Ph.D. thesis

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

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Introduction

Ischemia-reperfusion is associated with enhanced formation of reactive oxygen species (ROS), such as hydrogen-peroxide, superoxide and hydroxyl radicals. ROS can initiate lipid peroxidation, protein oxidation and the formation of DNA breaks. Single-strand DNA breaks are the obligatory triggers of PARP activation. In response to DNA damage, PARP becomes activated and, by cleaving NAD^+ as a substrate, catalyzes the building of homopolymers of adenosine diphosphate ribose units, resulting in a substantial depletion of intracellular NAD^+. As NADH functions as an electron carrier to the mitochondrial respiratory chain, NAD^+ depletion is coupled to a rapid fall in intracellular ATP levels. Thus, excessive activation of PARP leads to ATP depletion, which may ultimately cause cell death. As a consequence, inhibition of PARP can improve the recovery of different cells from oxidative injury.

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 capable of reducing the oxidative damage of cellular components without any obvious scavenger activity. Although necrosis is responsible for a large proportion of cell loss during cardiac ischemia-reperfusion, it has been proven that apoptosis also occurs. Therefore, apoptosis may provide a new target for cardioprotection during an evolving acute myocardial infarction in humans, even so since the apoptotic cells, in contrast to the necrotic ones are still alive, and consequently, can be rescued by reversing the apoptotic process.

Previous results indicate that the growth-factor-associated kinase Akt is phosphorylated following ischemia-reperfusion in a phosphoinositol-3-kinase (PI3-kinase)-dependent manner. PI3-kinase pathway is one of several signal transduction pathways implicated in cell survival. Akt, in turn, phosphorylates a number of downstream targets leading to the inactivation of glycogen synthase kinase-3 (GSK-3), the pro-apoptotic Bcl-2 family member Bad, caspase-9, Forkhead transcription factor, as well as to the activation of nuclear factor κB (NFκB) and endothelial nitric oxide synthase (eNOS). The overall impact of Akt action is thus a remarkable antiapoptotic effect, metabolic adjustment and vasodilation.

Previous results and our previous data demonstrate that PARP inhibitors protect hearts against ischemia-reperfusion injury.

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. We monitored myocardial energy metabolism, cardiac contractile function, and measured the infarct size. Furthermore, we studied the effect 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 the PARP inhibitors.

Materials and Methods

Chemicals

4-hydroxyquinazoline, 2,4-dinitrophenylhydrasine, LY294002 and thiobarbituric acid were purchased from Sigma-Aldrich Chemical Co. HO-3089 was synthesized in the Institute of Organic and Medicinal Chemistry (University of Pécs, Hungary). Wortmannin was purchased from Calbiochem. All other reagents were of the highest purity commercially available.

Animals

Male Wistar rats weighing 300-350 g were used for this study. Rats were handled in accordance with the *Guide for the Care and Use of Laboratory Animals*.

Heart perfusion

Rats were anaesthetized with 200 mg/kg ketamine intraperitoneally and heparinized with sodium heparin (100 IU/rat i.p.). Isolated rat hearts were perfused with a modified phosphate-free Krebs-Henseleit buffer according to the Langendorff method at a constant pressure of 70 mmHg, at 37ºC as described before. The perfusion medium contained 118 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose and 0.6 mM octanoic acid. 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 15 minutes), hearts were perfused for 10 min (baseline) and freeze-clamped to obtain ”normoxic” hearts, or were then subjected to 30-minute global ischemia by closing the aortic influx and reperfused for either 15, 45 or 90 minutes. PARP inhibitors and/or PI3-kinase inhibitors were administered (4OHQ in 100 μM and HO-3089 in 25 μM, wortmannin in 100 nM and LY294002 10 μM) into the medium at the beginning of baseline perfusion. During ischemia, hearts were submerged into perfusion buffer at 37ºC. At the end of the 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.

