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Effect of Poly(ADP-Ribose) Polymerase Inhibitors on the Ischemia-Reperfusion-Induced Oxidative Cell Damage and Mitochondrial Metabolism in Langendorff Heart Perfusion System

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ABSTRACT

Ischemia-reperfusion induces reactive oxygen species (ROS) formation, and ROS lead to cardiac dysfunction, in part, via the activation of the nuclear poly(ADP-ribose) polymerase (PARP, called also PARS and ADP-RT). ROS and peroxynitrite induce single-strand DNA break formation and PARP activation, resulting in NAD^+ and ATP depletion, which can lead to cell death. Although protection of cardiac muscle by PARP inhibitors can be explained by their attenuating effect on NAD^+ and ATP depletion, there are data indicating that PARP inhibitors also protect mitochondria from oxidant-induced injury. Studying cardiac energy metabolism in Langendorff heart perfusion system by ^{31}P NMR, we found that PARP inhibitors (3-aminobenzamide, nicotinamide, BGP-15, and 4-hydroxyquinazoline) improved the recovery of high-energy phosphates (ATP, creatine phosphate) and accelerated the reutilization of inorganic phosphate formed during the ischemic period, showing that PARP inhibitors facilitate the faster and more complete recovery of the energy production. Furthermore, PARP inhibitors significantly decrease the ischemia-reperfusion-induced increase of lipid peroxidation, protein oxidation, single-strand DNA breaks, and the inactivation of respiratory complexes, which indicate a decreased mitochondrial ROS production in the reperfusion period. Surprisingly, PARP inhibitors, but not the chemically similar 3-aminobenzoic acid, prevented the H_2O_2-induced inactivation of cytochrome oxidase in isolated heart mitochondria, suggesting the presence of an additional mitochondrial target for PARP inhibitors. Therefore, PARP inhibitors, in addition to their important primary effect of decreasing the activity of nuclear PARP and decreasing NAD^+ and ATP consumption, reduce ischemia-reperfusion-induced endogenous ROS production and protect the respiratory complexes from ROS induced inactivation, providing an additional mechanism by which they can protect heart from oxidative damages.

Reactive oxygen species (ROS) and peroxynitrite contribute to the ischemia- and reperfusion-induced cardiac injury (Xie and Wolin, 1996), and initiate lipid peroxidation (Pan and Hori, 1994), protein oxidation (Butterfield et al., 1997), and the formation of single-strand DNA breaks (Zingarelli et al., 1997; Szabados et al., 1999a). Single-strand DNA (ssDNA) breaks can activate the nuclear poly(ADP-ribose) polymerase (PARP, called also PARS and ADP-RT) (Lindahl et al., 1995; Jacobson and Jacobson, 1999), which ADP-ribosylates different nuclear proteins on the expense of cleaving NAD^+. If PARP activation exceeds a certain limit it can lead to cellular NAD^+ and ATP depletion, causing eventually cell death (Radons et al., 1994; Gilad et al., 1997; Grupp et al., 1999; Szabados et al., 1999a). Therefore, the inhibition of PARP can improve the recovery of different cells from oxidative damages (Mizumoto et al., 1993; Heller et al., 1995; Said et al., 1996; Thiemermann et al., 1997).

Mitochondria play a pivotal role in oxidative cell damages because oxidative damage can cause the release of cytochrome *c* from mitochondrial intermembrane space and can induce apoptotic cell death (Lenaz, 1998; Saikumar et al., 1998), or oxidant can partially inactivate respiratory complexes (Turrens et al., 1991), resulting in impaired energy metabolism and significant increase in mitochondrially produced ROS, which eventually induce cell death (Takeyama et al., 1993; Lemasters et al., 1998). The ROS-induced mitochondrial permeability transition can lead to intramitochondrial NAD^+ loss and the inhibition of mitochondrial NAD^+-

