Enhanced Akt activation by poly(ADP-ribose)polymerase inhibitors in postischemic hearts

Ph.D. thesis

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List of abbreviations

ASK1	apoptosis signal-regulating kinase-1
AP-1	activator protein-1
ATF-2	activator transcription factor-2
ERK	extracellular signal-regulated kinase
eIF2B	eukaryotic initiation factor 2B
eNOS	endothelial nitric oxide synthase
FKHR	forkhead transcription factor
GSK-3β	glycogen synthase kinase-3β
HR	heart rate
JNK	c-jun N-terminal kinase
LVDP	left ventricular developed pressure
NAD	nicotinamide adenine dinucleotide
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor-κB
NMR	nuclear magnetic resonance
PARP	poly(ADP-ribose) polymerase
PI3K	phosphatidylinositol-3-kinase
ROS	reactive oxygen species
RPP	rate-pressure product
TBARS	thiobarbituric acid reactive substances

Introduction

Role of poly(ADP-ribose) polymerase in myocardial ischemia-reperfusion injury

Acute coronary occlusion is the leading cause of morbidity and mortality in the Western world, and according to the World Health Organisation, will be the major cause of death in the world as a whole by the year 2020 (1). The impact of primary prevention strategies may be limited, particularly in the developing countries. There is therefore an urgent need for effective treatments, which will limit the extent of an evolving myocardial infarction during the acute phase, since infarct size is directly correlated with subsequent morbidity and mortality. Prolonged periods of myocardial ischemia can result in tissue injury and cell death. At present, reperfusion is the only means of salvaging ischemic myocardium and limiting infarct development. The two major clinical approaches to reperfusion are the use of thrombolytics and coronary angioplasty. Reperfusion, although essential for tissue salvage, (2) can, paradoxically, also lead to increased cell mortality, possibly as a result of the inflammatory response, the associated neutrophil accumulation, and oxidant formation (3). Ischemia-reperfusion is associated with enhanced formation of reactive oxygen species (ROS) and peroxynitrite, which initiate lipid peroxidation, protein oxidation, enzyme inactivation as well as DNA break formation (4). Poly(ADP-ribose) polymerase (PARP) is a protein-modifying and nucleotide-polymerizing enzyme that is present abundantly in the nucleus. In response to DNA damage PARP becomes activated and, using nicotinamide adenine dinucleotide (NAD^+) as a subsrate, it builds up homopolimers of adenosine diphosphate ribose units. This process rapidly depletes the intracellular NAD^+ and ATP energetic pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction and death (5,6). Accordingly, inhibition of PARP can improve the recovery of different cells from oxidative injury (7).

In heart tissue, a dominant fraction of energy production occurs in the mitochondria, therefore protection against oxidative damage of mitochondria can be a very important step in the normalization of cardiac energy production. Our previous data showed that PARP inhibitors were able to reduce the oxidative damage of cellular components without having any obvious scavenger activity (4). Although necrosis is

responsible for a large portion of cell loss during cardiac ischemia-reperfusion (8), it has been proven that apoptosis also occurs (9,10). Therefore, apoptosis may provide a new target for cardioprotection during evolving acute myocardial infarction in humans.

Signal transduction pathways during ischemia-reperfusion

Several protein kinase cascades and inflammatory reactions have recently become established as part and parcel of any external stress-related tissue injury such as ischemia-reperfusion, and other oxidative, metabolic, toxic as well as infectious insults. 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 phosphoinositol-3-kinase (PI3-kinase)-dependent manner (11). PI3-kinase pathway is one of several signal transduction pathways implicated in cell survival (12,13). Akt, in turn, phosphorylates a number of downstream targets leading to the inactivation of glycogen synthase kinase-3β (GSK-3β), the pro-apoptotic Bcl-2 family member Bad (14), caspase-9 (15), Forkhead transcription factor (13), as well as to the activation of nuclear factor-κB (NF-κB) (16), p70 ribosomal S6 kinase and endothelial nitric oxide synthase (eNOS) (17,18).

Inactivation of GSK-3β will allow glycogen synthase to build up cellular glycogen stores, eukaryotic initiation factor 2B (eIF2B) to launch the synthesis of various proteins as well as cyclin D1 to facilitate cell cycle progression (19). Two Bad molecules constituting a homodimer can contribute to the release of cytochrome c from the external side of the inner mitochondrial membrane into the cytoplasm, where the free cytochrome c triggers apoptotic cell death with the participation of caspase-9. Bad homodimer formation is prevented either through the dimerization with Bcl-2 anti-apoptotic molecule or by Bad phosphorylation, for instance by Akt, directing it toward degradation (20). Moreover, endothelial nitric oxide synthase activated by Akt forms nitric oxide causing vasorelaxation (17,18). The overall impact of Akt action is thus a remarkable antiapoptotic effect, metabolic adjustment and vasodilation, each of which inevitably promotes cell survival.

Wortmannin is a fungal metabolite having a sterol-type structure, passes into cells by simple diffusion, and irreversibly binds to and blocks the 110-kDa catalytic subunit of PI3-kinase (21,22). Previous studies have shown that wortmannin inhibits

superoxide release, adherence and chemotaxis of polymorphonuclear leucocytes (23-25). However, the effects of wortmannin have not yet been studied in myocardial ischemia/reperfusion injury in the presence of PARP inhibitors. LY294002 is another potent PI3-kinase inhibitor.

Mitogen-activated protein kinase (MAPK) cascades constitute an integrated part of stress-related signaling routes and include three distinct but interlinked pathways: extracellular signal-regulated kinase (ERK½), c-jun N-terminal kinase (JNK) and p38-MAPK (26,27). Although ERK is mainly involved in growth factor-induced mitogen signaling and cellular differentiation, the kinase can exhibit protective roles under circumstances of oxidative stress through the inhibition of apoptosis by downregulating JNK as well as caspase-3 (28). ERK is also brought into connection with delayed cytoprotection, as so its sustained activation seems to mediate late cardiomyocyte protection after simulated ischemia-reoxygenation (29).

Stress-responsive kinases, i.e. JNK and p38-MAPK, are implicated in triggering apoptotic cell death in response to oxidative or cytokine (TNF, Fas ligand) stress (26). Apoptotic signal-regulating kinase-1 (ASK-1) plays a pivotal role in the induction of both protein kinases regarding reactive oxygen species challenge (30). JNK and p38-MAPK are capable of initiating the apoptotic caspase cascade as well as the activation of the heterodimeric transcription factor activator protein-1 (AP-1). The aforementioned kinases can phosphorylate the components of AP-1, such as c-jun, c-fos and ATF-2, and as so elicit the expression of a diverse array of genes modulating apoptotic, inflammatory and stress responses (31). Nevertheless, p38-MAPK also has downstream substrates such as MAPKAPK2 (MAPK-activated protein kinase 2) and heat shock protein 27 are accounted for as elements of cytoprotection afforded by ischemic preconditioning (32).

Figure 1 gives an outline of the aforementioned signal transduction pathways that may participate in the mediation of ischemia-reperfusion injury.

