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Critical role of PI3-kinase/Akt activation in the PARP inhibitor induced heart function recovery during ischemia–reperfusion

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Abbreviations:
GSK-3β, glycogen synthase kinase-3β
IR, ischemia–reperfusion
NAD^+, nicotinamide adenine dinucleotide
PARP, poly(ADP-ribose) polymerase
PI3-kinase, phosphatidylinositol-3-kinase
ROS, reactive oxygen species
TBARS, thiobarbituric acid reactive substances

ABSTRACT

Poly(ADP-ribose) polymerase (PARP) inhibitors protect hearts from ischemia–reperfusion (IR)-induced damages by limiting nicotinamide adenine dinucleotide (NAD^+) and ATP depletion, and by other, not yet elucidated mechanisms. Our preliminary data suggested that PARP catalyzed ADP-ribosylations may affect signaling pathways in cardiomyocytes. To clarify this possibility, we studied the effect of a well-characterized (4-hydroxyquinazoline) and a novel (carboxaminobenzimidazol-derivative) PARP inhibitor on the activation of phosphatidylinositol-3-kinase (PI3-kinase)/Akt pathway in Langendorff-perfused hearts. PARP inhibitors promoted the restoration of myocardial energy metabolism (assessed by ^{31}P nuclear magnetic resonance spectroscopy) and cardiac function compared to untreated hearts. PARP inhibitors also attenuated the infarct size and reduced the IR-induced lipid peroxidation, protein oxidation and total peroxide concentration. Moreover, PARP inhibitors facilitated Akt phosphorylation and activation, as well as the phosphorylation of its downstream target glycogen synthase kinase-3β (GSK-3β) in normoxia and, more robustly, during IR. Blocking PI3-kinase by wortmannin or LY294002 reduced the PARP inhibitor-elicited robust Akt and GSK-3β phosphorylation upon ischemia–reperfusion, and significantly diminished the recovery of ATP and creatine phosphate showing the importance of Akt activation in the recovery of energy metabolism. In addition, inhibition of PI3-kinase/Akt pathway decreased the protective effect of PARP inhibitors on infarct size and the recovery of heart functions. All these data suggest that contrary to the original view, which considered preservation of NAD^+ and consequently ATP pools as the exclusive underlying mechanism for the cytoprotective effect of PARP inhibitors, the activation of PI3-kinase/Akt pathway and related processes are at least equally important in the cardioprotective effects of PARP inhibitors during ischemia–reperfusion.

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1. Introduction

Acute myocardial ischemia accounts for the highest percentage of morbidity and mortality in the Western world [1]. Persistent ischemia can result in cardiomyocyte death and lead to congestive heart failure. Coronary reperfusion utilizing thrombolytics and coronary angioplasty can partially rescue the ischemic myocardium and limit the development of an infarct. However, reperfusion, though prerequisite for tissue salvage, might also lead to increased cell mortality, possibly as a result of the inflammatory response, a burst of oxygen-free radical production and calcium overload [2,3].

Several studies have suggested that both neutrophils and reactive oxygen species (ROS) play important roles in ischemia–reperfusion (IR)-induced cardiac dysfunction [4–6]. High levels of ROS are generated from a variety of sources, such as the xantine oxidase system [4], the leakage of electrons from the mitochondrial respiratory chain [5,7], the cyclooxygenase pathway of arachidonic acid metabolism [6,8] and the respiratory burst of phagocytic cells [9,10]. In the heart, ROS can evoke cytotoxicity [11], cardiac stunning [12], arrhythmia [13], reduction of the calcium transient and contractility, elevated diastolic calcium levels [14] and intracellular ATP depletion [15].

During ischemia–reperfusion cycle ROS and peroxynitrite formation causes lipid peroxidation, protein oxidation as well as DNA breaks [16]. Poly(ADP-ribose) polymerase (PARP), a protein-modifying and nucleotide-polymerizing enzyme, is present abundantly in the nucleus. In response to DNA damage, PARP becomes activated and produces homopolimers of adenosine diphosphate-ribose units using nicotinamide adenine dinucleotide (NAD^+) as a substrate. This process rapidly depletes the intracellular NAD^+ and ATP pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction and death [17,18]. Accordingly, inhibition of PARP can improve the recovery of different cells from oxidative injury [19]. Our previous data showed that PARP inhibitors were able to reduce the oxidative damage of cellular components without having an obvious scavenger activity [16].

External stress-related tissue injury, such as ischemia–reperfusion can initiate protein kinase cascades and inflammatory reactions. Previous results indicate that the growth factor-associated kinase Akt (also known as protein kinase B) is phosphorylated following ischemia–reperfusion in cardiomyocytes in a phosphatidylinositol-3-kinase (PI3-kinase)-dependent manner [20]. However, some data suggest that Akt can be activated by a PI3-kinase-independent way, as well [21,22]. Akt kinase pathway is one of several signal transduction pathways implicated in cell survival [23,24]. Akt can phosphorylate a number of downstream targets leading to the inactivation of glycogen synthase kinase-3β (GSK-3β), the pro-apoptotic Bcl-2 family member Bad [25], caspase-9 [26] and Forkhead transcription factor (FKHR) [24], as well as to the activation of nuclear factor-κB (NF-κB) [27], p70 ribosomal S6 kinase and endothelial nitric oxide synthase (eNOS) [28,29]. PARP inhibitors have been shown to improve the survival of mice with lipopolysaccharide-induced septic shock in a PI3-kinase/Akt-dependent manner [30]. However, it needs to be elucidated whether the proven cardioprotective properties of PARP inhibitors in ischemia–reperfusion models are, at least in part, mediated via Akt signaling.

In the present study, we investigated the molecular mechanism by which PARP inhibitors promote the recovery of energy metabolism and heart function during ischemia–reperfusion, and provided evidence that PARP inhibitors activated PI3-kinase/Akt pathway in postischemic hearts. Furthermore, data presented here provide the first evidence that the activation of PI3-kinase/Akt pathway in postischemmic heart is responsible in a significant extent for the recovery of energy metabolism and heart function, as well as preservation of viable myocardium in ischemia–reperfusion, indicating a novel molecular mechanism in the cardioprotective effect of PARP inhibitors.

2. Materials and methods

2.1. PARP inhibition

The IC_{50} of 4-hydroxyquinazoline and HO-3089 was studied in an in vitro assay as described before [31].

2.2. Cell culture and MTT assay

H9c2(2-1) cardiomyoblasts (American Type Culture Collection number CRL-1446), a clonal line derived from embryonic rat heart, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM pyruvate in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Before reaching confluence, the cells were split, plated at low density in culture dishes (approximately 2×10^4 cells/well) and cultured for 24 h. Cardiomyocytes were then incubated without (negative control) and with 1 mM hydrogen peroxide for 3 h either untreated (positive control) or treated with 4-hydroxyquinazoline (in 5, 10, 50, 100 and 200 μM) or HO-3089 (in 0.02, 0.05, 0.1, 10 and 50 μM). At the end of the incubation period the survival of cells was determined by the MTT assay as described before [32]. Briefly, the cells were incubated for 3 h in fresh medium containing 0.5% of the water-soluble yellow mitochondrial dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT^+). The MTT^+ reaction was terminated by adding HCl to the medium at a final concentration of 10mM. The amount of water-insoluble blue formasan dye formed from MTT^+ was proportional to the number of live cells, and was determined with an Anthos Labtech 2010 ELISA reader at 550 nm wavelength after dissolving the blue formasan precipitate in 10% sodium dodecyl sulphate. All experiments were run in at least four parallels and repeated three times.