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ABBREVIATIONS: ROS, reactive oxygen species; ssDNA, single-strand DNA; PARP, poly(ADP-ribose) polymerase; BGP-15, *O*-(3-piperidino-2-hydroxy-1-propyl)nicotinic amidoxime; TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid.

linked substrate oxidation (Said et al., 1996), further damaging the mitochondrial energy production (Takeyama et al., 1993; Lemasters et al., 1998). It was reported previously that PARP activation contributes to mitochondrial injury during oxidant-induced cell death (Virag et al., 1998), and that a new PARP inhibitor (BGP-15) decreased the oxidative damage of myocardium after ischemia-reperfusion (Szabados et al., 2000). These data raise the possibility that PARP inhibitors may interfere with endogenous mitochondrial ROS formation by a different mechanism than antioxidants (Szabados et al., 2000).

In heart tissue, a dominant fraction of energy production occurs in the mitochondria, therefore protection against oxidative damage of mitochondria can be very important step in the normalization of cardiac energy production. In this work, we investigated the effect of PARP inhibitors and 3-aminobenzoic acid (an inactive chemical analog of the best studied PARP inhibitor) (Fig. 1) on the energy metabolism of heart during ischemia-reperfusion cycle by ^{31}P NMR spectroscopy. Furthermore, we investigated the effect of PARP inhibitors on the ischemia-reperfusion-induced oxidative cardiac damages (lipid peroxidation, protein oxidation, and ssDNA break formation), inactivation of respiratory enzymes, and direct effect of PARP inhibitors on isolated mitochondria to extend our knowledge about the molecular mechanisms by which PARP inhibitors can protect mitochondria and cardiomyocytes from ischemia-reperfusion-induced damages.

Materials and Methods

Chemicals. 3-Aminobenzamide, 3-aminobenzoic acid, 4-hydroxyquinazoline, nicotinamide, H_2O_2, NAD^+, and dihydrorhodamine123 were purchased from Sigma-Aldrich Chemical Co. (Budapest, Hungary); malondialdehyde-bis(diethylacetal) was obtained from Merck (Darmstadt, Germany). BGP-15 was a gift from N-Gene Research Laboratories, Inc. (Budapest, Hungary). All other reagents were of the highest purity commercially available.

Animals. The hearts of adult male Wistar rats weighing 300 to 350 g were used for Langendorff heart perfusion experiments. All animal experiments were conducted in conformity with the guiding principles in the care and use of animals.

Heart Perfusion. Rats were anesthetized with 200 mg/kg ketamine intraperitoneally and heparinized with sodium heparin (100 IU/rat i.p.). Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 mm Hg, at 37°C as described before (Szabados et al., 1999a,b). The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 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 without or together with PARP inhibitors [3 mM nicotinamide, 3 mM 3-aminobenzamide, or 40 mg/l (113.9 μM) BGP-15]. The perfusate was adjusted to pH 7.4 and bubbled with 95% O_2, 5% CO_2 through a glass oxygenator. After a washout (nonrecirculating period of 15 min), hearts were either perfused under normoxic conditions for the given time, or were subjected to 25-min global ischemia by closing the aortic influx and reperfused for 15 min. During ischemia, hearts were submerged into perfusion buffer at 37°C. At the end of the perfusion, hearts were freeze-clamped.