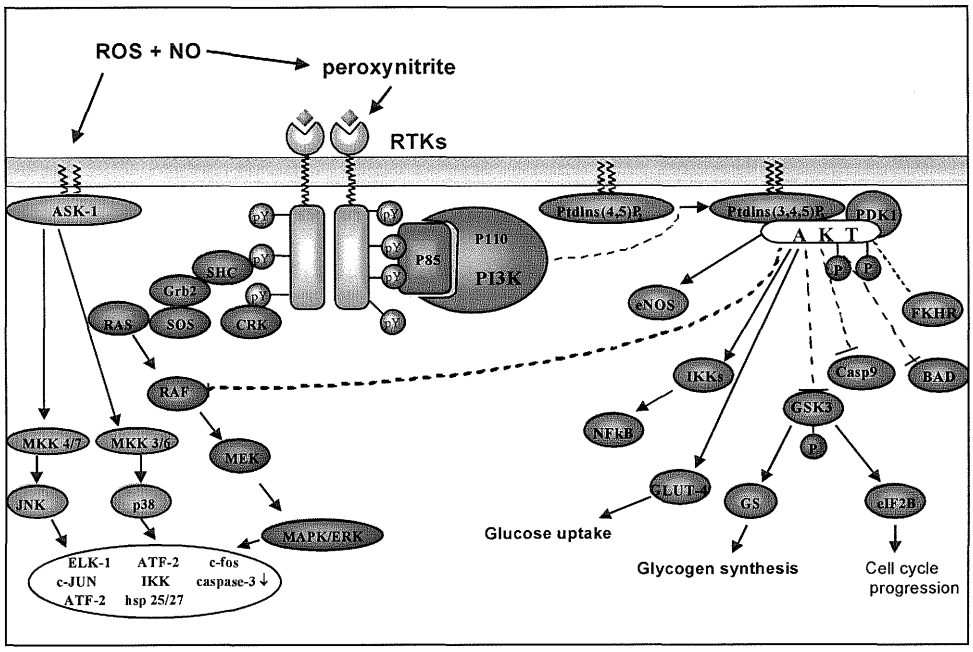


Figure 1. Illustration of the complexity of some ischemia-reperfusion-related signal transduction pathways and their possible connection to reactive oxygen species formation. *Peroxynitrite formed from superoxide anion and nitric oxide can activate receptor tyrosine kinases, which, in turn, leads to the activation of PI3-kinase recruiting Akt to the proximity of cell membrane and facilitating Akt phosphorylation. As a consequence, Akt phosphorylates a number of substrates resulting in antiapoptotic effects, metabolic adjustment and nitric oxide formation by eNOS. On the other hand, active receptor tyrosine kinases may also culminate in ERK activation (caspase-3 and JNK downregulation). Reactive oxygen species can induce p38-MAPK and JNK activation through ASK-1, as well as, having basically proapoptotic impacts.*

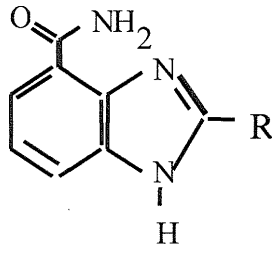
Study objectives

In this study we investigated the molecular mechanism of a known PARP inhibitor (4-hydroxyquinazoline) and an experimental compound exhibiting scavenger and PARP inhibitor properties (HO-3089) on the cardiac pathophysiology under conditions of ischemia-reperfusion in an isolated heart perfusion system, including the monitoring of myocardial energy metabolism, cardiac contractile function, as well as measuring the infarct size. Furthermore, we studied the effects of PARP inhibitors on the ischemia-reperfusion-induced oxidative myocardial injury, i.e. lipid peroxidation, protein oxidation, and total peroxide concentration. We found that ischemia-reperfusion activated Akt, therefore we have assessed the ability of PARP inhibitors to influence the phosphorylation of Akt. Finally, we used PI3-kinase inhibitors to see how they could affect the cardioprotective impacts of PARP inhibitors.

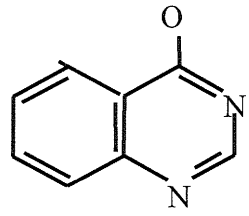
Materials and Methods

Chemicals

4-hydroxyquinazoline, LY294002, thiobarbituric acid and 2,4-dinitrophenylhydrazine were purchased from Sigma-Aldrich Chemical Co. (Budapest, Hungary). HO-3089 was a kind gift of Prof. Kalman Hideg. Figure 2 shows the chemical structure of both examined PARP inhibitors. Wortmannin was purchased from Calbiochem (Darmstadt, Germany). All other reagents were of the highest purity commercially available.



HO-3089



4OHQ

Figure 2. Chemical structure of the applied PARP inhibitors

PARP inhibition

The IC_{50} of 4-hydroxyquinazoline and HO-3089 was studied in an *in vitro* assay as described before (30).

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 2mM pyruvate in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Before reaching confluence, the cells were split, plated at low density in culture dishes (approx. 2×104 cell/well) and cultured for 24 hours. Cardiomyocytes were then incubated without (negative control) and with 1mM hydrogen peroxide for three hours 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 (33). Briefly, the cells were incubated for 3 hours 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 550nm wavelength after dissolving the blue formasan precipitate in 10% sodium dodecyl sulphate. All experiments were run in at least 4 parallels and repeated 3 times.

Animals

Hearts of adult male Wistar rats weighing 300-350 g were used for Langendorff heart perfusion experiments. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pecs, Medical School.

Heart perfusion

Rats fed with standard diet were heparinized with sodium heparin (100 IU /rat IP) and anesthetized with ketamine (200 mg/kg IP). Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 Hgmm, at 37°C as described before (33). The perfusion medium was a modified phosphate free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.25 $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose and 0.6 mM octanoic acid without or with PARP inhibitors (100 μM 4-hydroxyquinazoline or 25 μM HO-3089), and/or wortmannin (100 nM) or LY294002 (10 μM). The perfusate was adjusted to pH 7.40 and bubbled with 95% O_2/5% CO_2 through a glass oxygenator. After a washout (non-recirculating period of 10 minutes) hearts were perfused under normoxic conditions for 10 minutes and were subjected to a 30–minute global ischemia by closing the aortic influx and reperfused for 15, 45, or 90 minutes. The above mentioned compounds were administered into the perfusion medium at the beginning of normoxic perfusion. During ischemia hearts were

submerged into perfusion buffer at 37°C. At the end of each perfusion hearts were freeze-clamped.

NMR spectroscopy

NMR spectra were recorded with a Varian ^{UNITY}INOVA 400 WB instrument. ^{31}P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z•SPEC® 20-mm broadband probe (Nalorac Co., Martinez, CA, USA) applying GARP-1 proton decoupling ($\gamma B2$= 1.2 kHz) during acquisition. Field homogeneity was adjusted by following the ^{1}H signal ($w½$ = 10-15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each FID. 45° flip angle pulses were employed after a 1.25 s recycle delay, and transients were acquired over a 10 kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16,384. Under the above described circumstances the relative concentrations of the species can be taken proportional to the peak areas, because interpulse delays exceeded 4-5 x T1 values of the metabolites to be analyzed in ^{31}P experiments. The pH value in the myocardium was calculated by the inorganic phosphate-chemical shift (δ) from the creatine phosphate peak according to the following equation: $pH= 6.77 + \log [(\delta-3.23)/(5.70-\delta)]$.

