The Role of Akt and Mitogen-Activated Protein Kinase Systems in the Protective Effect of Poly(ADP-Ribose) Polymerase Inhibition in Langendorff Perfused and in Isoproterenol-Damaged Rat Hearts

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ABSTRACT

Blocking poly(ADP-ribosylation) of nuclear proteins protects the heart from ischemia-reperfusion injury. In addition, activation of Akt and mitogen-activated protein kinase (MAPK) cascades also plays a pivotal role in the survival of cardiomyocytes during ischemia-reperfusion; however, the potential interplay between these pathways is yet to be elucidated. We therefore tested the hypothesis whether poly(ADP-ribose) polymerase (PARP) inhibition can modulate Akt and MAPK signaling of ischemic-reperfused rat hearts. A novel PARP inhibitor, L-2286 [2-[(2-piperidin-1-ylethyl)thio]quinazolin-4(3H)-one] was administered during ischemia-reperfusion in Langendorff perfused rat hearts and in isoproterenol-induced myocardial infarction. Thereafter, the cardiac energy metabolism, oxidative damage, and the phosphorylation state of Akt and MAPK cascades were monitored. L-2286 exerted significant protective effect against ischemia-reperfusion-induced myocardial injury in both experimental models. More importantly, L-2286 facilitated the ischemia-reperfusion-induced activation of Akt, extracellular signal-regulated kinase, and p38-MAPK in both isolated hearts and in vivo cardiac injury. By contrast, isoproterenol-induced rapid c-Jun N-terminal kinase activation was repressed by L-2286. Here, we provide evidence for the first time that PARP inhibition beneficially modulates the cardiac Akt and MAPK signaling in ex vivo and in vivo ischemia-reperfusion models. We therefore propose that this novel mechanism may contribute to the cardioprotective properties of PARP inhibitors.

Enhanced activation of poly(ADP-ribose) polymerase (PARP) enzyme is a major contributor to oxidative stress-induced cell dysfunction and tissue injury (Virag and Szabo, 2002; Szabo et al., 2004). Reactive oxygen species and peroxynitrite formation expedites the ischemia-reperfusion-induced cardiac injury and causes lipid peroxidation, protein oxidation, and single-strand DNA brakes (Habon et al., 2001; Halmo$si$ et al., 2001). Single-strand DNA brakes can activate the nuclear PARP, which ADP-ribosylates different nuclear proteins at the expense of cleaving NAD$^{+}$ and ATP depletion, ultimately resulting in cell death (Habon et al., 2001; Halmo$si$ et al., 2001; Virag and Szabo, 2002; Szabo et al., 2004). We and other investigators have already shown that PARP inhibitors can efficiently reduce oxidative myocardial damage during ischemia-reperfusion both in isolated heart perfusion and in vivo myocardial infarction models (Zingarelli et al., 1997; Bowes et al., 1998; Docherty et al., 1999; Halmo$si$ et al., 2001). Recent studies, however, have

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ABBREVIATIONS: L-2286, 2-[(2-piperidin-1-ylethyl)thio]quinazolin-4(3H)-one; PARP, poly(ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3-kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HQ, hydroxyquinoline; MQ, 2-merkapto-4(3H)-quinazolinone; TBARS, thiobarbituric acid reactive substances; ISO, isoproterenol hydrochloride; TTC, triphenyltetrazolium chloride; LDH, lactate dehydrogenase; CK, creatine kinase; GSK, glycogen synthase kinase.
challenged the original dogma that protection by PARP inhibitors relies exclusively on the preservation of NAD$^+$ and ATP stores and suggested that PARP inhibitors may modify the activation state of signaling routes and gene expression (Kovacs et al., 2004; Szabo et al., 2004; Veres et al., 2004; Zingarelli et al., 2004). It is noteworthy that PARP enzyme was reported to alter the function of a variety of transcription factors and to interfere with the expression of several proinflammatory genes by direct protein–protein interaction or by poly(ADP-ribosyl)ation (Szabo et al., 2004; Veres et al., 2004). Nevertheless, to date, limited information is available on how PARP inhibition influences the signaling pathways during myocardial ischemia-reperfusion and how these changes may contribute to the cardioprotective properties of PARP inhibitors.