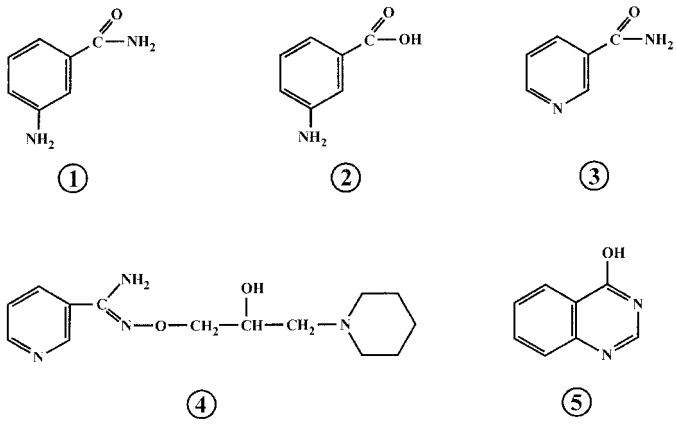

Fig. 1. Chemical structure of the studied PARP inhibitors and 3-aminobenzoic acid. 1, 3-aminobenzamide; 2, 3-aminobenzoic acid; 3, nicotinamide; 4, BGP-15; and 5, 4-hydroxyquinazoline.

Assay of NAD^+. The concentration of NAD^+ in the neutralized perchloric acid extract of the cardiac muscle was measured by using alcohol dehydrogenase reaction as described previously (Skuta et al., 1999).

Determination of DNA Single-Strand Breaks. Single-strand DNA breaks were determined by the alkaline fluorescence analysis of DNA unwinding as described previously (Birnboim and Jevcak, 1981). DNA samples were prepared from normoxic and ischemic perfused hearts. To estimate the quantity of undamaged double-stranded DNA, samples were divided into three sets of tubes. DNA fluorescence was determined under different conditions. To determine F values, DNA was kept at pH 12.4 to permit partial unwinding of DNA. To determine F_{min}, DNA was kept at pH 12.4, but at the beginning of the incubation period the DNA sample was sonicated for 60 s. To determine F_{max} the DNA sample was kept at pH 11.0, which is below the pH needed to induce unwinding. Solutions were incubated for 30 min at 0°C followed by 15-min incubation at 15°C. Unwinding was stopped by adjusting the pH to pH 11.0. Fluorescence was measured after the addition of the dye ethidium bromide (0.67 μg/ml), with an excitation wavelength of 520 nm and an emission wavelength of 590 nm by a PerkinElmer luminescence spectrometer. Results are expressed as D (percentage of double-stranded DNA) = $(F - F_{min})/(F_{max} - F_{min}) \times 100$.

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 (Serbinova et al., 1992). Cardiac tissue was homogenized in 6.5% trichloroacetic acid and a reagent containing 15% trichloroacetic acid, 0.375% TBA, 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.

Determination of Protein Carbonyl Content. Fifty milligrams of freeze-clamped perfused 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 using the 2,4-dinitrophenylhydrazine method (Butterfield et al., 1997; Szabados et al., 2000).

Isolation of Mitochondria. The hearts of sacrificed rats were quickly removed and immersed in an ice-cold isolation buffer containing 150 mM KCl, 5 mM Tris, 1 mM EDTA, pH 7.4. Hearts were cut into small pieces, homogenized in a Teflon homogenizer in the isolation buffer, and centrifuged for 10 min at 500g. The supernatant was then centrifugated for 10 min at 18,000g. After removing the supernatant, the pellet was carefully suspended in 1 ml of isolation buffer.

Incubation of Mitochondria with Hydrogen Peroxide and PARP Inhibitors. Aliquots of the suspended mitochondria were incubated in a buffer containing 150 mM KCl, 5 mM 4-morpholinepropanesulfonic acid, 1 mM EDTA, 1 mM succinate, pH 7.4 with 0.5 mM H_2O_2 and in the treated groups with a PARP inhibitor for 15 min at 37°C. To stop the hydrogen peroxide-induced injury of mitochondria, dithioerythritol (final concentration 4 mM) was added to

the medium. The samples were then centrifuged for 5 min at 15,000*g*. The pellet was suspended in 400 μl of isolation buffer and sonicated for 15 s. The samples were frozen at −80°C.

Measurement of Mitochondrial Enzyme Activity. Citrate synthase (Sumegi et al., 1985), NADH:cytochrome *c* oxidoreductase (Sumegi et al., 1990), and cytochrome oxidase (Sumegi et al., 1990) were measured as described previously.