Determination of heart function

A latex balloon was inserted into the left ventricle through the mitral valve and filled to achieve an end-diastolic pressure of 8-12 mmHg. All measurements were performed at the same balloon volume. Hearts were selected on the basis of the stability of high-energy phosphates (assessed by NMR) during a control period of 15 minutes before the experiment. The length of normoxia, ischemia, and reperfusion were 15, 30, and 45 minutes, respectively. PARP inhibitors were added to the perfusion medium after the 15-minute control period. Functional data of rat hearts (LVDP – left ventricular developed pressure, RPP – rate-pressure product, HR – heart rate, and dP/dt) were monitored during the entire perfusion.

Myocardial infarct size

Hearts was removed from the Langendorff apparatus. Both the auricles and the aortic root were excised out and ventricles were kept overnight at −4°C. Frozen ventricles

were sliced into uniform sections of about 2-3 mm thickness. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC) at 37°C in 0.2 M Tris buffer (pH=7.40) for 30 min (34). The normal myocardium was stained brick red, while the infracted portion remained unstained. Infarct size was measured by volume and weight method as described earlier (35).

Determination of total peroxide concentration

Hundred milligrams of heart tissue were homogenized 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 the samples, peroxide concentrations of the supernatants were measured by means of Biomedica OxyStat assay (Biomedica GmbH, Wien, Austria).

Lipid peroxidation

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined using a modification of a described method (36). Cardiac tissue was homogenized in 6.5% trichloroacetic acid and a reagent containing 15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25% HCl was added, mixed thoroughly, heated for 15 min in a boiling water bath, cooled, centrifuged, and the absorbance of the supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Using malondialdehyde standard, TBARS were calculated as nmol/g wet tissue.

Determination of protein carbonyl content

Fifty milligrams of freeze-clamped perfused heart tissue were homogenized with 1 ml of 4% perchloric acid and the protein content was collected by centrifugation. The protein carbonyl content was determined by using the 2,4-dinitrophenylhydrazine method (37).

Western blot analysis

Fifty milligrams of heart samples (hearts with both 15 and 45 minutes of reperfusion) were homogenized in ice-cold Tris buffer (50 mM, pH=8.0) and harvested in 2x

concentrated SDS-polyacrylamide gel electrophoretic sample buffer. Proteins were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. After blocking (2 hours with 3% non-fat milk in Tris buffered saline) membranes were probed overnight at 4°C with antibodies recognizing the following antigens: phospho-specific Akt-1 / protein kinase B-α Ser^{473} (1:1000 dilution; Cell Signaling Technology, Beverly, USA), non-phosphorylated Akt / PKB (1:1000), phospho-specific glycogen synthase kinase (GSK)-3β Ser^{9} (1:1000). Membranes were washed six times for 5 minutes in Tris buffered saline (pH=7.5) containing 0.2% Tween (TBST) prior to addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution, BioRad, Budapest, Hungary). Membranes were washed six times for 5 minutes in TBST and the antibody-antigen complexes were visualized by means of enhanced chemiluminescence. The results of Western blots were quantified by means of Scion Image Beta 4.02 program.

Statistical analysis

Statistical analysis was performed by analysis of variance and all of the data were expressed as the mean ± S.E.M. Significant differences were evaluated by use of unpaired Student's *t* test and *P* values below 0.05 were considered to be significant.

Results

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., 1981). 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 hours), HO-3089 in the nanomolar, while 4-hydroxyquinazoline in the micromolar concentration range (Fig. 1C-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.

Fig. 3 A.

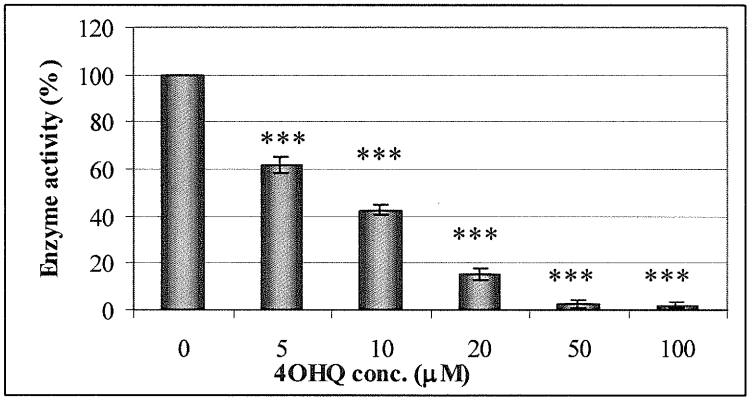


Fig. 3 B.

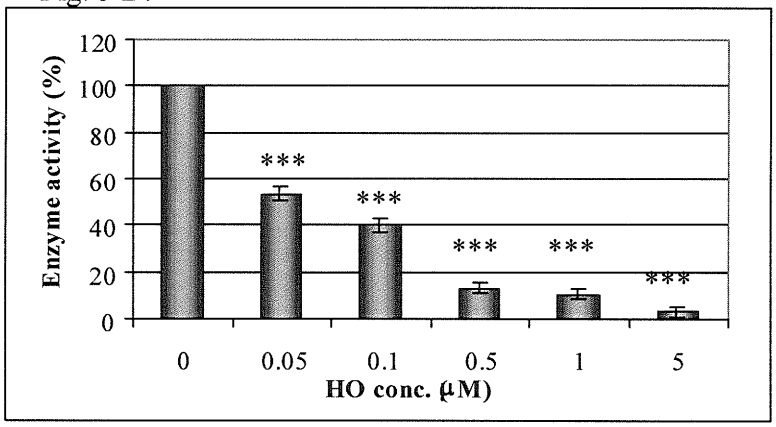


Fig. 3 C.

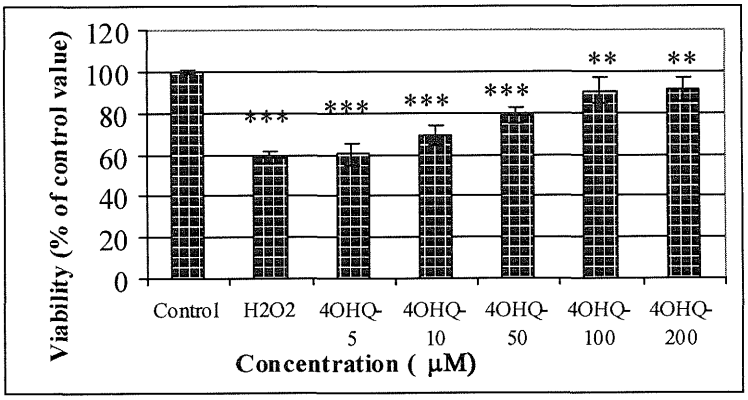


Fig. 3 D.

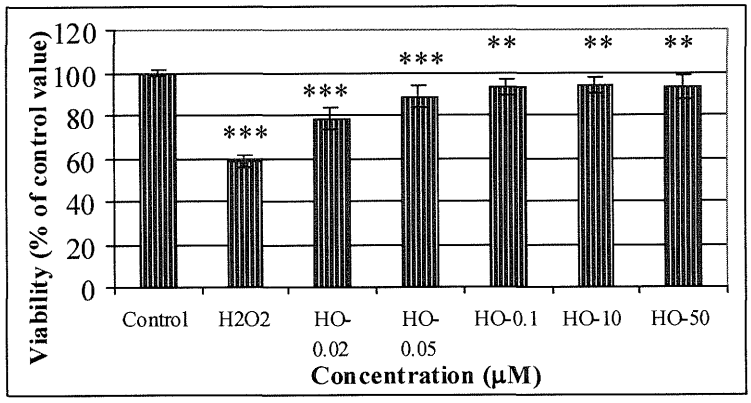


Fig. 3 E.