To elucidate the role of protein kinase signaling in the mechanism of cardioprotection afforded by PARP inhibitors, we used two experimental models of myocardial ischemia and reperfusion. First, we investigated the effect of the novel PARP inhibitor L-2286 on the recovery of energy metabolism in Langendorff perfused hearts during ischemia-reperfusion cycle, and then L-2286 was tested in vivo in isoproterenol-induced myocardial infarction model. As known, subcutaneous administration of the β-adrenoceptor agonist isoproterenol produces graded myocardial cell death and rapidly impairs left ventricular function, at least partially, through free radical generation (Grimm et al., 1998; Manikandan et al., 2002). However, to our knowledge no studies have evaluated the intracellular signaling cascades during isoproterenol-induced myocardial infarction.

Hypoxia-reoxygenation as well as other oxidative insults influence tissue survival partially via differential regulation of protein kinase cascades and inflammatory reactions (Toth et al., 2004; Becker, 2004). Phosphatidylinositol-3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) [including extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38-MAPK] signaling networks have all been shown to alter their activation state in response to oxidant injury and therefore could potentially participate in cell fate decisions (Shimizu et al., 1998; Armstrong, 2004). Signaling through Akt and ERK seems to be prosurvival in nature associated with growth factor receptor stimulation (Haenenlow and Yellon, 2004). On the other hand, JNK and p38-MAPK activation was linked to apoptosis; but depending on the context and duration of activation, they can exert opposite effects as well (Lin, 2002; Steenbergen, 2002).

In this work, we provide evidence for a new molecular mechanism of the cardioprotective effect of PARP inhibition. Our quinazolinoine derivate PARP inhibitor L-2286 (Fig. 1) facilitated the recovery of myocardial energy metabolism and activated the PI3K/Akt pathway and MAP kinase cascades in ischemic-reperfused Langendorff hearts. Furthermore, differential regulation of PI3K/Akt and MAP kinase cascades are described in vivo isoproterenol-induced myocardial infarction and demonstrated the novel cardioprotective mechanism of PARP inhibitors.

**Materials and Methods**

**Cell Viability Assay.** Cell viability was tested by the diazo dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)), which provides an estimate of cellular reducing power, thereby metabolic activity. H9c2 rat embryonic cardiomyoblast line (American Type Culture Collection, Manassas, VA) was cultured in 96-well microtiter plates in a humidified 95% O$_2$/5% CO$_2$ atmosphere at 37°C. Cells were treated for 4 h with 0.3 mM H$_2$O$_2$ in the presence or absence of 4-hydroxyquinazoline (HQ), 2-merkaptop-4(3H)-quinazolinoine (MQ) (Sigma-Aldrich, Budapest, Hungary), or L-2286. After removing the medium, MTT was added in a concentration of 0.1 mg/ml. After 4-h incubation, a solution containing 10% SDS and 10 mM HCl was added. After solving the blue crystals overnight, absorbance of each sample was read by a multwell spectrophotometer at 540-nm wavelength. Cell viability was calculated as the percentage of absorbance in each sample versus control. The individual 50% effective concentrations (EC$_{50}$) were determined in each assay and expressed in millimolar, and maximal protection afforded by HQ, MQ, and L-2286 was measured and expressed in the percentage of the control.

**Heart Perfusion.** Adult male CFY-strain Sprague-Dawley rats weighing 300 to 380 g were used for the Langendorff heart perfusion experiments or the myocardial infarction model. The animals were housed in solid-bottomed polypropylene cages and received commercial rat diet and water ad libitum. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pecs Medical School. Rats were anesthetized with 200 mg/kg ketamine hydrochloride intraperitoneally (Richter Gedeon Ltd., Budapest, Hungary) and heparinized with sodium heparin (100 IU/rat i.p.; Biochemie GmbH, Kundl, Austria). Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 mm Hg, at 37°C as described previously (Halmosi et al., 2001). The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$, 11 mM glucose, and 0.6 mM octanoic acid, and, in the treated group, L-2286 in 10 and 20 μM concentrations. The perfusate was adjusted to pH 7.40 and bubbled with 95% O$_2$ and 5% CO$_2$ through a glass oxygenator. After a washout, nonrecirculating period of 10 min, hearts were either perfused under normoxic conditions for 10 min or were subjected to a 30-min global ischemia by closing the aortic influx and reperfused for 15 min. The experimental compound was administered into the perfusion medium at the beginning of normoxic perfusion. During ischemia hearts were submersed into perfusion buffer at 37°C. Hearts were freeze-clamped at the end of each perfusion.