2.3. Heart perfusion

Male Wistar rats weighing 300–350 g were heparinized with sodium heparin (100 IU, i.p.) and anesthetized with ketamine (200 mg/kg, i.p.). The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, and was approved by the Animal Research Review Committee of the University of Pecs. Hearts were perfused via the aorta according to the Langendorff

method at a constant pressure of 70 mmHg at 37 °C as described before [16]. The perfusion medium was a modified phosphate-free Krebs–Henseleit buffer without or with PARP inhibitors (100 μM 4-hydroxyquinazoline or 25 μM HO-3089), and/or wortmannin (100 nM) or LY294002 (10 μM). The aforementioned compounds were administered into the perfusion medium at the beginning of a normoxic perfusion period. After a 15-min normoxic perfusion, hearts were exposed to 30-min global ischemia followed by 15, 45 or 90-min reperfusion. During ischemia, the hearts were submerged into the perfusion buffer at 37 °C. Hearts were freeze-clamped at the end of each perfusion. Myocardial energy metabolism was continuously detected by means of a ^{31}P nuclear magnetic resonance spectroscope as described earlier [16]. Functional performance of the hearts was monitored by placing a balloon catheter into the left ventricle [33]. Myocardial infarct size was determined by triphenyl tetrazolium chloride staining as described before [34,35].

2.4. Myocardial oxidative damage

Lipid peroxidation was assessed by measuring the amount of thiobarbituric acid reactive substances (TBARS) [36], while the quantity of protein-bound aldehyde groups served as assessment for protein oxidation [37]. Total peroxide concentration was determined by homogenizing 100 mg of heart tissue with a Teflon-glass homogenizer in ice-cold MOPS (50 mM) and EDTA (1 mM) buffer. Homogenates were than bubbled with argon gas, sonicated, then Tween 20 was added to a final concentration of 1%, and the samples were homogenized again by sonication. After centrifuging, peroxide concentration of the supernatants were measured by means of Biomedica OxyStat assay (Biomedica GmbH, Wien, Austria).

2.5. Western blot analysis

Heart samples were prepared and Western blot was performed as described before [33]. Membranes were probed overnight at 4 °C with primary antibodies recognizing the following antigens: phospho-specific Akt-1 Ser^{473} (1:1000 dilution), non-phosphorylated Akt (1:1000), phospho-specific glycogen synthase kinase-3β Ser^{9} (1:1000; Cell Signaling Technology, Beverly, USA) and anti-poly(ADP-ribose) (Alexis Biochemicals, Nottingham, UK). Membranes were washed six times for 5 min in Tris-buffered saline (pH 7.5) containing 0.2% Tween (TBST) prior to addition of goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000; BioRad, Budapest, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence on conventional films. After scanning, results were quantified by means of Scion Image Beta 4.2 program. All experiments were performed at least four times.

2.6. Statistical analysis

All data were expressed as the means ± S.E.M. Statistical analysis was performed by analysis of variance and unpaired Student's *t*-test. Differences with *p*-values below 0.05 were considered to be significant.

3. Results

3.1. 4-Hydroxyquinazoline and HO-3089 inhibit poly-ADP-ribosylation in vitro and in vivo

Under our experimental conditions 4-hydroxyquinazoline had an IC_{50} = 8 μM for poly(ADP-ribose) polymerase (Fig. 1A), which is in accordance with previous data (Fishbein et al. [34]). The novel PARP inhibitor HO-3089 had an IC_{50} = 0.06 μM (Fig. 1B). These PARP inhibitors indeed improved the survival of H9c2 cells during oxidative stress (1 mM H_2O_2 for 4 h), HO-3089 in the nanomolar, while 4-hydroxyquinazoline in the micromolar concentration range (Fig. 1C and D). In perfused hearts, both PARP inhibitors decreased the self poly-ADP-ribosylation of PARP, detected by Western blotting utilizing an anti-poly(ADP-ribose) antibody (Fig. 1E), indicating their PARP inhibitory properties.

3.2. Protection by PARP inhibitors against ischemia–reperfusion injury in Langendorff-perfused hearts

Energy metabolism of Langendorff-perfused hearts was monitored in the magnet of an NMR spectroscope capable of monitoring changes in high-energy phosphate intermediates. Ischemia induced a rapid decrease in creatine phosphate and ATP levels and a fast elevation of inorganic phosphate. Under our experimental conditions, high-energy phosphate intermediates only partially recovered in untreated hearts during the 15-min reperfusion phase. On the other hand, HO-3089 and, moreover, 4-hydroxyquinazoline facilitated the recovery of creatine phosphate (expressed as % of the normoxic level: 61.2 ± 5.7% for 4OHQ-treated and 49.1 ± 5.4% for HO-3089-treated versus 24.2 ± 5.1% for untreated hearts; Fig. 2A) and ATP (Fig. 2B). According to these data, both PARP inhibitors could significantly improve the final recovery of high-energy phosphate intermediates. We tested the PARP inhibitors at the concentration range of 25–500 μM for 4-hydroxyquinazoline and 6.25–200 μM for HO-3089. Although, both PARP inhibitors had significant protective effect on the energy metabolism of the heart during ischemia–reperfusion even at the lowest concentration used (data not shown), we have observed the maximal protective effect of the substances at the concentrations of 100 and 25 μM, respectively. For this reason, we used these concentrations throughout the heart perfusion experiments. PARP inhibitors also promoted the faster and more complete reutilization of inorganic phosphate during reperfusion (expressed as % of the value at the end of ischemic period: 27.8 ± 3.2% for 4OHQ-treated and 31.5 ± 4.1% for HO-3089-treated versus 53.7 ± 2.9% for untreated hearts; Fig. 2C). The intracellular pH markedly fell by the end of the ischemic period from 7.41 ± 0.04 to 5.81 ± 0.04 in untreated hearts and to 6.21 ± 0.04 in 4-hydroxyquinazoline and 6.18 ± 0.7 in HO-3089-treated hearts. Fifteen minutes of reperfusion brought about a slight recovery of the pH in untreated hearts (5.94 ± 0.06), whereas this recovery was much improved in the presence of the PARP inhibitors (6.85 ± 0.05 for 4OHQ and 6.6 ± 0.07 for HO-3089; Fig. 2D).