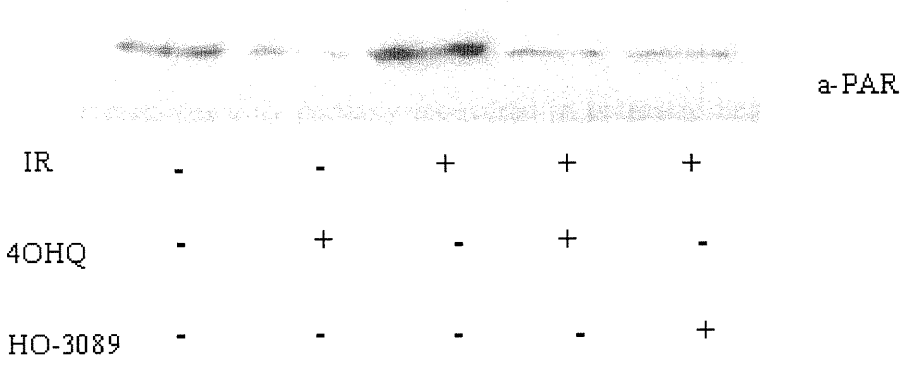


Figure 3. Poly(ADP-ribose) polymerase inhibitory effect of 4-hydroxyquinazoline and HO-3089 in vitro and in isolated hearts. A-B: *Effect of 4-hydroxyquinazoline (A) and HO-3089 (B) on the activity of isolated poly(ADP-ribose) polymerase activity.* ***C-D:*** *Cytoprotective effect of 4-hydroxyquinazoline (C) and HO-3089 (D) in H9C2 cells in the presence of 1 mM H_2O_2.* ***E:*** *Inhibitory effect of 4-hydroxyquinazoline and HO-3089 on the self-ADP-ribosylation of PARP in Langendorff-perfused rat hearts during ischemia-reperfusion*

Values given as means ± SEM for five experiments.

IR: ischemia-reperfusion without treatment; 4OHQ: 4-hydroxyquinazoline treatment; HO: HO-3089 treatment, using the indicated μM concentrations.

*** Significant difference from samples without the PARP inhibitor ($p<0.01$).*

**** Significant difference from samples without the PARP inhibitor ($p<0.001$).*

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. 4 A.) and ATP (Fig. 4 B.). According to these data, both PARP inhibitor 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. 4 C.). 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 min 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. 4 D.).

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 HO-3089. At the end of the normoxic period, LVDP was 135.2±16.4 mmHg, RPP was $3.4\pm0.15\times10^4$ mmHg/min, dP/dt_{max} was 1310±196 mmHg/s and the average heart rate was 217±19 beats/min. As Fig. 4 E. 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. 4 F.). In accordance with our previous reports all the significant changes in the function and metabolism of the perfused hearts occurred within 15 minutes and there were no significant changes afterwards up to one hour of reperfusion.

Fig. 4 A.

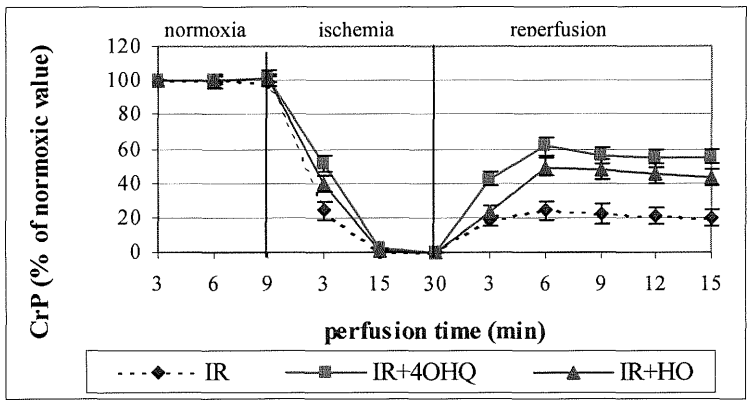


Fig. 4 B.

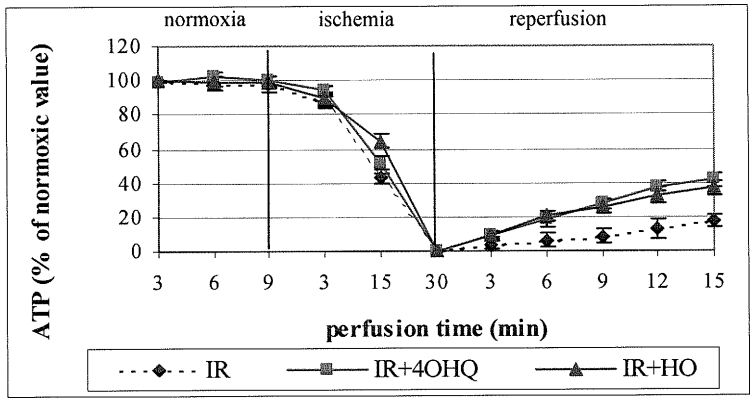


Fig. 4 C.

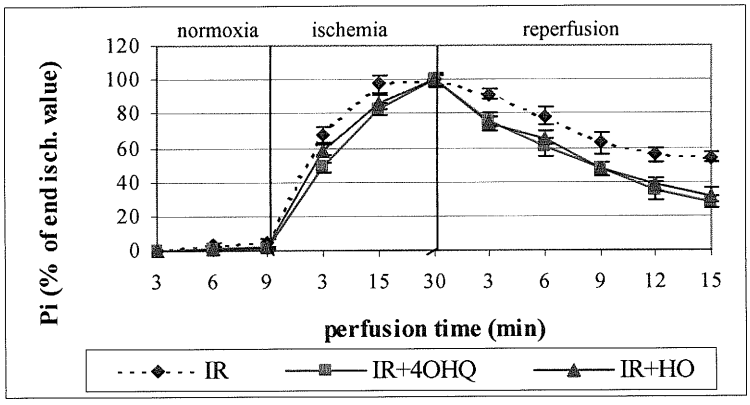


Fig. 4 D.

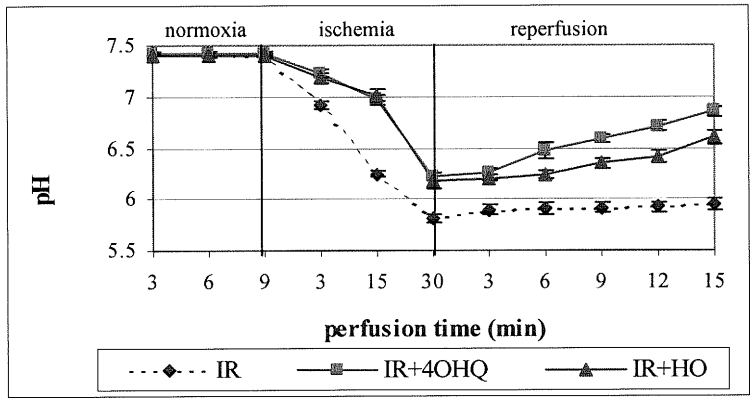


Fig. 4 E.