Determination of Mitochondrial ROS Production. Mitochondria were incubated in a buffer containing 150 mM KCl, 1 mM EDTA, 5 mM 4-morpholinepropanesulfonic acid, 1 mM succinate, pH 7.4 and in the presence of PARP inhibitors and 3-aminobenzoic acid (0.2, 1, 3 mM). The mitochondrial suspension was stirred and the ROS production was continuously monitored by following the oxidation of dihydrorhodamine123 to rhodamine123 in a PerkinElmer fluorescence spectroscope at an excitation wavelength of 496 nm and an emission wavelength of 536 nm (Szabados et al., 1999).

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), applying proton decoupling during acquisition. Field homogeneity was adjusted by following the proton 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. Pulses of 45° flip angle were used 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 to 5× the T1 values of the metabolites to be analyzed in ^{31}P experiments.

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 the PARP Inhibitors on Ischemia-Reperfusion-Induced Lipid Peroxidation. Lipid peroxidation induced by ischemia-reperfusion in Langendorff perfused heart was characterized by the formation of TBA reactive substances. Under our experimental conditions, ischemia-reperfusion increased the amount of TBA reactive substances compared with the normoxic conditions ($p < 0.01$) (Table 1). In normoxic hearts, PARP inhibitors did not have significant effects on TBA-reactive substance formation (Table 1). When ischemia-reperfusion occurred in the presence of PARP inhibitors, the formation of TBA reactive substances was significantly lower than in the hearts subjected to ischemia-reperfusion without PARP inhibitors (Table 1), indicating that PARP inhibitors prevented the ischemia-reperfusion-induced lipid peroxidation. However, 3-aminobenzoic acid, an inactive structural analog of the PARP inhibitor 3-aminobenzamide, could not prevent the ischemia-reperfusion-induced lipid peroxidation (Table 1).

Dose response of PARP inhibitors on the ischemia-reperfusion-induced lipid peroxidation is shown in Fig. 2, indicating that although in different concentration range, PARP inhibitors protect the heart from lipid peroxidation in a concentration-dependent manner. The IC_{50} values were 226, 513, 29, and 35 μM for 3-aminobenzamide, nicotinamide, BGP-15, and 4-hydroxyquinazoline, respectively.

Hydrogen peroxide as an externally added oxidant induced lipid peroxidation in Langendorff perfused hearts, which was determined from the quantity of TBA reactive substances formation (Table 1). The presence of PARP inhibitors in the perfusion media could significantly decrease the quantity of TBA reactive substances formation in perfused hearts (Table 1). These effects of PARP inhibitors could not be due to their antioxidant property, because under our experimental conditions the PARP inhibitors did not inhibit the H_2O_2-induced (chemical) oxidation of dihydrorhodamine123 to rhodamine123 (data not shown).

Effect of PARP Inhibitors on Ischemia-Reperfusion-Induced Protein Oxidation. ROS formation in ischemia-reperfusion cycle can induce the oxidation of proteins in the cardiomyocytes, which can be characterized by the quantity of protein-bound aldehyde groups (Butterfield et al., 1997; Skuta et al., 1999). Table 2 shows that ischemia-reperfusion significantly increased the quantity of protein-bound aldehyde groups. However, the presence of PARP inhibitors during ischemia-reperfusion cycle prevented the increase in the quantity of protein-bound aldehyde groups (Table 2). However, 3-aminobenzoic acid could not prevent the ischemia-reperfusion-induced protein oxidation (Table 2).

The protective effect of PARP inhibitors was also seen when protein oxidation was induced by externally added H_2O_2, but in these cases PARP inhibitors could only partially protect heart proteins from oxidative damages (Table 2).