**NMR Spectroscopy.** NMR spectra were recorded with a Varian INOVA 400 WB instrument. $^3$P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z-Spec in a 20-mm broadband probe (Nalorac Co., Martinez, CA), applying proton decoupling ($\gamma B_2 = 1.2$ kHz) during acquisition. Field homogeneity was adjusted by regular checking of the half-width of the $^1$H signal (w1/2 = 10–15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each free induction decay. We used 45° flip angle pulses after a 1.25-s recycle delay and transients were acquired over a 10-kHz spectral width of 0.25 s, and the acquired data points (5000) were zero filled to 16 K. Under the above-mentioned conditions, spectra are well resolved and the chemical shifts were referenced to a 10% suspension of 1H,2H pyridine-d$_5$ in D$_2$O.
circumstances the relative concentrations of the species can be taken proportional to the peak areas, because interpulse delays exceeded 4-5xT1 values of the metabolites to be analyzed in 31P experiments.

Lipid Peroxidation and Protein Carbonyl Content. Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined using a modification of a described method (Serbinova et al., 1992). 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 nanomoles per gram of wet tissue.

To measure protein carbonyl content, 50 mg of frozen heart tissue was homogenized with 1 ml of 4% perchloric acid, and the protein content was collected by centrifugation. The protein carbonyl content was determined by means of the 2,4-dinitrophenylhydrazine-method (Butterfield et al., 1997).

Myocardial Infarction Model. Control rats received physiological saline (1 ml/kg) intraperitoneally. On the other hand, myocardial infarct was induced by subcutaneous injection of 80 mg/kg hydrochloride (ISO) (Sigma-Aldrich). ISO solutions were prepared with sterile distilled water immediately before injection. ISO-treated animals were assigned into two groups: the ISO group received repeated injections of saline and the ISO + L-2286 group received L-2286 10 min before (10 mg/kg) and every hour for 5 h (3 mg/kg) after ISO administration. Electrocardiogram was detected before and hourly (for 5 h) after ISO administration (electrocardiograph; Schiller AG Baar, Switzerland).

Infarct Size Measurement. Twenty-four hours after the ISO administration, animals were sacrificed and hearts were removed and kept overnight at −20°C. Frozen ventricles were sliced into 2- to 3-mm thick sections and then incubated in 1% triphenyltetrazolium chloride ( TTC) (Sigma-Aldrich) at 37°C in 0.2 M Tris buffer, pH 7.4, for 30 min. Although the normal myocardium was stained brick red, the infarcted areas remained unstained. Size of the infarcted area was estimated by the volume and weight method (Sharma and Singh, 2000).

Serum Necroenzyme Determination. Serum lactate dehydrogenase (LDH) and creatine kinase (CK) levels were determined from blood samples collected 24 h after ISO administration. Myocardial enzyme activities were measured by standard methods as described previously (Bergmeyer and Bernt, 1974; Forster et al., 1974).

Western Blot Analysis. For Western blot analysis heart samples were taken after each perfusion and from animals sacrificed 0.5, 2, 4, and 24 h after ISO administration. Fifty milligrams of heart samples were homogenized in ice-cold 50 mM Tris buffer, pH 8.0 (containing protease inhibitor cocktail, 1:1000; Sigma-Aldrich) and harvested in 2× concentrated SDS-polyacrylamide gel electrophoretic sample buffer. Proteins were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. After blocking (2 h with 3% nonfat milk in Tris-buffered saline), membranes were probed overnight at 4°C with antibodies recognizing the following antigens: phospho-specific Akt-I/protein kinase B-α Ser473 (1:1000), nonphosphorylated Akt/PKB (1:1000), phospho-specific glycogen synthase kinase (GSK)-3β Ser9 (1:1000), phospho-specific extracellular signal-regulated kinase (ERK1/2) Thr183-Tyr185 (1:1000), phospho-specific JNK (1:1000; Cell Signaling Technology (CST), Beverly, MA), and N-terminal domain of actin (1:10,000; Sigma-Aldrich). Membranes were washed six times for 5 min in Tris-buffered saline, pH 7.5, containing 0.2% Tween and the antibody-antigen complexes were visualized by means of enhanced chemiluminescence. The results of Western blots were quantified by means of Scion Image (ver. 4.02 beta; Scion Corporation, Frederick, MD).