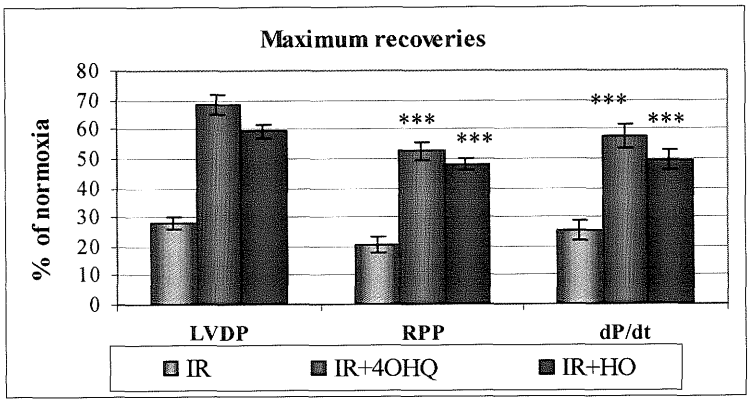


Fig. 4 F.

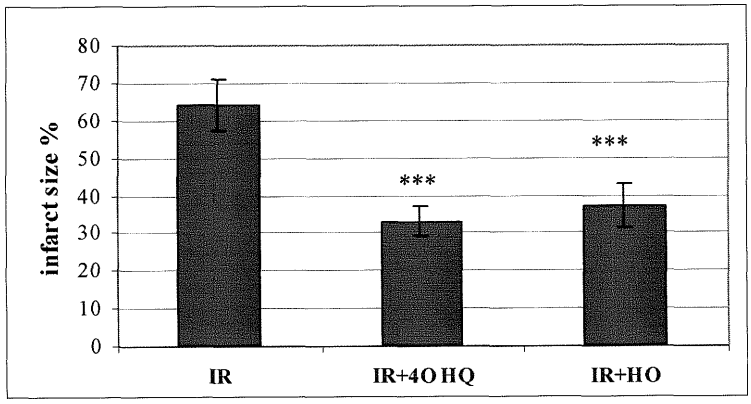


Figure 4. Salutary effects of PARP inhibitors on the energy metabolism, heart function and infarct size of postichemic hearts. *Time-course of creatine phosphate (CrP; **A**), ATP (**B**), inorganic phosphate (Pi; **C**), and pH levels (**D**) during ischemia-reperfusion. **E:** Maximal percentage recovery 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 ± SEM 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 ($p<0.001$).*

Attenuation of cardiac oxidative damage by PARP inhibitors

Under our experimental conditions, ischemia-reperfusion increased the amount of thiobarbituric acid reactive substances (TBARS) compared to the normoxic conditions (Fig. 5 A.). 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 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. 5 B. 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. 5 C.).

Fig. 5 A.

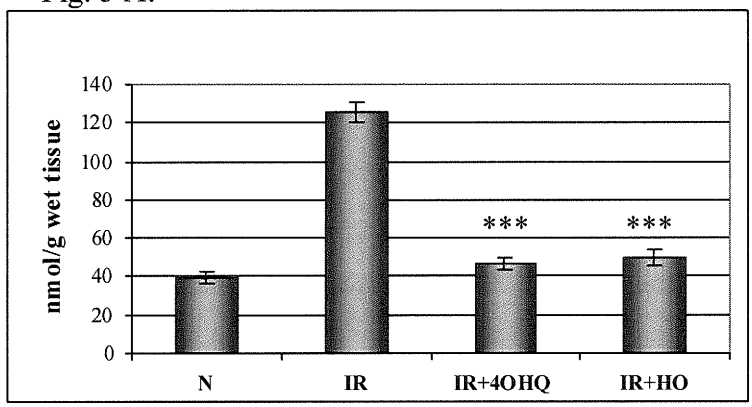


Fig. 5 B.

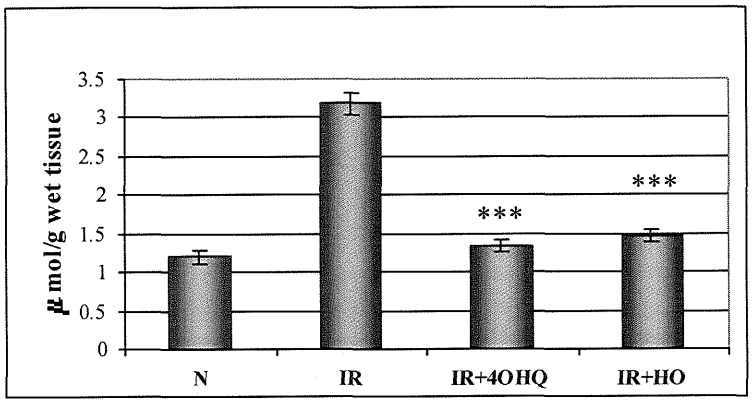


Fig. 5 C.

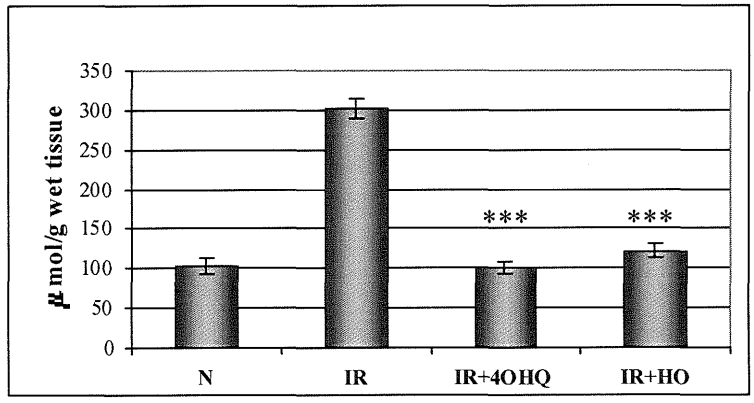


Figure 5. Effect of PARP inhibitors on oxidative stress and Akt pathway of postichemic hearts. *Lipid peroxidation (**A**), protein carbonyl content (**B**), and total peroxide concentration (**C**) of perfused hearts.*

Values are given as means ± SEM for five experiments.

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 ($p<0.001$).*

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 followed by 15-minute 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. 6.). 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.

Fig. 6.