TABLE 1

Effect of PARP inhibitors on the ischemia-reperfusion and hydrogen peroxide-induced lipid peroxidation in Langendorff perfused rat hearts

Lipid peroxidation (TBA reactive substances) was measured as detailed under *Materials and Methods* in Langendorff heart perfusion system either in reperfused heart (25 min of ischemia followed by 15 min of reperfusion) or in normoxic heart perfused with 1 mM H_2O_2 for 30 min. Concentrations of the applied chemicals were 3 mM for 3-aminobenzamide (3-AB), 3-aminobenzoic acid (3-ABA) and nicotinamide, 0.113.9 mM for BGP-15, and 0.1 mM for 4-hydroxyquinazoline (4-HQ). Values are mean ± S.E.M. for five experiments.

Inhibitor	Thiobarbituric Acid Reactive Substances			
	Normoxic	Ischemia-Reperfusion	No Addition	H_2O_2 Added
	nmol/mg of wet tissue			
None	39.78 ± 1.59	70.71 ± 4.66	38.57 ± 2.65	66.53 ± 3.45
3-AB	38.82 ± 2.03	51.05 ± 2.98†	37.19 ± 1.85	56.23 ± 2.67*
3-ABA	41.32 ± 3.42	68.84 ± 3.51	39.54 ± 2.49	67.45 ± 2.14
Nicotinamide	39.25 ± 2.47	42.15 ± 1.76†	38.17 ± 1.71	53.17 ± 1.53*
BGP-15	39.72 ± 3.41	47.39 ± 2.31†	39.08 ± 2.82	55.59 ± 1.34*
4-HQ	38.25 ± 2.23	46.54 ± 1.87†	38.09 ± 1.96	56.12 ± 2.12*

Difference from ischemia-reperfusion * $p < 0.05$, † $p < 0.01$.
Difference from H_2O_2-treated group * $p < 0.05$.

Dose response of PARP inhibitors on the ischemia-reperfusion-induced protein oxidation is shown in Fig. 3, indicating that PARP inhibitors, in different concentration range, protect heart proteins against oxidation in a concentration-dependent manner. The IC_{50} values were 239, 478, 36, and 22 μM for 3-aminobenzamide, nicotinamide, BGP-15, and 4-hydroxyquinazoline, respectively.

Effect of PARP Inhibitors on Ischemia-Reperfusion-Induced Single-Strand DNA Breaks Formation and NAD^+ Catabolism. Ischemia-reperfusion increased ROS formation in perfused hearts, which can contribute to the formation of single-strand DNA breaks. Under normoxic conditions, most of the DNA was undamaged, but ischemia-reperfusion induced large amounts of single-strand DNA breaks, and the quantity of undamaged DNA decreased to under 30% (Fig. 4). In the presence of PARP inhibitors, ischemia-reperfusion increased only slightly the amount of ssDNA breaks (Fig. 4), and the amount of undamaged DNA was significantly higher than in postischemic hearts and not significantly lower than the normoxic values (Fig. 4). The inactive analog of 3-aminobenzamide, 3-aminobenzoic acid, could not decrease the ischemia-reperfusion-induced ssDNA breaks (Fig. 4).

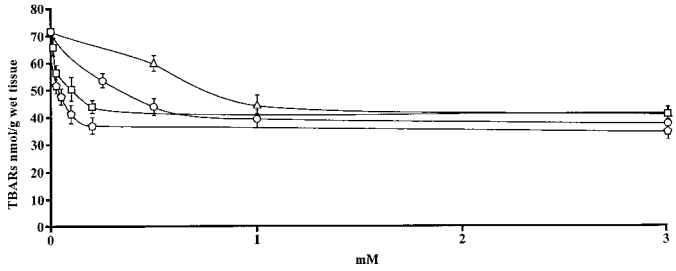

Fig. 2. Dose response of PARP inhibitors on the ischemia-reperfusion-induced lipid peroxidation in Langendorff perfused rat hearts. Lipid peroxidation (TBA reactive substances) was measured in Langendorff heart perfusion system in reperfused hearts (25 min of ischemia followed by 15 min of reperfusion) as detailed under *Materials and Methods*. Concentrations of the applied chemicals are as indicated in the figure. Values are mean ± S.E.M. for five experiments. A significant decrease was observed in lipid peroxidation: 3-aminobenzamide (○) at 0.25 mM, $p < 0.05$, at higher concentrations, $p < 0.01$; nicotinamide (△) at 1 and 2 mM, $p < 0.01$; BGP-15 (⬠) at 0.025 mM, $p < 0.05$, at higher concentrations, $p < 0.01$; and 4-hydroxyquinazoline (□) at 0.01 mM and higher concentrations, $p < 0.01$.

