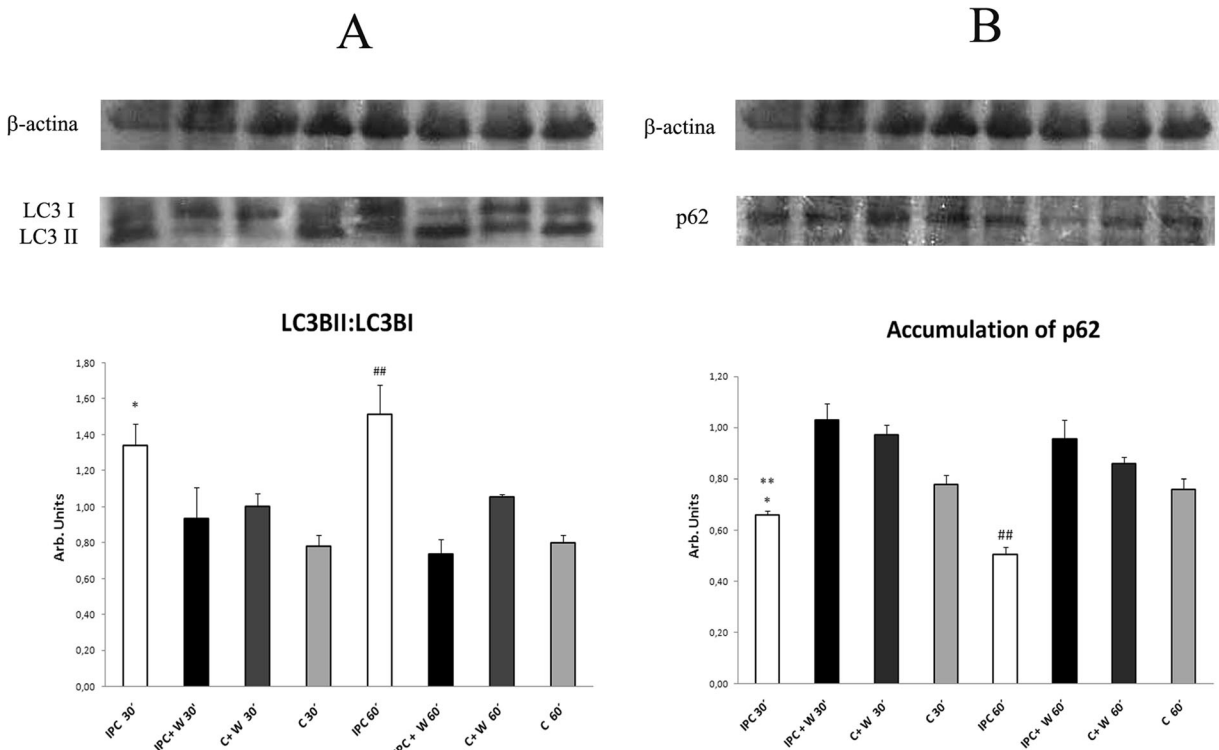

Fig. 2 Creatine kinase release from hearts subjected to ischemia, with or without IPC in the presence or absence of W, during the first 10 min of reperfusion. Values are mean±SEM ($n = 8$) *$p < 0.05$ vs. C, C+W, IPC+W

A

B

Fig. 3 **a** Western blot showing LC3-II and LC3-I expression in rat heart and average values for the LC3-II/LC3-I ratio. Values are mean ± SEM ($n = 4$). *$p < 0.05$ vs. C, C + W, IPC + W 30-min reperfusion; ##$p < 0.01$ vs. C, C + W, IPC + W 60-min reperfusion. **b** Western blot showing average values for p62 expression normalized to β-actin protein levels. Values are mean ± SEM ($n = 5$). *$p < 0.05$ vs. C 30-min reperfusion; **$p < 0.01$ vs. C + W, IPC + W 30-min reperfusion; ##$p < 0.01$ vs. C, C + W, IPC + W 60-min reperfusion. In both charts, *IPC* ischemic preconditioning; *W* wortmannin; *C 30'* control hearts subjected to 25 min of ischemia and 30 min of reperfusion; *IPC 30'* hearts subjected to ischemic preconditioning, 25 min of ischemia, and 30 min of reperfusion;