Statistical Analysis. Statistical analysis was performed by analysis of variance, and all data are 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

L-2286 Protected against H2O2-Induced Cytotoxicity in H9c2 Cells. MTT assay demonstrated a dose-dependent protective effect of L-2286 against H2O2-induced cytotoxicity in H9c2 cells, starting at a concentration as low as 0.1 μM (Fig. 2). Under these experimental conditions the efficacy of L-2286 (EC50 = 0.41 μM) surpassed that of other basic quinazolines, such as 4-hydroxyquinazoline (211.6 μM) and 2-merkapto-4(3H)-quinazolinone (178.6 μM). Maximal protection provided by L-2286 was also better than that seen in cells treated with HQ or MQ (Table 1).

L-2286 Promoted the Postischemic Recovery of Myocardial Energy Stores. Energy metabolism of Langendorff perfused hearts was monitored in the magnet of a 31P NMR spectroscope enabling the detection of changes in high-energy phosphate intermediates. Ischemia induced a rapid decrease in ATP and creatine phosphate levels and a fast evolution of inorganic phosphate (Fig. 3, A–C). In our experimental setting, high-energy phosphate intermediates recovered only partially in untreated hearts during the 15-min reperfusion phase. On the other hand, L-2286 facilitated the recovery of creatine phosphate and ATP in both 10 and 20 μM concentrations (Fig. 3, A and B). Consistent with the high-energy phosphate data, L-2286 also promoted the faster and more complete reutilization of inorganic phosphate during reperfusion (Fig. 3C).

L-2286 Attenuated the Ischemia-Reperfusion-Induced Lipid Peroxidation and Protein Oxidation. In our current study, ischemia-reperfusion increased the
amount of TBARS compared with the normoxic hearts (Fig. 3D). However, 10 μM L-2286 administration significantly reduced the formation of TBARS (p < 0.01) compared with untreated hearts, indicating that L-2286 prevented the ischemia-reperfusion-related lipid peroxidation. On the other hand, Fig. 3E shows that ischemia-reperfusion markedly elevated the level of protein oxidation; however, L-2286 significantly attenuated (p < 0.01) the increase in the quantity of protein-bound aldehyde groups.

Enhanced Akt-1 and MAP Kinase Activation upon L-2286 Treatment during Ischemia-Reperfusion. The moderate phosphorylation of Akt-1 under normoxic conditions in our study increased after ischemia-reperfusion; nevertheless, L-2286 treatment further enhanced the activation of Akt-1 (Fig. 4A). GSK-3β was not phosphorylated during normoxia but became moderately phosphorylated after ischemia-reperfusion and strongly phosphorylated upon L-2286 treatment. Because GSK-3β is phosphorylated by Akt-1, leading to its inactivation, the marked phosphorylation of GSK-3β in treated hearts is in accordance with enhanced Akt-1 activation in the same tissue samples (Fig. 4A). It is interesting that L-2286 also brought about Akt-1 as well as GSK-3β phosphorylation during the 10-min normoxic perfusion, which is in clear contrast with the untreated normoxic condition where moderate or no phosphorylation was observed.

In addition, L-2286 promoted the phosphorylation of ERK, p38-MAPK as well as JNK, both in normoxic and ischemic-reperfused hearts (Fig. 4B). Ischemia-reperfusion by itself only slightly increased the phosphorylation of ERK, p38-MAPK, and JNK.