It is well known that ssDNA breaks activate PARP, which stimulates intracellular NAD^+ catabolism; therefore, it is expectable that ischemia-reperfusion cycle decreased NAD^+ content of perfused hearts. Figure 5 shows that ischemia-reperfusion significantly decreased the NAD^+ content of hearts. The presence of PARP inhibitors in the perfusate during ischemia-reperfusion cycle partially protected the loss of NAD^+ in postischemic hearts (Fig. 5). However, 3-aminobenzoic acid could not protect hearts from the ischemia-reperfusion-induced loss of NAD^+.

Effect of PARP Inhibitors on Energy Metabolism of Perfused Hearts during Ischemia-Reperfusion. Energy metabolism of Langendorff perfused hearts was monitored in the magnet of NMR spectroscope, making it possible to detect changes in high-energy phosphorus intermediates (Fig. 6). Ischemia induced a rapid decrease in ATP and creatine phosphate levels and a fast evolution of inorganic phosphate. Under our experimental conditions, high-energy phosphate intermediates recovered only partially in 15-min reperfusion phase, and 3-aminobenzamide facilitated the recovery of ATP and creatine phosphate (Fig. 6). However, 3-aminobenzoic acid did not improve the recovery of ischemic heart (data not shown), indicating that the inhibition of PARP was responsible for the improved recovery.

In Fig. 7, the time dependence of ATP, creatine phosphate, and inorganic phosphate levels is shown during ischemia-reperfusion cycle using four PARP inhibitors. These data show that each PARP inhibitor improved significantly the final recovery of high-energy phosphate intermediates, and the rate of recovery was increased in the presence of PARP inhibitors (Fig. 7). In accord with the high-energy phosphate data, inorganic phosphate levels were decreased in the reperfusion phase (Fig. 7), and PARP inhibitors increased the rate of inorganic phosphate utilization.

Dose response of PARP inhibitors on the recovery of creatine phosphate was studied in Langendorff perfused hearts (Fig. 8.) These data show that PARP inhibitors in a concentration-dependent manner promoted the recovery of creatine phosphate in perfused hearts. The IC_{50} values were 239, 485, 32, and 19 μM for 3-aminobenzamide, nicotinamide, BGP-15, and 4-hydroxyquinazoline, respectively. Recovery of ATP showed similar dose response as creatine phosphate (data not shown), and IC_{50} values were 301, 476, 48, and 26 μM for 3-aminobenzamide, nicotinamide, BGP-15, and 4-hydroxyquinazoline, respectively.

TABLE 2

Effect of PARP inhibitors on the ischemia-reperfusion and hydrogen peroxide-induced protein oxidation in Langendorff perfused rat hearts

Protein oxidation (protein carbonyl content with 2,4-dinitrophenylhydrazine) was measured as detailed under *Materials and Methods* in Langendorff heart perfusion system either in reperfused heart (25 min of ischemia followed by 15 min of reperfusion) or in normoxic heart perfused with 1 mM H_2O_2 for 30 min. Concentrations of the applied chemicals were 3 mM for 3-aminobenzamide (3-AB), 3-aminobenzoic acid (3-ABA) and nicotinamide, 0.114 for mM BGP-15, and 0.1 mM for 4-hydroxyquinazoline (4-HQ). Values are mean ± S.E.M. for five experiments.