C + W 30' control hearts treated with wortmannin and subjected to 25 min of ischemia and 30 min of reperfusion; *IPC + W 30'* hearts subjected to ischemic preconditioning in the presence of wortmannin, 25 min of ischemia, and 30 min of reperfusion; *C 60'* control hearts subjected to 25 min of ischemia and 60 min of reperfusion; *IPC 60'* hearts subjected to ischemic preconditioning, 25 min of ischemia, and 60 min of reperfusion; *C + W 60'* control hearts treated with wortmannin and subjected to 25 min of ischemia and 60 min of reperfusion; *IPC + W 60'* hearts subjected to ischemic preconditioning in the presence of wortmannin, 25 min of ischemia, and 60 min of reperfusion

endocytosis, endosome fusion, and endosomal protein sorting [4–6, 16]. Another process involving membrane traffic is autophagy. As mentioned before, autophagy is an evolutionarily conserved process crucial for normal tissue homeostasis, but its role in (I/R) injury is still unclear [30]. In this regard, given that distinct classes of PI3K control the autophagic pathway in opposite directions, different roles of kinases in autophagy have been suggested. While stimulation of class I PI3K activity inhibits autophagy at the sequestration step, activity of class III PI3K is required to trigger autophagic sequestration [26].

In order to further confirm whether autophagy was modulated by IPC, the levels of LC3-II/LC3-I and p62

protein expression were examined. A significant increase in the LC3-II/LC3-I ratio was observed, suggesting that IPC could involve the formation of autophagosomes. The extent of degradation of p62 demonstrated that not only did IPC encourage greater autophagosomes formation but also that these structures were fused with lysosomes for degradation of vacuolar content [24]. Huang et al. also reported that ischemic preconditioning in isolated rat hearts mediates cardioprotection against I/R injury through an autophagy-dependent pathway involving p62 recruitment to mitochondria [14]. In all cases, W, a nonspecified inhibitor of autophagy, overturned the established differences between IPC and C hearts. This allowed consideration of the correlation between
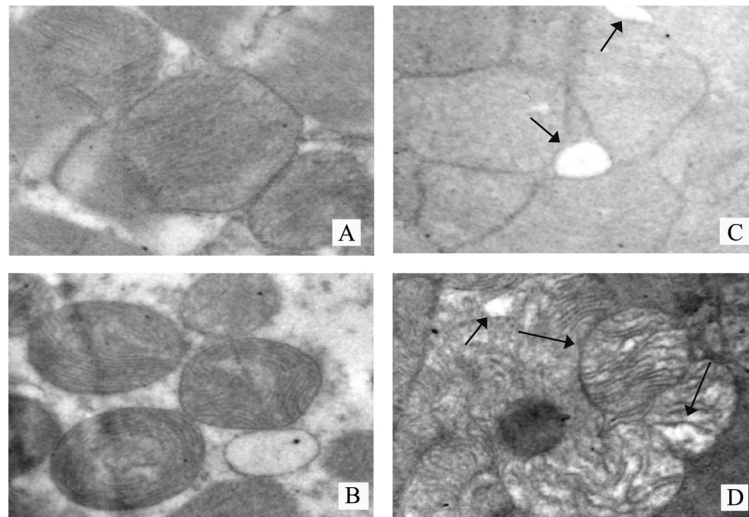
Fig. 4 Representative electron micrographs (×30000) obtained from hearts subjected to **a** 60-min stabilization period, 25 min of ischemia, and 60 min of reperfusion; **b** 20-min stabilization period, 40-min of preconditioning, 25 min of ischemia, and 60 min of reperfusion; **c** 60-min stabilization period treated with W (W was added to the perfusion medium 45 min before sustained ischemia and maintained until the end of ischemia.), 25 min of ischemia, and 60 min of reperfusion; **d** 20-min stabilization period treated with W (W was added to the perfusion medium 5 min before preconditioning and maintained until the end of ischemia), 40 min of preconditioning, 25 min of ischemia, and 60 min of reperfusion. *Arrows* point out the separation between peaks and clearance, as well as the presence of vacuoles

autophagy and functional recovery during reperfusion in IPC hearts.