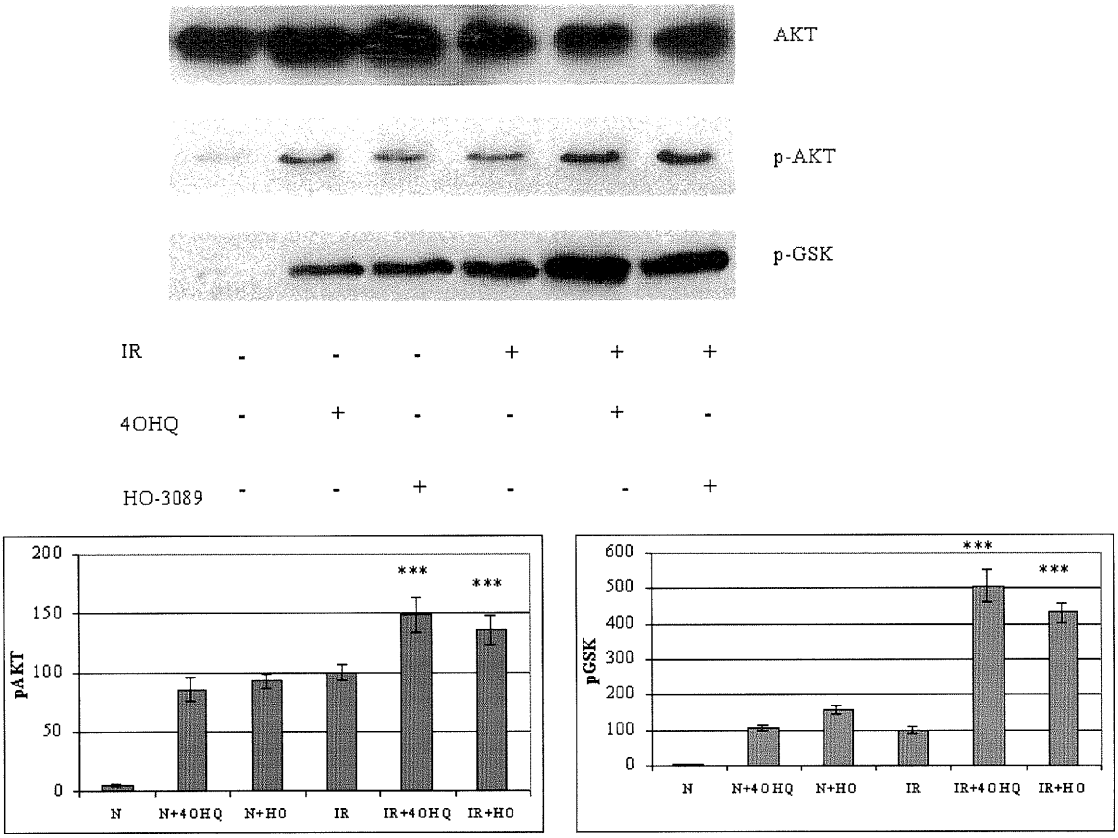


Figure 6. Effect of PARP inhibitors on Akt pathway of postichemic hearts. *Phosphorylation state of Akt and GSK 3β are shown on a representative blot of five experiments. Phospho-Akt and phospho-GSK signals were normalised to total Akt protein content. For comparison, the total Akt contents are also 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 ($p<0.001$).*

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. 7 A-B.) and inorganic phosphate levels. Furthermore, the PARP inhibitor-induced functional improvement was also significantly attenuated in the presence of PI3-kinase inhibitors (Fig. 7 C-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 HO3089 + wortmannin and 47.2±2.6% in HO-3089 + LY294002-treated hearts, Fig. 7 F.).

Fig. 7 A.

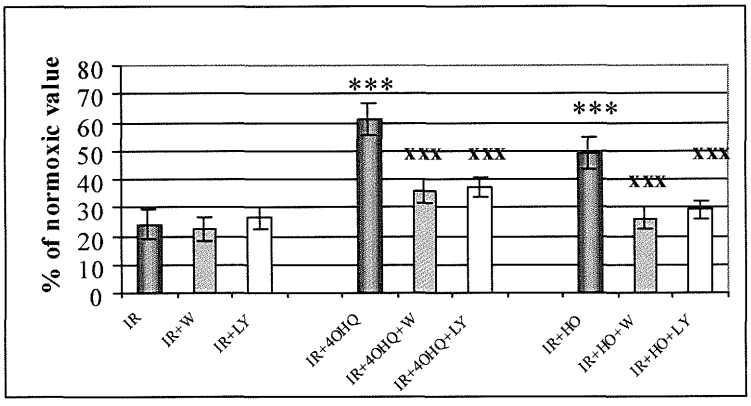


Fig.7 B.

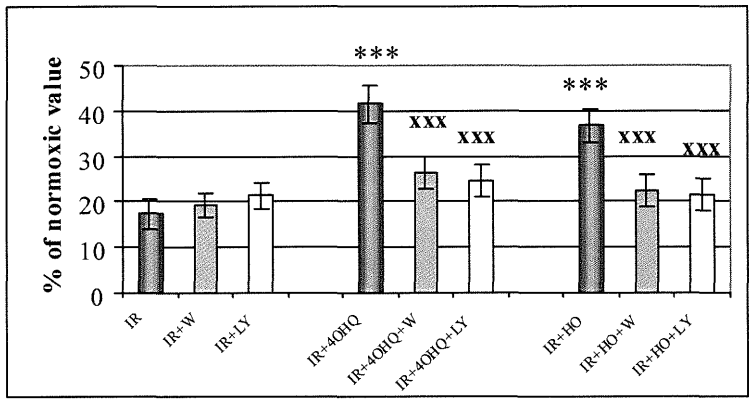


Fig. 7 C.

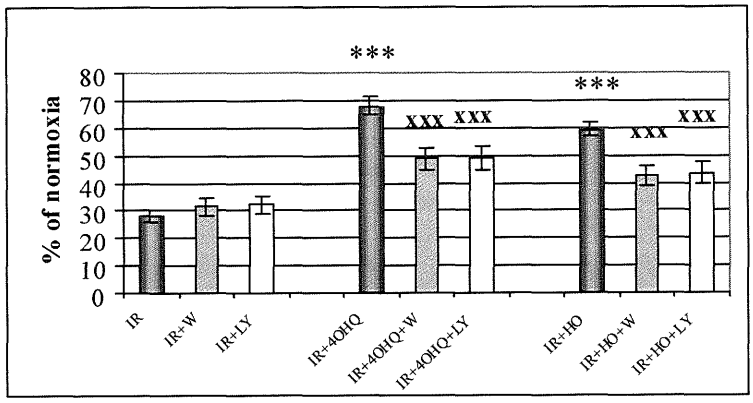


Fig. 7 D.

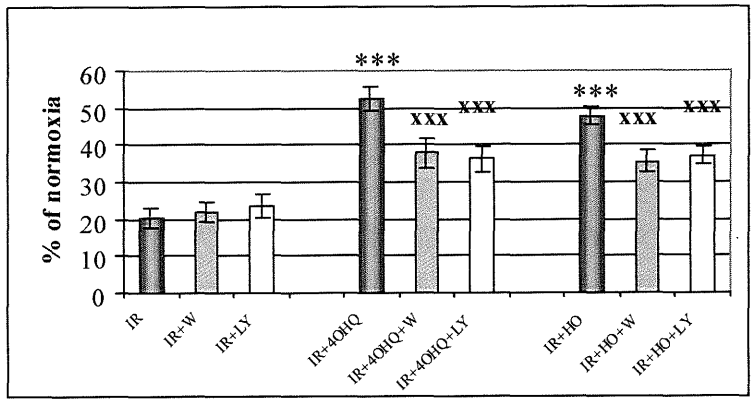


Fig. 7 E.

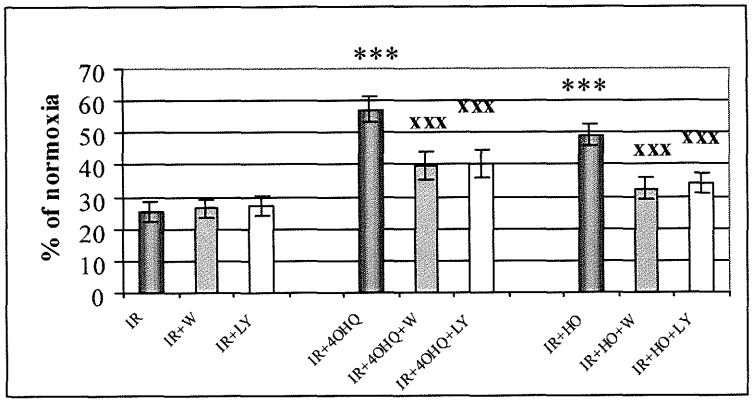


Fig. 7 F.