To evaluate the effect of PARP inhibitors on the postischemic myocardial functional recovery, isolated hearts were perfused in the absence or presence of 100 μM 4OHQ or 25 μM

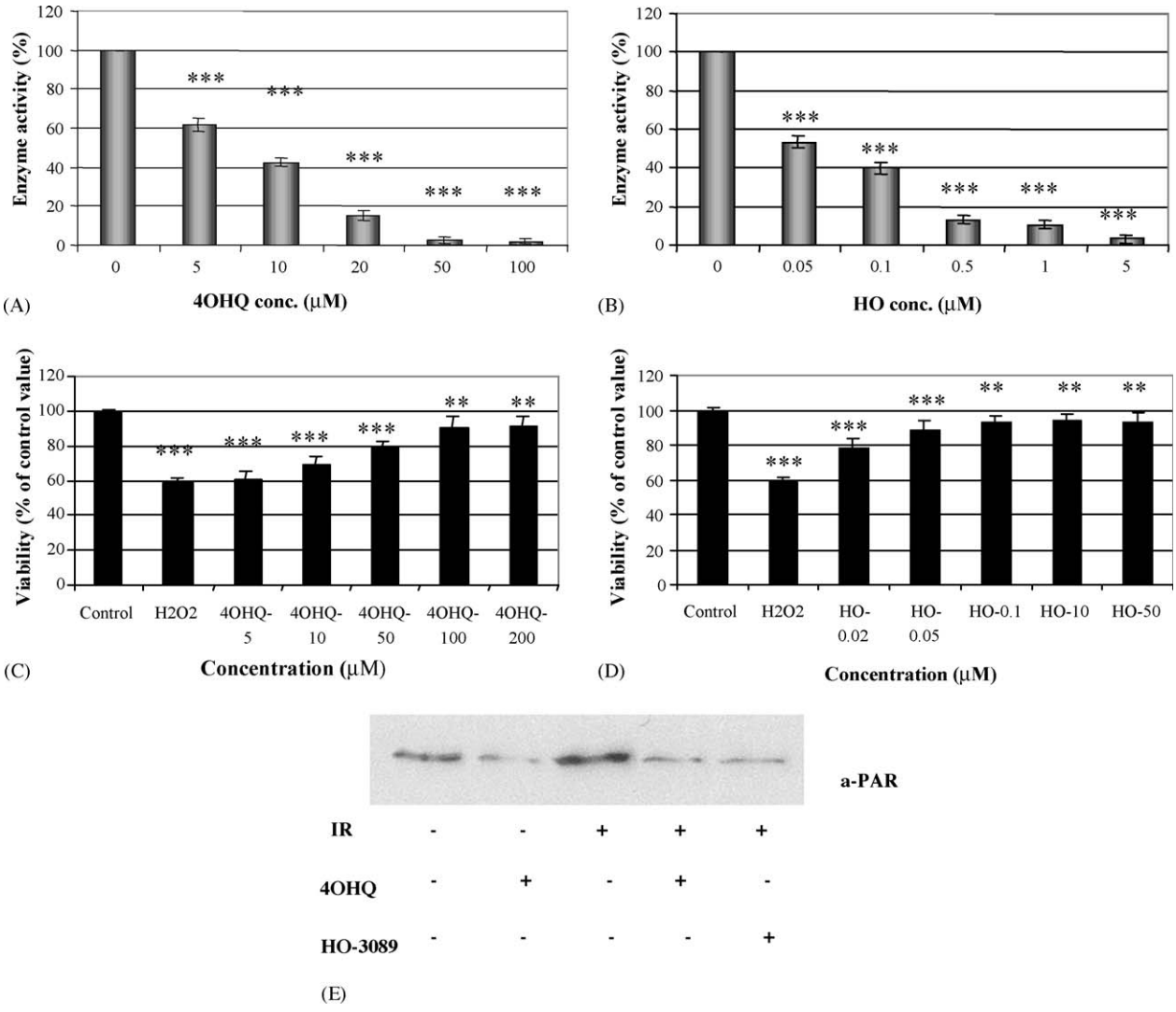


Fig. 1 – Poly(ADP-ribose) polymerase inhibitory effect of 4-hydroxyquinazoline and HO-3089 in vitro and in isolated hearts. Effect of 4-hydroxyquinazoline (A) and HO-3089 (B) on the activity of isolated poly(ADP-ribose) polymerase activity, cytoprotective effect of 4-hydroxyquinazoline (C) and HO-3089 (D) in H9c2 cells in the presence of 1 mM H_2O_2, inhibitory effect of 4-hydroxyquinazoline and HO-3089 on the self-ADP-ribosylation of PARP in Langendorff-perfused rat hearts during ischemia–reperfusion (E). Values given as means ± S.E.M. for five experiments. IR, ischemia–reperfusion without treatment; 4OHQ, 4-hydroxyquinazoline treatment; HO, HO-3089 treatment; using the indicated micromolar concentrations. Significant difference from samples without the PARP inhibitor ($^{**}p < 0.01$ and $^{***}p < 0001$).

HO-3089. At the end of the normoxic period, LVDP was 135.2 ± 16.4 mmHg, RPP was 3.4 ± 0.15 × 10^4 mmHg/min, dP/dt_{max} was 1310 ± 196 mmHg/s and the average heart rate was 217 ± 19 beats/min. As Fig. 2E demonstrates, both PARP inhibitors significantly improved the recovery of all parameters indicating that the preservation of energy metabolism resulted in a better functional performance. Triphenyl tetrazolium chloride staining of the myocardium after 90 min of postischemic reperfusion revealed that PARP inhibitors were capable of significantly diminishing the infarct size compared to untreated cases (expressed as % of the total area: 33.1 ± 4.2% for 4OHQ and 37.2 ± 5.7% for HO-3089 compared to 64.2 ± 6.8% for untreated; Fig. 2F). In accordance with our previous reports [31,33] all the significant changes in the function and metabolism of the perfused hearts occurred within 15 min and there were no significant changes afterwards up to 1 h of reperfusion.

3.3. *Attenuation of cardiac oxidative damage by PARP inhibitors*

Under our experimental conditions, ischemia–reperfusion increased the amount of thiobarbituric acid reactive substances compared to the normoxic conditions (Fig. 3A). In normoxic hearts, PARP inhibitors did not have significant effect on TBARS. However, during IR the formation of TBARS was significantly lower in the presence of PARP inhibitors than in hearts subjected to IR alone (46.3 ± 3.2 nM/g for 4-OHQ and 49.5 ± 4.1 nM/g for HO-3089 versus 125.2 ± 5.4 nM/g for untreated) indicating that PARP