On the other hand, electron microscopy examination revealed that mitochondria from hearts subjected to IPC showed greater preservation of their structure than other groups that showed greater separation between peaks and clearance [35]. Under these experimental conditions, IPC preserved structural integrity of mitochondria at the end of reperfusion [33]. Moreover, hearts subjected to IPC showed increased mitochondrial ATP synthesis, which demonstrated the impact of IPC on the conservation of mitochondria in cardiac tissue. The heart depends almost entirely on mitochondrial supply of ATP for the maintenance of its function.

Taken together, these results and the results obtained with electron microscopy and ATP synthesis capacity would establish that upregulation of autophagy by IPC would be associated with the cardioprotection elicited in hearts subjected to IPC. The cardioprotection exerted by IPC could be connected to the removal of damaged mitochondria by mitophagy, a selective form of autophagy. This process would start with the recruitment of Parkin, a specific protein that ubiquitinates multiple outer mitochondrial membrane proteins and recruits

Fig. 5 The effects of wortmannin (W) on mitochondrial ATP synthesis in C and IPC hearts. Values are mean ± SEM ($n = 8$) and are expressed as nmol/min/ug protein. $*p < 0.05$ vs. C, C + W, IPC + W
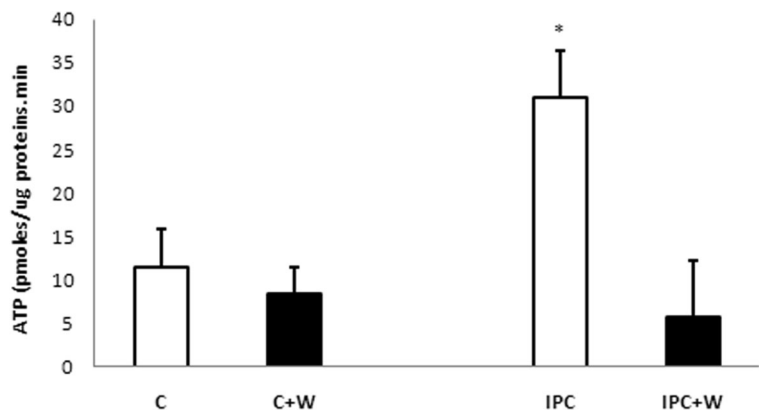
the autophagic machinery [10]. Mitophagy is key in keeping the cell healthy, promoting turnover of damaged mitochondria, and preventing accumulation of dysfunctional ones, which can lead to cellular degeneration. Mitochondria undertake multiple critical functions in a cell: they supply all the necessary biological energy, regulate the cellular redox state, produce most of the cellular reactive oxygen species (ROS), buffer cellular Ca^{2+}, and initiate cellular apoptosis. In response to cellular environment, mitochondria can send death signals and decide the cell fate. Mitochondria can be degraded by both non-selective autophagy and mitophagy, a selective type of autophagy activated in cardiomyocytes after stress such as I/R in order to allow cells to adapt to stress by selective clearance of damaged mitochondria.

Even though wortmannin is an inhibitor of PI3K kinases with opposite effects on autophagy, it is widely used as autophagy inhibitor in this experimental model. As in addition to its action as an inhibitor of autophagy wortmannin also exerts other effects on cellular functions, it is not possible to posit that the detrimental effects of this agent on the IPC rat heart are caused solely by the inhibition of autophagy. Future studies are necessary to investigate the possible role played by mitophagy in the beneficial effects of IPC and to elucidate the pathway involved in and producing the results obtained in the present study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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