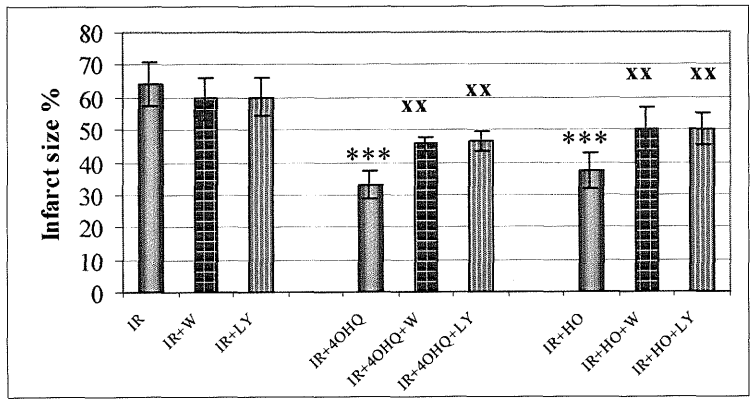


Figure 7. PI3-kinase inhibitors antagonize the protective effects of PARP inhibitors on energy metabolism, heart function and infarct size of postichemic hearts. *Maximal percentage recovery of creatine phosphate (CrP; **A**) and ATP levels (**B**) during ischemia-reperfusion. Maximal percentage recovery 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 ± SEM 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 ($p<0.001$)*

^{xx} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.01$)

^{xxx} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.001$)

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; 52.5±3.1% for HO3089 + wortmannin- and 58.7±4.3% for HO-3089 + LY294002- versus 49.5±4.1 for HO3089-treated hearts) (Fig. 8 A.). 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. 8 B. and C.).

Fig. 8 A.

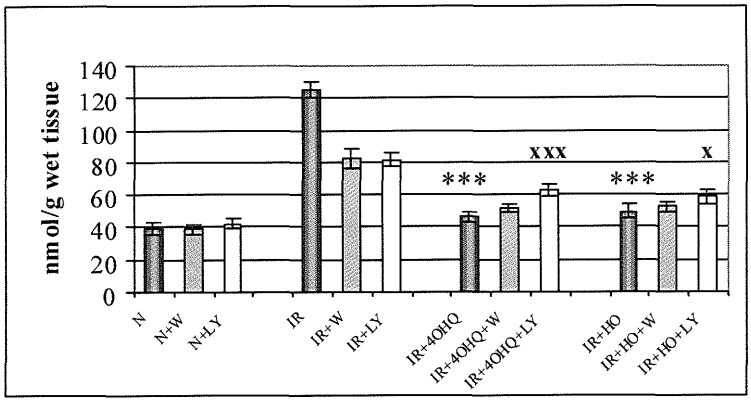


Fig. 8 B.

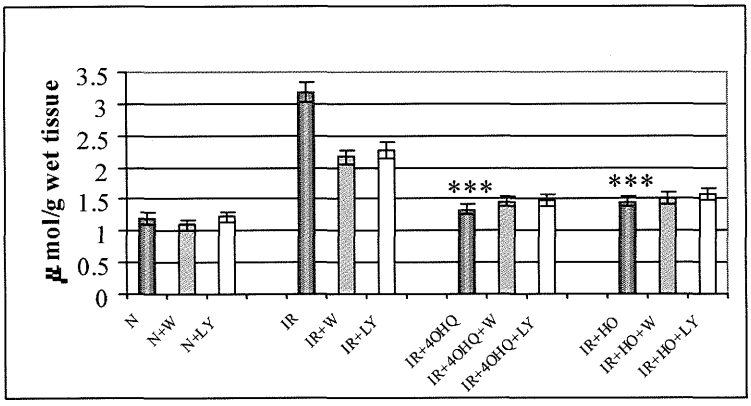


Fig. 8 C.

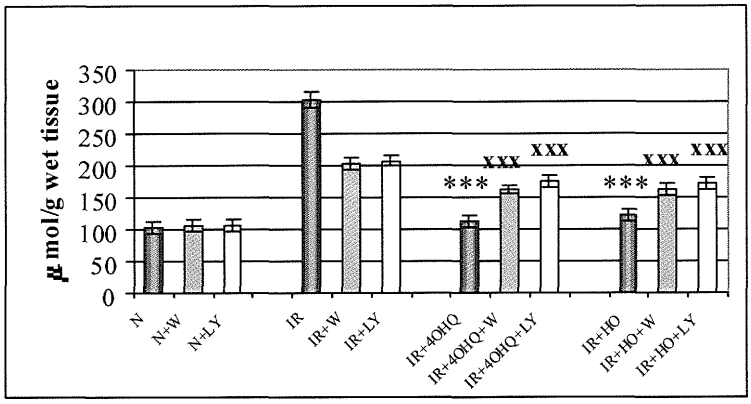


Figure 8. PI3-kinase inhibitors antagonize the effects of PARP inhibitors on oxidative stress in postichemic hearts. *Lipid peroxidation (**A**), protein carbonyl content (**B**), and total peroxide concentration (**C**) of perfused hearts.*

Values are given as means ± SEM 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 ($p<0.001$)*

^{x} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.05$)

^{xxx} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.001$)

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 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. 9-10.). 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. 9-10.).

Fig. 9.