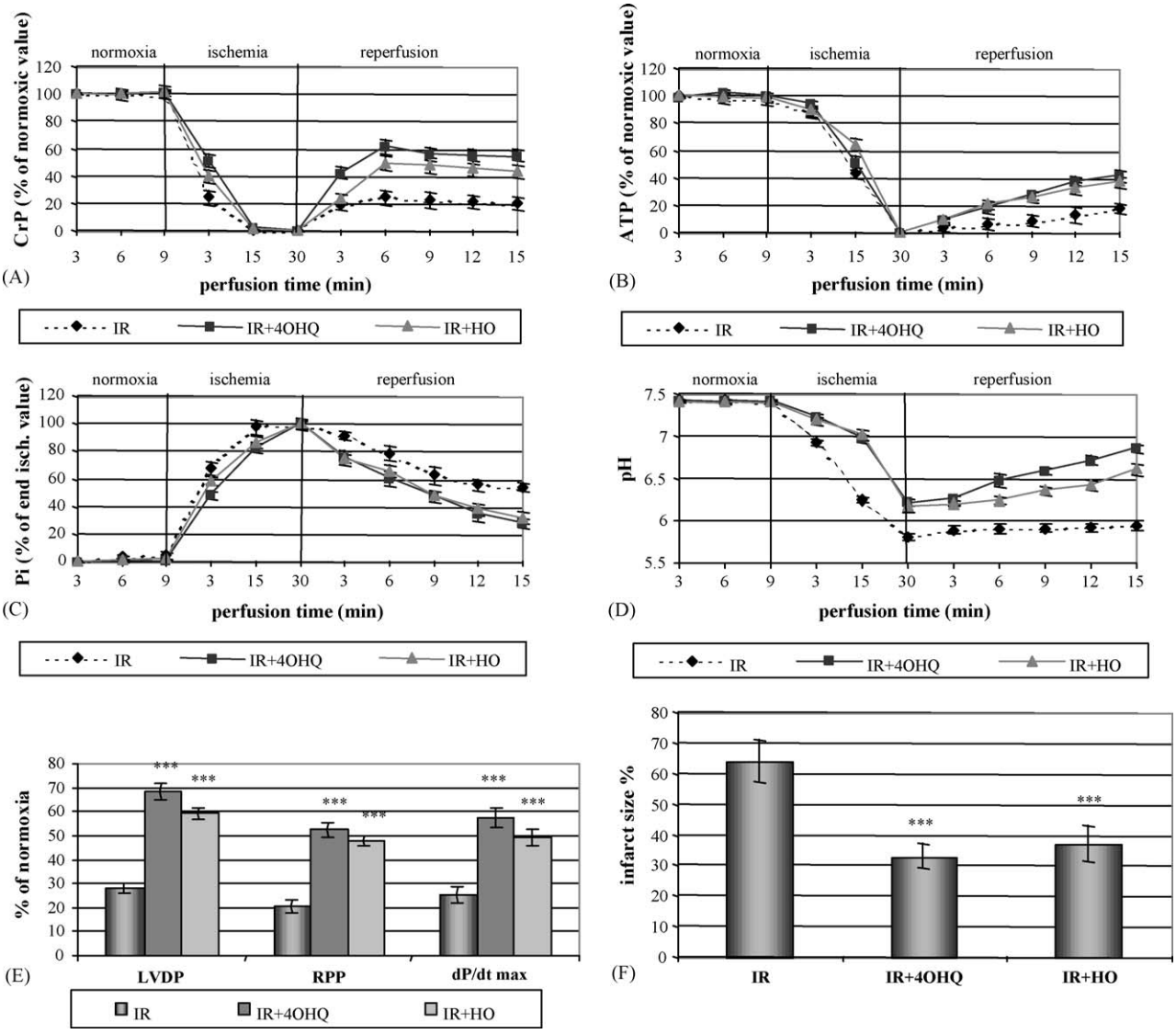


Fig. 2 – Salutary effects of PARP inhibitors on the energy metabolism, heart function and infarct size of postichemic hearts. Time-course of creatine phosphate (CrP; A), ATP (B), inorganic phosphate (Pi; C) and pH levels (D) during ischemia–reperfusion. (E) Maximal percentage recovery of left ventricular developed pressure (LVDP), rate–pressure product (RPP) and dP/dt_{max} during the 45-min reperfusion period after ischemia. (F) The infarct size after ischemia–reperfusion. Values are given as means ± S.E.M. for five experiments. (A–D) Values measured in the presence of the PARP inhibitors were significantly different ($p < 0.001$) from those of the untreated hearts for every time points of the reperfusion phase. IR + 4OHQ, ischemia–reperfusion in the presence of 100 μM 4-hydroxyquinazoline; IR + HO, ischemia–reperfusion in the presence of 25 μM HO-3089. Significant difference from untreated IR hearts ($^{***}p < 0.001$).

inhibitors attenuated the ischemia–reperfusion-induced lipid peroxidation.

Reactive oxygen species formation in IR cycle can also trigger the oxidation of proteins, which can be characterized by the quantity of protein-bound aldehyde groups. Fig. 3B shows that IR significantly elevated the level of protein oxidation, and that the administration of PARP inhibitors during the IR cycle prevented the increase in protein-bound aldehyde groups (1.34 ± 0.08 μM/g for 4OHQ and 1.46 ± 0.08 μM/g for HO-3089 versus 3.18 ± 0.15 μM/g for untreated).

Total peroxide concentrations of the heart samples show a direct correlation between free radicals and circulating biological peroxides, thus allow the characterization of the oxidative state of the sample. Here, we have found that IR increased the total peroxide concentration compared to normoxic conditions. Administration of PARP inhibitors significantly lowered the amount of total peroxide concentration (112.3 ± 8.4 μM/g for 4OHQ and 121.8 ± 8.4 μM/g for HO-3089 versus 301.8 ± 12.3 μM/g for untreated; Fig. 3C).

3.4. *Akt activation by PARP inhibitors in ischemia–reperfusion*

The phosphorylation of Akt-1 (Ser^{473}) was undetectable under normoxic conditions in our study. However, ischemia fol-