L-2286 Inhibited the Isoproterenol-Induced Myocardial Cell Loss. ISO administration results in compromised

<table>
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<th>Compound</th>
<th>HQ</th>
<th>MQ</th>
<th>L-2286</th>
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<tr>
<td>EC_{50} (μM)</td>
<td>211.6 ± 3.2*</td>
<td>178.6 ± 6.9*</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Maximal protection (%)</td>
<td>55.7 ± 2.6*</td>
<td>76.2 ± 4.9*</td>
<td>85.7 ± 3.7</td>
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* Significant difference from L-2286-treated samples (p < 0.001).
Fig. 4. L-2286 promotes Akt-1, ERK, p38-MAPK, and JNK phosphorylation in ischemic-reperfused hearts. Western blot analysis of Akt-1 and GSK-3β phosphorylation (A) as well as ERK, p38-MAPK, and JNK phosphorylation (B). Actin is shown as loading control. N, normoxic perfusion; IR, ischemia-reperfusion. Representative immunoblots from five experiments and densitometric evaluation are demonstrated. *, significant difference from normoxic sample (p < 0.01); †, significant difference from IR sample (p < 0.01).
cell membrane integrity and causes necroenzyme release from cardiomyocytes. In comparison with the control group, ISO administration significantly increased CK and LDH release from the injured cardiomyocytes \( (p < 0.01) \). By contrast, L-2286 treatment significantly reduced CK and LDH levels in the serum \( (p < 0.05) \) (Fig. 5, A and B). ECG monitoring of the heart rate revealed that L-2286 did not interfere with the tachycardia elicited by ISO administration (Table 2).

As the TTC staining in five consecutive samples demonstrated, ISO administration caused a large, 21 \% infarct of the ventricles (Fig. 5C). In the meantime, L-2286 treatment significantly reduced the infarct size to 8.9 \% \( (p < 0.05) \). TTC staining of control hearts rendered no appreciable infarcted area.

**L-2286 Enhanced Akt-1, ERK, and p38-MAPK but Reduced JNK Phosphorylation in Isoproterenol-Induced Myocardial Injury.** Although ISO administration rapidly increased Akt phosphorylation, cotreatment with L-2286 could slightly further enhance phosphorylation (Fig. 6A). Subsequent elevation in GSK-3β phosphorylation was delayed until 4 h after ISO administration, but L-2286 triggered a more pronounced phosphorylation than ISO by itself (Fig. 6A).

Although ISO administration led to higher ERK phosphorylation only after 24 h, L-2286 cotreatment accelerated this process immediately after ISO injection (Fig. 6B). Likewise, the initial p38-MAPK phosphorylation level rather diminished after ISO treatment and recovered only after 24 h (Fig. 6B). By contrast, the ISO-induced decrease in p38-MAPK phosphorylation was prevented by L-2286; therefore, the ISO + L-2286 hearts exhibited higher phosphorylation levels than the ISO hearts at each examined time point (Fig. 6B). Furthermore, JNK became highly phosphorylated within the first 4 h after ISO injection to ultimately return to baseline at 24 h (Fig. 6B). In contrast to our ischemia-reperfusion data, L-2286 reduced the ISO-induced JNK phosphorylation throughout the entire experiment (Fig. 6B).

**Discussion**

Our study gave first insight into the mechanism of cardioprotection by a novel PARP inhibitor L-2286 in isolated ischemic-reperfused hearts and in vivo isoproterenol-