Inhibitor	Protein Carbonyl Content			
	Normoxic	Ischemia-Reperfusion	No addition	H_2O_2 added
	nmol carbonyl/mg of protein			
None	1.22 ± 0.14	2.47 ± 0.07	1.21 ± 0.13	2.67 ± 0.12
3-AB	1.21 ± 0.07	1.59 ± 0.13*	1.20 ± 0.07	1.75 ± 0.12†
3-ABA	1.22 ± 0.09	2.43 ± 0.09	1.23 ± 0.08	2.71 ± 0.22
Nicotinamide	1.20 ± 0.11	1.45 ± 0.15*	1.20 ± 0.11	1.81 ± 0.21†
BGP-15	1.20 ± 0.12	1.52 ± 0.16*	1.22 ± 0.19	1.82 ± 0.19†
4H-ISO	1.21 ± 0.09	1.49 ± 0.11*	1.21 ± 0.07	1.78 ± 0.13†

Difference from ischemia-reperfusion group * $p < 0.01$.
Difference from H_2O_2-treated group † $p < 0.01$.

Protecting Effect of PARP Inhibitors against Ischemia-Reperfusion-Induced Damage of Respiratory Complexes. Under our experimental conditions, ischemia-reperfusion caused a partial inactivation of respiratory complexes (Table 3), which could be the consequence of mitochondrial ROS formation during reperfusion (Ambrosio et al., 1993; Vanden Hoek et al., 1998; Szabados et al., 1999b). Our data (Figs. 2 and 3; Tables 1 and 2) indicated that PARP inhibitors decreased the oxidative damage for different components of heart during ischemia-reperfusion cycle; therefore, it is reasonable to assume that PARP inhibitors may attenuate the inactivation of respiratory complexes. Using four different PARP inhibitors, we found that cytochrome oxidase activity was almost completely protected from the ischemia-reperfusion-induced partial inactivation (Table 3).

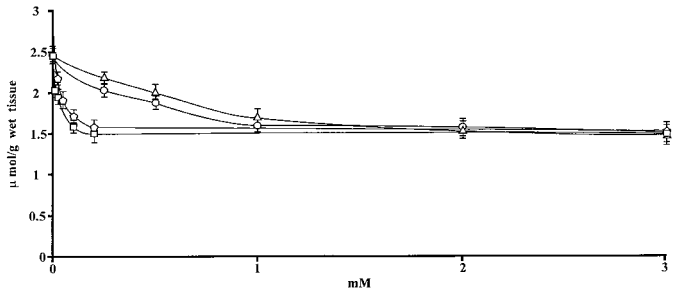

Fig. 3. Dose response of PARP inhibitors on the ischemia-reperfusion-induced protein oxidation in Langendorff perfused rat hearts. Protein oxidation (protein carbonyl content with 2,4-dinitrophenylhydrazine) was measured in Langendorff heart perfusion system in reperfused hearts (25 min of ischemia followed by 15 min of reperfusion) as detailed under *Materials and Methods*. Concentrations of the applied chemicals are as indicated in the figure. Values are mean ± S.E.M. for five experiments. Significant decrease was seen in protein oxidation: 3-aminobenzamide (○) at 0.5 mM, $p < 0.05$, at higher concentrations, $p < 0.01$; nicotinamide (△) at 5 mM, $p < 0.05$, at higher concentrations, $p < 0.01$; BGP-15 (⬠) at 0.0 mM, $p < 0.05$, at higher concentrations, $p < 0.01$; and 4-hydroxyquinazoline (□) at 0.01 and 0.02 mM, $p < 0.05$, at higher concentrations, $p < 0.01$.