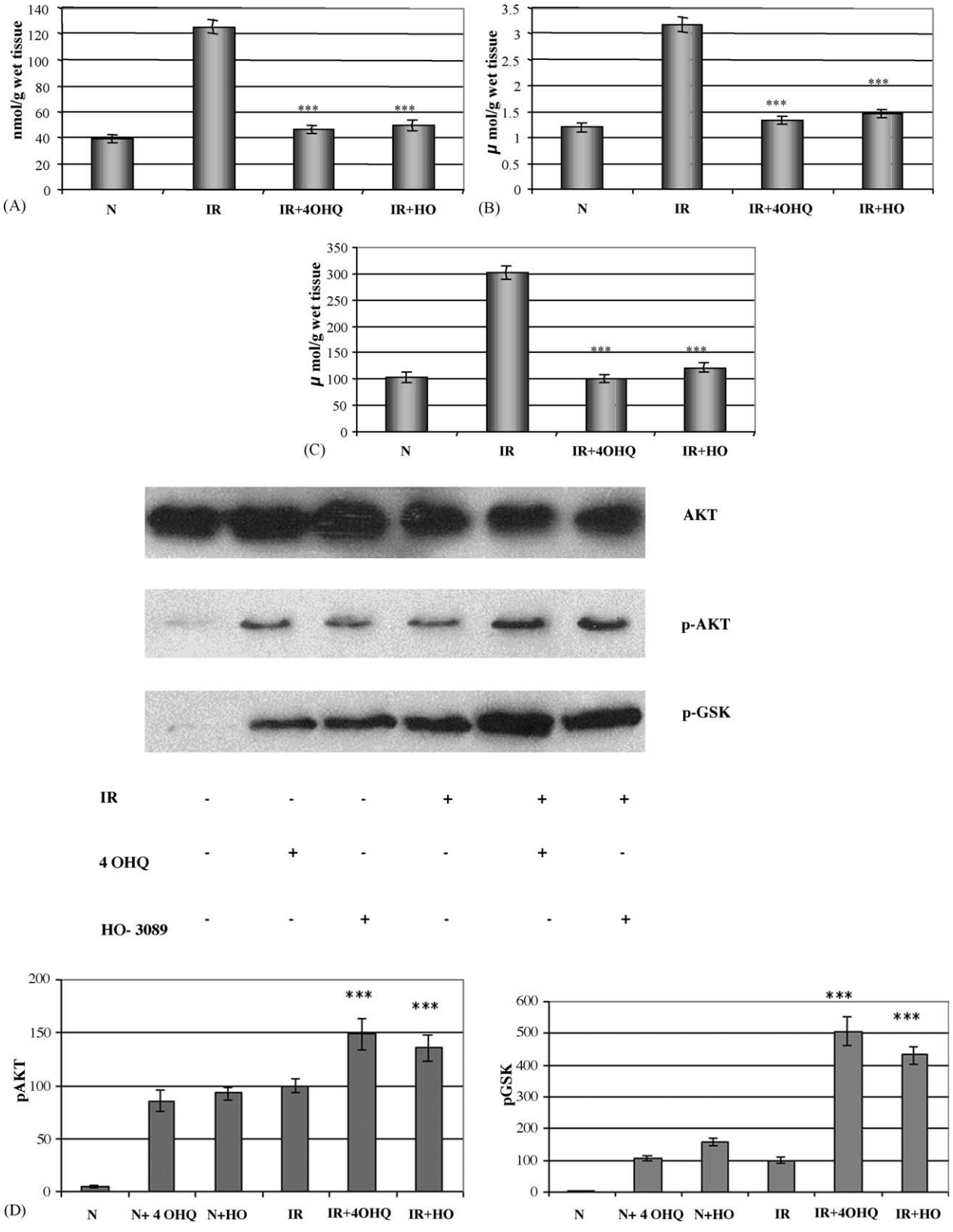


Fig. 3 – Effect of PARP inhibitors on oxidative stress and Akt pathway of postichemic hearts. Lipid peroxidation (A), protein carbonyl content (B) and total peroxide concentration (C) of perfused hearts. (D) Phosphorylation state of Akt and GSK-3β are shown on a representative blot of five experiments. Values are given as means ± S.E.M. for five experiments. Phospho-Akt and phospho-GSK signals were normalised to total Akt protein content. For comparison, the total Akt contents are also presented. N, normoxia; IR, ischemia–reperfusion without treatment; IR + 4OHQ, ischemia–reperfusion in the presence of 100 μM 4-hydroxyquinazoline; IR + HO, ischemia–reperfusion in the presence of 25 μM HO-3089. Significant difference from untreated IR hearts ($^{***}p < 0.001$).