### Table 2

<table>
<thead>
<tr>
<th>Time after ISO administration</th>
<th>ISO</th>
<th>ISO + L-2286</th>
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<tbody>
<tr>
<td>0 min</td>
<td>391.8 ± 9.2</td>
<td>396.2 ± 8.6</td>
</tr>
<tr>
<td>5 min</td>
<td>403.2 ± 7.4</td>
<td>403.2 ± 7.4</td>
</tr>
<tr>
<td>15 min</td>
<td>451.8 ± 6.9*</td>
<td>454.6 ± 7.2*</td>
</tr>
<tr>
<td>30 min</td>
<td>538.9 ± 8.2*</td>
<td>536.9 ± 7.2*</td>
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<tr>
<td>1 h</td>
<td>555.6 ± 6.3*</td>
<td>560.3 ± 5.8*</td>
</tr>
<tr>
<td>2 h</td>
<td>439.2 ± 6.4*</td>
<td>439.2 ± 6.4*</td>
</tr>
<tr>
<td>4 h</td>
<td>405.6 ± 7.5</td>
<td>405.6 ± 7.5</td>
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* Significant difference from 0 time point \( (p < 0.01) \).
Fig. 6. L-2286 increases Akt-1, ERK, and p38-MAPK, whereas it suppresses JNK phosphorylation in isoproterenol-induced myocardial injury. Western blot analysis of Akt-1 and GSK-3β phosphorylation (A) as well as ERK, p38-MAPK, and JNK phosphorylation (B) 0, 0.5, 2, 4, and 24 h after ISO administration. Actin is shown as loading control. Representative immunoblots from five experiments and densitometric evaluation are demonstrated. *, significant difference from control untreated sample (p < 0.01); †, significant difference from L-2286-treated samples (p < 0.01).
induced myocardial injury. PARP inhibition was first shown to beneficially influence the activation of Akt signaling pathway and the MAPK cascade in two animal models of myocardial ischemia and reperfusion. Increased PARP activation induced by oxidative stress after ischemia-reperfusion contributes to myocardial cell death and subsequent inflammatory reactions (Szabo et al., 2004). Therefore, PARP inhibition was shown to exert protection against oxidative injury in cell cultures and ex vivo and in vivo animal models of regional or global cardiac, brain or renal ischemia-reperfusion (Halmosi et al., 2001; Virag and Szabo, 2002; Szabo et al., 2004). Therefore, PARP gene ablation or pharmacological inhibition of PARP improved the functional and metabolic recovery of postischemic myocardium and reduced infarct size in various models (Zingarelli et al., 1997, 2004; Docherty et al., 1999). In the current report, PARP inhibition was achieved by a novel compound, L-2286, which was derived from 2-mercapto-4(3H)-quinazolinone by alkylation with 1-(2-chloroethyl)piperidine (Kulcsar et al., 2003). L-2286 was chosen because in vitro PARP assay, it exhibited significantly better PARP inhibitory activity than 4-hydroxyquinazoline or 2-merkapto-4(3H)-quinazolinone (Kulcsar et al., 2003). Accordingly, in H9c2 cells L-2286 provided significantly better protection against 

H2O2-induced oxidative injury than the basic quinazolines (already in nanomolar concentrations).

Most notably, we demonstrated that L-2286 was able to promote the postischemic recovery of myocardial energy metabolism in Langendorff heart perfusion system. L-2286 helped preserve the high-energy phosphate intermediates and facilitated the rapid and more complete consumption of inorganic phosphate during reperfusion. Reutilization of the latter bears crucial importance because excessive amounts of inorganic phosphate, calcium, and reactive oxygen species are the most potent triggers of mitochondrial permeability transition (Toth et al., 2003). The improved metabolic recovery in the presence of L-2286 was accompanied by decreased myocardial oxidative damage (i.e., lipid peroxidation and protein oxidation).

In accordance with our Langendorff studies, L-2286 treatment also significantly attenuated the isoproterenol-induced myocardial damage in vivo. This was proved by reduced cardiac necroenzyme (CK and LDH) release and smaller infarct size in ISO + L-2286–treated compared with ISO-treated animals. It is noteworthy that isoproterenol, a β-adrenergic agonist induces extensive cardiomyocyte necrosis by its positive inotropic and chronotropic effect, ranging from patchy subendocardial necrosis to transmural infarction (Teerlink et al., 1994; Grimm et al., 1998). In addition, ISO administration was reported to enhance free radical formation, which might result in both acute and chronic deterioration of hemodynamic variables (Teerlink et al., 1994; Manikandan et al., 2002; Chattopadhyay et al., 2003). Together, the novel PARP inhibitor L-2286 conferred protection in various models with oxidative challenge, including cultured cells, ischemic-reperfused hearts, and in vivo cardiac injury.