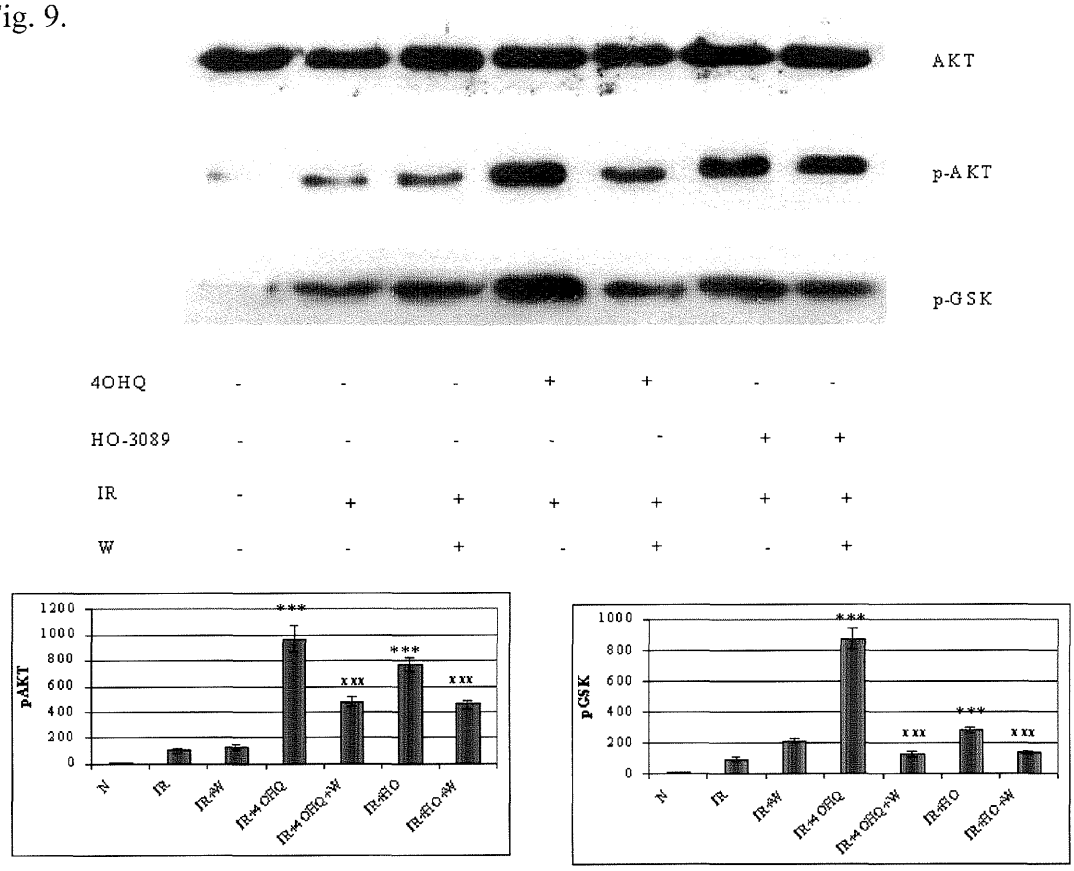


Figure 9. Wortmannin antagonize the effects of PARP inhibitors Akt phosphorylation in postichemic hearts. *The phosphorylation state of Akt and GSK 3β upon treatment with PARP inhibitors and/or wortmannin.*

Values are given as means ± SEM for four 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.

****Significant difference from untreated IR hearts ($p<0.001$)*

^{xx} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.01$)

^{xxx} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.001$)

Fig. 10.

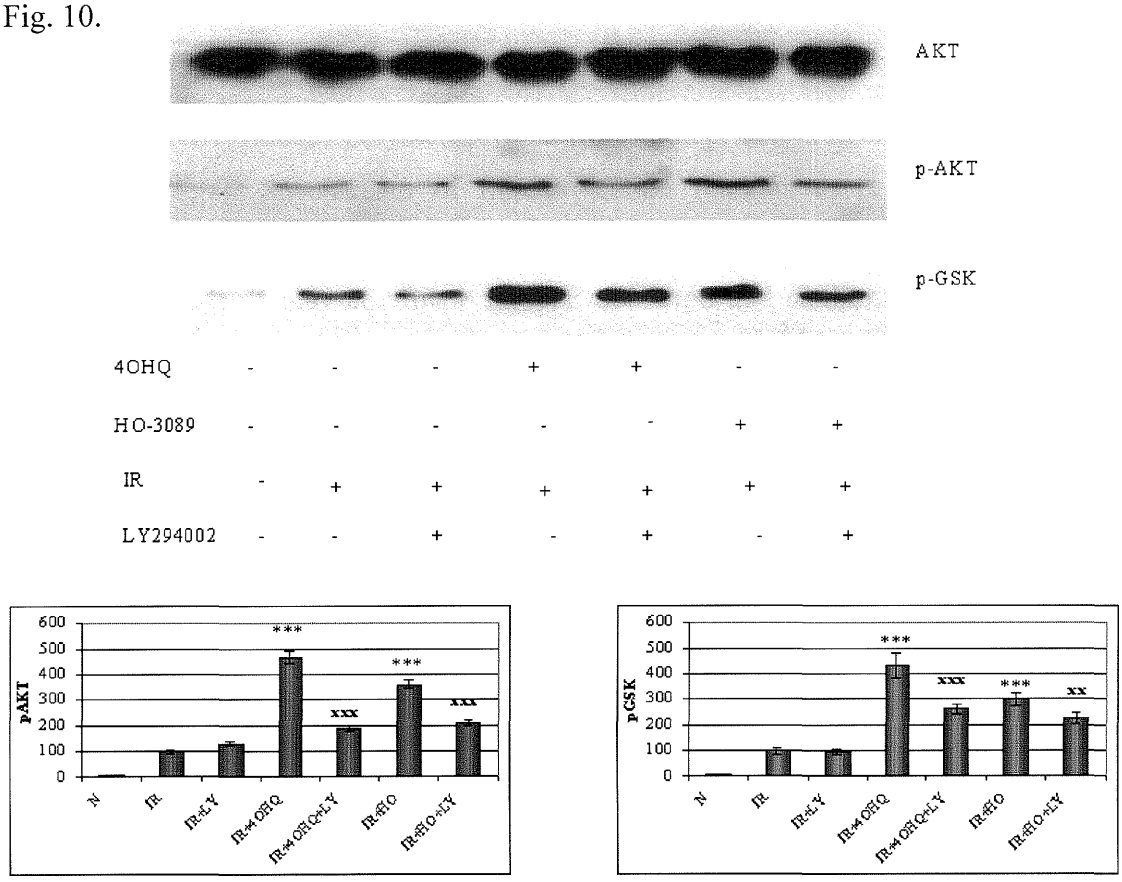


Figure 10. LY294002 antagonize the effects of PARP inhibitors on oxidative stress and Akt phosphorylation in postichemic hearts. *The phosphorylation state of Akt and GSK 3β upon treatment with PARP inhibitors and/or PI3-kinase inhibitors.*

Values are given as means ± SEM for four 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+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 ($p<0.001$)*

^{xx} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.01$)

^{xxx} Significant difference from the PARP inhibitor-treated IR hearts ($p<0.001$)

Discussion

Poly(ADP-ribose) polymerase inhibitors protect hearts against IR injury (4,7), 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 (4, 7, 30), and decrease the degree of ROS production, which is predominantly a mitochondrial process in postischemic myocardium (4). 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 (38). 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 (13). 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 down-stream 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 5-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.

Conclusions

Our data provide the first insight into how PARP inhibitors can influence the intracellular signal transduction pathways. Both examined PARP inhibitors preserved cardiac energy metabolism as well as cardiac contractile function during ischemia-reperfusion, and also attenuated the oxidative injury of the myocardium. Moreover, PARP inhibitor administration prompted the activation of Akt and the subsequent inactivation of glycogen synthase kinase-3β both during normoxic perfusion and ischemia-reperfusion. The phosphatidylinositol-3-kinase inhibitors wortmannin and LY294002 partially abrogated the beneficial effects of PARP inhibitors, concerning the myocardial energetics, contractile function, infarct size, oxidative damages as well as Akt activation. Taken together, protective effects of the examined PARP inhibitors may be in part attributable to their ability to upregulate the prosurvival Akt protein kinase cascade.

Although little is known about the precise triggers of ischemia-reperfusion-induced signaling pathways, it has been proposed that oxidative stress, mediated by ROS, may play an important role in this process. Our findings suggest that Akt activation occurs as a response to PARP inhibitor treatment and could play an important role in promoting cell survival. However, further studies are required to delineate the role of Akt activation and the detailed signaling mechanisms under conditions of various treatment agendas.

These promising results further add to our understanding of the executive mechanisms of how these compounds may confer their remarkable cardioprotection and attract additional efforts to elucidate the precise interplay between the molecules and the signaling elements.

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I. Papers serving as basis for the Ph.D. thesis

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II. Book chapters

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III. Additional papers

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IV. Published abstracts

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