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 was 10, 30, and 45 minutes, respectively. PARP inhibitors were added to the perfusion medium after the 15-minute control period. Functional data of the hearts (LVDP – left ventricular developed pressure, RPP – rate pressure product and dP/dt) were monitored during the entire perfusion.

Infarct size

Hearts were removed from the Langendorff apparatus. Both the auricles and the aortic root were excised and the 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. The normal myocardium was stained brick red, while the infarcted portion remained unstained. Infarct size was measured by the volume and weight method.

Lipid peroxidation and protein oxidation

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS) using malondialdehyde standard. The protein carbonyl content was determined by using the 2,4-dinitrophenylhydrazine method.

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).

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; Cell Signaling Technology, Beverly, USA), phospho-specific glycogen synthase kinase (GSK)-3β Ser^{9} (1:1000; Cell Signaling Technology, Beverly, USA). 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

Effect of PARP inhibitors on the energy metabolism, contractile function, and cell death of hearts during ischemia-reperfusion

Energy metabolism of Langendorff perfused hearts was monitored in the magnet of NMR spectroscope enabling to detect changes in the level of high-energy phosphate intermediates. Ischemia induced a rapid decrease in creatine phosphate and ATP levels and a fast evolution of inorganic phosphate. Under our experimental conditions, high-energy phosphate intermediates only partially recovered in untreated hearts during the 45-minute reperfusion phase; on the other hand, HO-3089 and moreover 4-hydroxyquinazoline facilitated the recovery of creatine phosphate and ATP. These data show that each PARP inhibitor could significantly improve the final recovery of high-energy phosphate intermediates (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). 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).

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. 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).

Effect of PARP inhibitors on cardiac oxidative damage and ischemia-reperfusion-related Akt phosphorylation

Lipid peroxidation induced by ischemia-reperfusion in Langendorff perfused hearts was characterized by the formation of thiobarbituric acid reactive substances (TBARS). Under our

experimental conditions, ischemia-reperfusion increased the amount of TBARS compared to the normoxic conditions. In normoxic hearts, PARP inhibitors did not have significant effects on TBARS. When ischemia-reperfusion was performed in the presence of PARP inhibitors, the formation of TBARS was significantly lower than in the hearts subjected to ischemia-reperfusion alone, indicating that PARP inhibitors prevented the ischemia-reperfusion-induced lipid peroxidation.

Reactive oxygen species formation in ischemia-reperfusion cycle can also trigger the oxidation of proteins, which can be characterized by the quantity of protein-bound aldehyde groups. Ischemia-reperfusion significantly elevated the level of protein oxidation; nevertheless, the administration of PARP inhibitors during ischemia-reperfusion cycle prevented the increase in the protein-bound aldehyde groups.

In addition, under our experimental conditions, moderate Akt kinase phosphorylation occurred as a result of ischemia-reperfusion. On the other hand, 4–hydroxyquinazoline and HO-3089 treatment could further enhance Akt activation.

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 and inorganic phosphate levels. Furthermore, the PARP inhibitor-induced functional improvement was also significantly attenuated in the presence of PI3-kinase inhibitors.

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).

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). 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). 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.

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

Discussion

Poly(ADP-ribose) polymerase inhibitors protect hearts against IR injury, but the molecular mechanism of this protection remains to be elucidated. Excessive activation of PARP can cause NAD^+ and 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, and decrease the degree of ROS production, which is predominantly a mitochondrial process in postischemic myocardium. Recent works reported the existence of mitochondrial poly(ADP-ribose) polymerases which could be blocked with PARP-1 inhibitors. 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. Similar data were also seen in neuronal cells. 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. 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. Therefore, the mitochondrial protective effect of PARP inhibitors can be mediated via the PI3-kinase/Akt/BAD pathway.

Here, we characterized the PARP-inhibitory property of well established and a novel PARP inhibitor 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 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.

Publications of the author

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

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Impact factor: 2.993

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II. Book chapters

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III. Additional papers

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