Reports from our laboratory and other laboratories have recently challenged the notion that the protection by PARP inhibition is solely attributable to the preservation of cellular NAD+ and ATP pools (Halmsi et al., 2001; Kovacs et al., 2004; Veres et al., 2004; Zingarelli et al., 2004). By contrast, these results suggested that PARP blockade might also modulate a diverse array of signaling cascades and gene expression. In this respect, PARP inhibition has been shown to suppress the activation of JNK, activator protein-1, and nuclear factor-κB in inflammatory processes and cardiac ischemia-reperfusion (Szabo et al., 2004; Veres et al., 2004) and interfere with the expression of several proinflammatory genes such as the inducible NO synthase and intercellular adhesion molecule-1 (Ha et al., 2002; Zingarelli et al., 2003, 2004; Szabo et al., 2004; Veres et al., 2004). Furthermore, we have demonstrated that phosphatidylinositol-3-kinase-dependent Akt activation contributes to the PARP inhibitor–related protection in septic shock (Veres et al., 2004).

Our present findings demonstrated that PARP inhibitors could promote Akt activation during cardiac ischemia-reperfusion. We found enhanced L-2286–triggered Akt and GSK-3β phosphorylation not only in isolated hearts, but also in isoproterenol-induced cardiac injury. To our knowledge, this is the first ex vivo and in vivo report, which attributes a critical role to Akt in the cardioprotection conferred by PARP inhibitors. The PI3K/Akt pathway is one of several prosurvival signaling routes, which is activated as an adaptive response to cellular stress (Hausenloy and Yellon, 2004). Among others, ischemia-reperfusion itself can expedite Akt signaling in cardiac myocytes, as shown in our study. However, L-2286 administration further increased Akt activation independently of cardiac injury, presumably exerting antiapoptotic and favorable metabolic effects.

Thereafter, we demonstrated the differential activation of mitogen-activated protein kinases upon ischemia-reperfusion, which was clearly mediated by concomitant L-2286 treatment, strongly depending on the context and timing of the deleterious insult. First, although ERK phosphorylation was enhanced upon ischemia-reperfusion of isolated hearts, isoproterenol in vivo caused a transient fall followed by a delayed increase in ERK activity. This is in accordance with reports showing that ERK activity reduced during ischemia ensued by its recovery during reperfusion in rat hearts (Bogoyevitch et al., 1996; Mizukami et al., 1997; Omura et al., 1999). Based on our ECG recordings, subcutaneous administration of isoproterenol provoked faster heart rate for at least 4 h, implicating a prolonged “ischemic” period matching the duration of lower ERK activity. Most importantly, L-2286 not only promoted ERK phosphorylation in normoxic and ischemic-reperfused isolated hearts but also accelerated that in vivo covering the most vulnerable, first 4 h immediately after isoproterenol administration. Although ERK is mainly involved in growth factor-induced signaling, it can play protective roles in oxidative stress via blocking apoptosis (Hausenloy and Yellon, 2004). Indeed, sustained activation of ERK during reoxygenation was shown to render delayed cytoprotection, probably by down-regulating caspase-3 and JNK activity (Hong et al., 2001; Martindale and Holbrook, 2002). In summary, the ability of L-2286 to elicit robust ERK activation in both ex vivo and in vivo ischemia-reperfusion may promote cardiac myocyte survival.

Second, phosphorylation of p38-MAPK exhibited a similar dynamics to that of ERK (i.e., a slight activation upon postischemic reperfusion in the Langendorff heart and a
protracted activation 24 h after isoproterenol administration). Our findings are thus consistent with reports where p38-MAPK was not activated by ischemia alone but was stimulated by reperfusion in rat hearts (Bogoyevitch et al., 1996; Yin et al., 1997). It is remarkable that, independent of the model used, p38-MAPK phosphorylation was enhanced 2- to 3-fold in the presence of L-2286. The role of p38-MAPK in myocardial ischemia-reperfusion injury is controversial. Several data suggest that the activation of p38-MAPK is not protective during myocardial ischemia-reperfusion, but rather that protection by PARP inhibitors exclusively rely on the activation of JNK activity. The PARP inhibition-induced alterations in signaling further challenge the original dogma that protection by PARP inhibitors exclusively rely on the preservation of NAD+ as well as ATP stores.

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