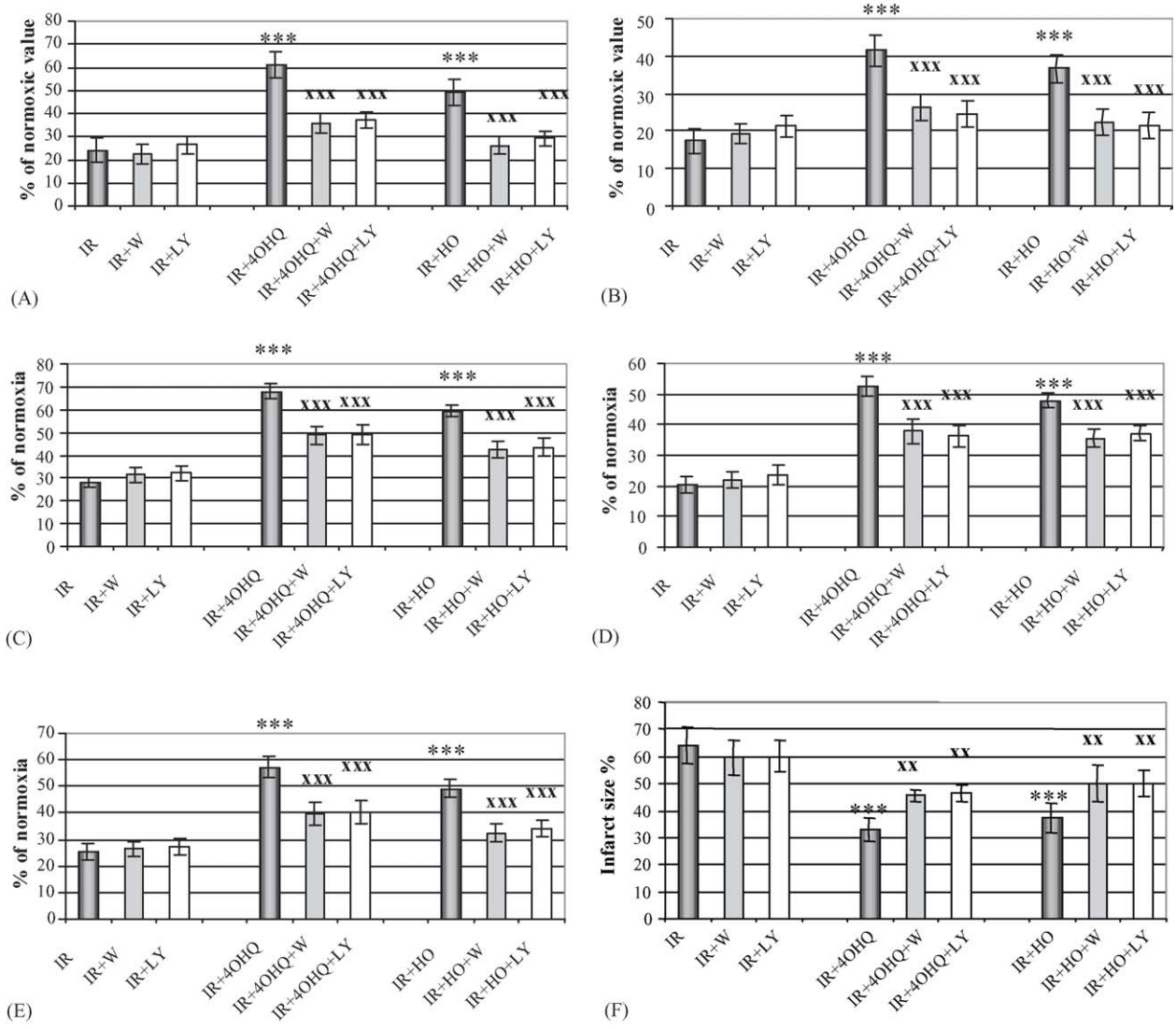


Fig. 4 – PI3-kinase inhibitors antagonize the protective effects of PARP inhibitors on energy metabolism, heart function and infarct size of postischemic hearts. Maximal percentage recovery of creatine phosphate (CrP; A) and ATP levels (B) during ischemia–reperfusion. Maximal percentage recovery of left ventricular developed pressure (LVDP; C), rate–pressure product (RPP; D) and dP/dt_{max} (E) during the 45-min reperfusion period after ischemia. (F) Infarct size after ischemia–reperfusion. Values are given as means ± S.E.M. for five experiments IR, ischemia–reperfusion without treatment; IR + 4OHQ, ischemia–reperfusion in the presence of 100 μM 4-hydroxyquinazoline; IR + HO, ischemia–reperfusion in the presence of 25 μM HO-3089; IR + W, ischemia–reperfusion in the presence of 100 nM wortmannin; IR + W + 4OHQ, ischemia–reperfusion in the presence of 100 nM wortmannin and 100 μM 4-hydroxyquinazoline; IR + W + HO, ischemia–reperfusion in the presence of 100 nM wortmannin and 25 μM HO-3089; IR + LY, ischemia–reperfusion in the presence of 10 μM LY294002; IR + LY + 4OHQ, ischemia–reperfusion in the presence of 10 μM LY294002 and 100 μM 4-hydroxyquinazoline; IR + LY + HO, ischemia–reperfusion in the presence of 10 μM LY294002 and 25 μM HO-3089. Significant difference from untreated IR hearts ($^{***}p < 0.001$). Significant difference from the PARP inhibitor-treated IR hearts ($^{xx}p < 0.01$ and $^{xxx}p < 0.001$).

lowed by 15-min reperfusion induced Akt phosphorylation, which was further increased in the presence of both PARP inhibitors (4-hydroxiquinazoline and HO-3089). The enhanced phosphorylation indicates an activation of Akt-1, which is highly increased by PARP inhibitors in postischemic myocardium (Fig. 3D). Under the same experimental conditions, PARP inhibitors strongly enhanced the phosphorylation of GSK-3β, a downstream target of Akt, showing the increased catalytic activity of Akt in postischemic hearts in the presence of PARP inhibitors.

3.5. *PI3-kinase inhibitors interfere with the cardioprotection by PARP inhibitors*

To test whether the observed Akt activation contributes to the cardioprotective effect of the PARP inhibitors, we treated

hearts with PI3-kinase inhibitors. When added by itself, 100 nM wortmannin or 10 μM LY294002 did not alter the recovery of high-energy phosphates and the elevation of inorganic phosphate during ischemia–reperfusion. On the other hand, both agents significantly reduced the beneficial effect of PARP inhibitors on creatine phosphate, ATP (Fig. 4A and B) and inorganic phosphate levels. Furthermore, the PARP inhibitor-induced functional improvement was also significantly attenuated in the presence of PI3-kinase inhibitors (Fig. 4C–E).

When applied alone, wortmannin and LY294002 did not affect the infarct size in hearts exposed to IR (59.6 ± 6.5 and 60.1 ± 5.8%, respectively). However, co-administration of PARP inhibitors and PI3-kinase inhibitors during IR led to an increase in infarct sizes as compared to those in hearts treated with the PARP inhibitors alone (from 36.1 ± 2.2% in 4OHQ-treated to 42.5 ± 3.4% in 4OHQ + LY294002 and to 41.6 ± 2.9% in 4OHQ + wortmannin-treated hearts; and from 33.4 ± 3.1% in HO-3089-treated to 45.3 ± 2.7% in HO-3089 + wortmannin and 47.2 ± 2.6% in HO-3089 + LY294002-treated hearts; Fig. 4F).

PI3-kinase inhibitors administered by themselves could lower the IR-induced increase in TBARS (82.4 ± 5.7 nM/g in wortmannin-treated and 81.4 ± 3.9 nM/g in LY294002-treated hearts versus 125.2 ± 5.4 nM/g in untreated hearts). On the other hand, the level of TBARS decreased to almost normoxic values in hearts treated with the PARP inhibitors (46.3 ± 3.2% for 4OHQ-treated and 49.5 ± 4.1% for HO-3089-treated versus 39.3 ± 3.2% for untreated normoxic hearts). When the PARP inhibitors were administered together with PI3-kinase inhibitors, the latter partially antagonised the effect of the former resulting in higher TBARS values than with the PARP inhibitors alone (51.3 ± 2.3% for 4OHQ + wortmannin and 62.8 ± 3.4% for 4OHQ + LY294002 versus 46.3 ± 3.2% for 4OHQ-treated hearts; and 52.5 ± 3.1% for HO-3089 + wortmannin and 58.7 ± 4.3% for HO-3089 + LY294002 versus 49.5 ± 4.1 for HO-3089-treated hearts) (Fig. 5A). Similarly to the TBARS data, the protein oxidation and total peroxide concentrations of the heart samples after IR were reduced by wortmannin and LY294002, but the PARP inhibitors had more pronounced effect decreasing protein oxidation and total peroxide concentrations to almost normoxic levels, and the PI3 inhibitors partially antagonised the effect of the PARP inhibitors (Fig. 5B and C).

When added alone, wortmannin and LY294002 did not significantly affect the moderate IR-induced phosphorylation (activation) of Akt-1 indicating that IR activates Akt-1 through

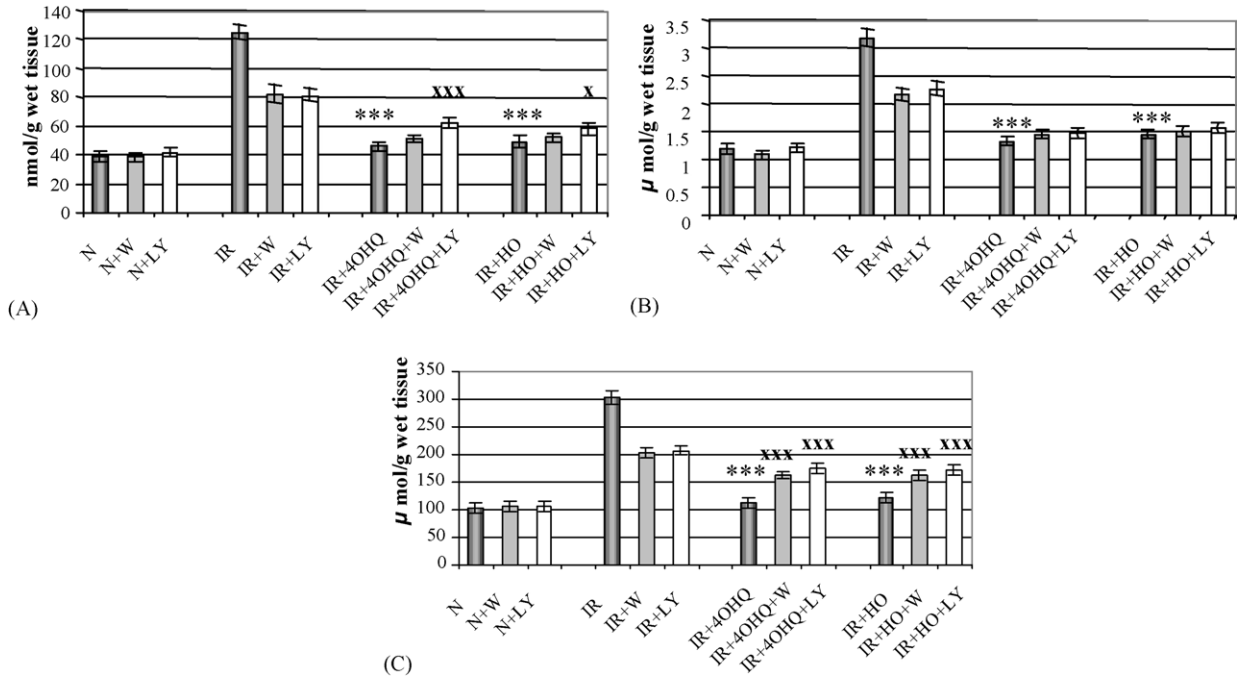
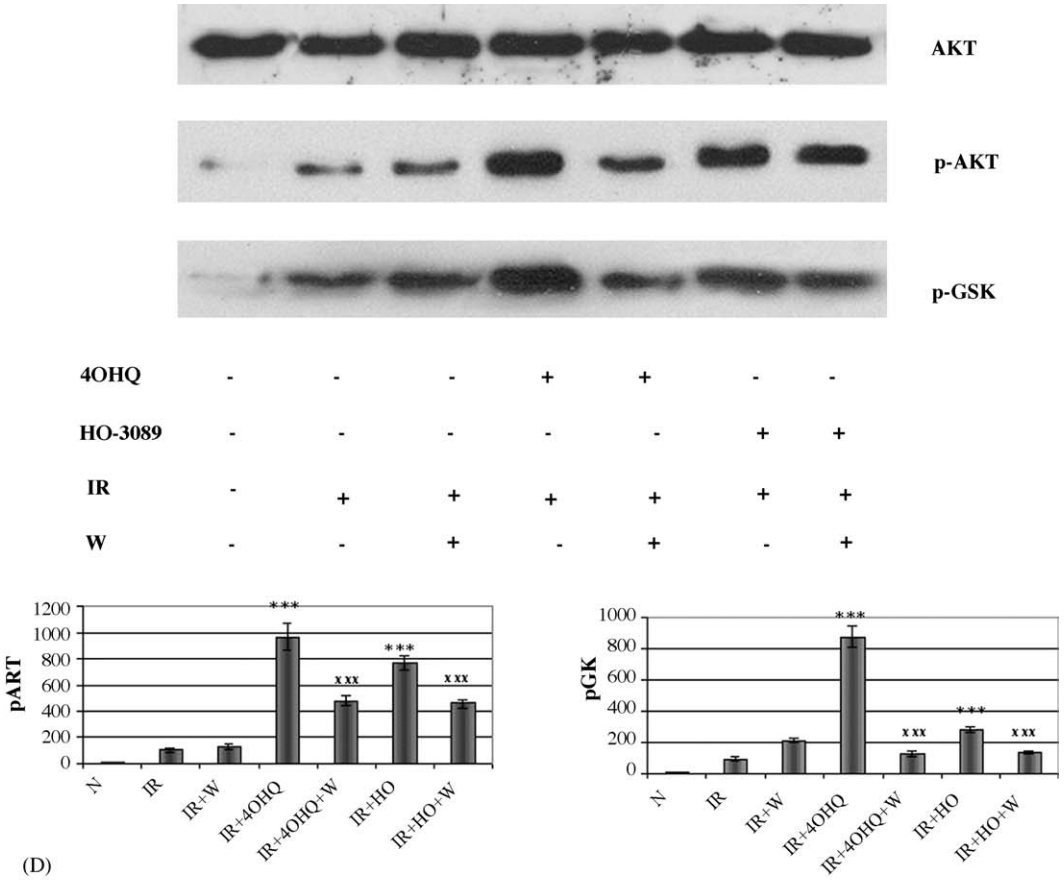


Fig. 5 – PI3-kinase inhibitors antagonize the effects of PARP inhibitors on oxidative stress and Akt phosphorylation in postichemic hearts. Lipid peroxidation (A), protein carbonyl content (B) and total peroxide concentration (C) of perfused hearts. (D and E) The phosphorylation state of Akt and GSK-3β upon treatment with PARP inhibitors and/or PI3-kinase inhibitors. Values are given as means ± S.E.M. for four (D and E) or five (A–C) experiments. Phospho-Akt and phospho-GSK signals were normalised to total Akt protein content. For comparison, the total Akt contents are also presented. IR, ischemia–reperfusion without treatment; IR + 4OHQ, ischemia–reperfusion in the presence of 100 μM 4-hydroxyquinazoline; IR + HO, ischemia–reperfusion in the presence of 25 μM HO-3089; IR + W, ischemia–reperfusion in the presence of 100 nM wortmannin; IR + W + 4OHQ, ischemia–reperfusion in the presence of 100 nM wortmannin and 100 μM 4-hydroxyquinazoline; IR + W + HO, ischemia–reperfusion in the presence of 100 nM wortmannin and 25 μM HO-3089; IR + LY, ischemia–reperfusion in the presence of 10 μM LY294002; IR + LY + 4OHQ, ischemia–reperfusion in the presence of 10 μM LY294002 and 100 μM 4-hydroxyquinazoline; IR + LY + HO, ischemia–reperfusion in the presence of 10 μM LY294002 and 25 μM HO-3089. Significant difference from untreated IR hearts ($^{***}p < 0.001$). Significant difference from the PARP inhibitor-treated IR hearts ($^{x}p < 0.05$, $^{xx}p < 0.01$ and $^{xxx}p < 0.001$).



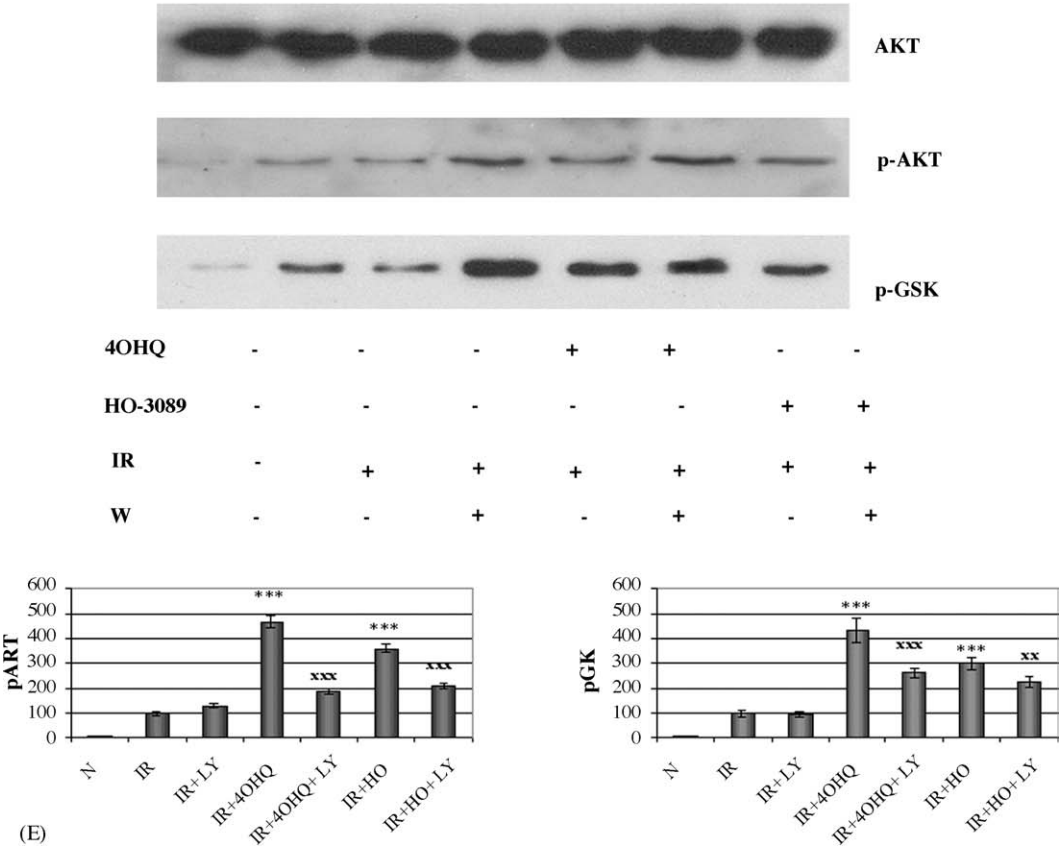


Fig. 5. (Continued).

a PI3-kinase-independent pathway. However, the administration of PARP inhibitors together with PI3-kinase inhibitors significantly increased Akt-1 phosphorylation, although these increases were much smaller than those observed in case of the PARP inhibitors alone (Fig. 5D and E). In addition, the ischemia–reperfusion-triggered slight increase in GSK-3β phosphorylation was not blocked by wortmannin or LY294002. Similarly to the Akt phosphorylation, the co-administration of PARP inhibitors and PI3-kinase inhibitors significantly attenuated GSK-3β phosphorylation compared to the effect of the PARP inhibitors alone (Fig. 5D and E).

4. Discussion

Poly(ADP-ribose) polymerase inhibitors protect hearts against IR injury [16,19], but the molecular mechanism of this protection remains to be elucidated. Since excessive activation of PARP can decompose NAD^+ to protein-bound ADP-ribose units and nicotinamide, it may culminate in ATP depletion and cardiomyocyte necrosis. In addition, during IR a considerable fraction of cardiac myocytes die in apoptotic cell death, but the role of PARP in this process is also unknown. Furthermore, we and others showed that PARP inhibitors protect mitochondria in postischemic heart [16,19,31], and decrease the degree of ROS production, which is predominantly a mitochondrial process in postischemic myocardium [16]. Recent works reported the existence of mitochondrial poly(ADP-ribose) polymerases which could be blocked with PARP-1 inhibitors [38]. Although, this might be involved in mitochondrial protection, several other pathways should also be considered.

We have previously demonstrated that PARP inhibitors induced the phosphorylation and activation of Akt in the liver, lung and spleen of lipopolysaccharide-treated mice, raising the possibility that the protective effect of PARP inhibition was, at least partially, mediated through the PI3-kinase/Akt pathway [30]. Similar data were also seen in neuronal cells [39]. These observations indicate that the protective effect of PARP inhibitors involve far more complexity than it is expected merely from NAD^+ and ATP depletion, because Akt kinase can phosphorylate several regulatory proteins, including GSK-3β, caspase-9, BAD or FKHR [24]. Phosphorylation and so inactivation of pro-apoptotic BAD protein contribute to the stabilization of mitochondrial membrane system and may prevent the release of pro-apoptotic proteins, i.e. cytochrome c or apoptosis-inducing factor [40]. Therefore, the mitochondrial-protective effect of PARP inhibitors can be mediated via the PI3-kinase/Akt/BAD pathway. Moreover, Akt can also phosphorylate and inactivate caspase-9, which can result in the blockade of cytochrome *c*/Apaf-1/caspase-9/caspase-3 pathway [41], further emphasizing the potential importance of Akt activation in the protective effects of PARP inhibitors.

Here, we characterized the PARP inhibitory property of well-established and a novel PARP inhibitor in vitro, in cell culture and in perfused hearts. These PARP inhibitors improved the recovery of creatine phosphate, ATP and pH, and the reutilization of inorganic phosphate in hearts subjected to ischemia–reperfusion. The PARP inhibitors limited the oxidative myocardial damage, which was characterized by decreased lipid peroxidation, total peroxide content and protein oxidation. Furthermore, the favorable changes in cardiac energetics were accompanied by improved recovery of functional performance and reduced infarct size.

Under the same experimental conditions, PARP inhibitors elicited Akt phosphorylation. We showed that this phosphorylation event was associated with Akt activation, because the downstream Akt substrate, GSK-3β was simultaneously phosphorylated. Although, these data demonstrated the activation of Akt upon PARP inhibitor administration, they did not provide evidence that Akt activation played a considerable role in the protective effect of PARP inhibitors.

PI3-kinase inhibitors significantly, albeit not completely, diminished the Akt and GSK-3β phosphorylation in the presence of PARP inhibitors indicating that these compounds could penetrate the heart and that a significant portion of Akt phosphorylation occured via the PI3-kinase pathway. Inhibition of the PI3-kinase/Akt pathway in the presence of PARP inhibitors significantly reduced the recovery of creatine phosphate, ATP and pH, and the reutilization of inorganic phosphate suggesting that Akt activation significantly contributed to the restoration of energy homeostasis of the reperfused myocardium. This phenomenon might be explained by the beneficial effects of Akt on the preservation of mitochondrial membrane integrity. In accordance with this view, PI3-kinase inhibitors compromised the protective effect of PARP inhibitors on infarct size and on the recovery of heart function. Wortmannin or LY294002 alone did not exert significant effect on the recovery of postischemic energy metabolism, although these compounds attenuated myocardial oxidative damage with an unknown mechanism. Furthermore, PI3-kinase inhibition hardly influenced Akt phosphorylation, even five-fold concentrations of wortmannin or LY294002 failed to completely block Akt phosphorylation during IR. Thus, the low phosphorylation level of Akt seen in postischemic hearts may occur in a PI3-kinase-independent way. In contrast, PARP inhibitor-elicited Akt phosphorylation overwhelmingly occurred through PI3-kinase, because PI3-kinase inhibition could block this event. Since decreased Akt activation significantly reduced the protective effects of PARP inhibitors, we suggest that Akt activation and subsequent events contribute to a significant extent to the cardioprotective effect of PARP inhibitors in postischemic hearts.

In conclusion, we provided evidences for undermining the original view that cytoprotection by PARP inhibitors rely exclusively on the preservation of NAD^+ and consequently the ATP stores in oxidative stress. Our data established that Akt activation and related processes are at least equally important in the cardioprotective effects of PARP inhibitors during ischemia–reperfusion.

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