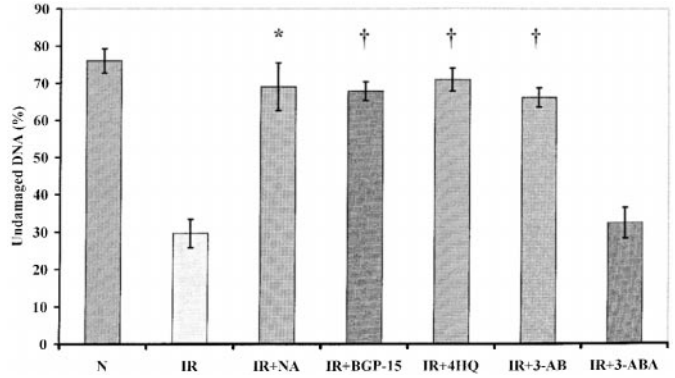

Fig. 4. Effect of PARP inhibitors on the ischemia-reperfusion-induced single-strand DNA breaks in Langendorff perfused rat hearts. Single-strand DNA breaks (determined by the alkaline fluorescence analysis of DNA unwinding) was measured in Langendorff heart perfusion system in normoxic or in reperfused hearts (25 min of ischemia followed by 15 min of reperfusion) as detailed under *Materials and Methods*. N, normoxic; IR, ischemia-reperfusion; IR + NA, ischemia-reperfusion in the presence of 3 mM nicotinamide; IR + 3-AB, ischemia-reperfusion in the presence of 3 mM 3-aminobenzamide; IR + 3-ABA, ischemia-reperfusion in the presence of 3 mM 3-aminobenzoic acid; IR + BGP-15, ischemia-reperfusion in the presence of 0.114 mM BGP-15; and IR + 4HQ, ischemia-reperfusion in the presence of 0.1 mM 4-hydroxyquinazoline. Values are mean ± S.E.M. for five experiments. Difference from ischemia-reperfusion, $^{*}p < 0.05$, $^{\dagger}p < 0.01$.

Ischemia-reperfusion also partially inactivated the NADH:cytochrome *c* oxidoreductase activity (complex I-III), and PARP inhibitors could partially protect NADH:cytochrome *c* oxidoreductase activity in postischemic heart mitochondria (Table 3). Under the same experimental conditions, H_2O_2 or PARP inhibitors did not affect citrate synthase activity (data not shown).

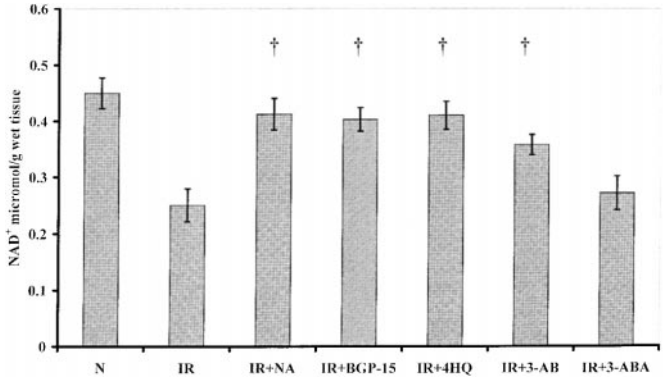

Fig. 5. Effect of PARP inhibitors on the ischemia-reperfusion-induced NAD^+ loss in Langendorff perfused rat hearts. The NAD^+ content using an alcohol dehydrogenase method was measured as detailed under *Materials and Methods* after 15 min of reperfusion. N, normoxic; IR, ischemia-reperfusion; IR + NA, ischemia-reperfusion in the presence of 3 mM nicotinamide; IR + 3-AB, ischemia-reperfusion in the presence of 3 mM 3-aminobenzamide; IR + 3-ABA, ischemia-reperfusion in the presence of 3 mM 3-aminobenzoic acid; IR + BGP-15, ischemia-reperfusion in the presence of 0.114 mM BGP-15; and IR + 4HQ, ischemia-reperfusion in the presence of 0.1 mM 4-hydroxyquinazoline. Values are mean ± S.E.M. for five experiments. Difference from ischemia-reperfusion $^{\dagger}p < 0.05$.

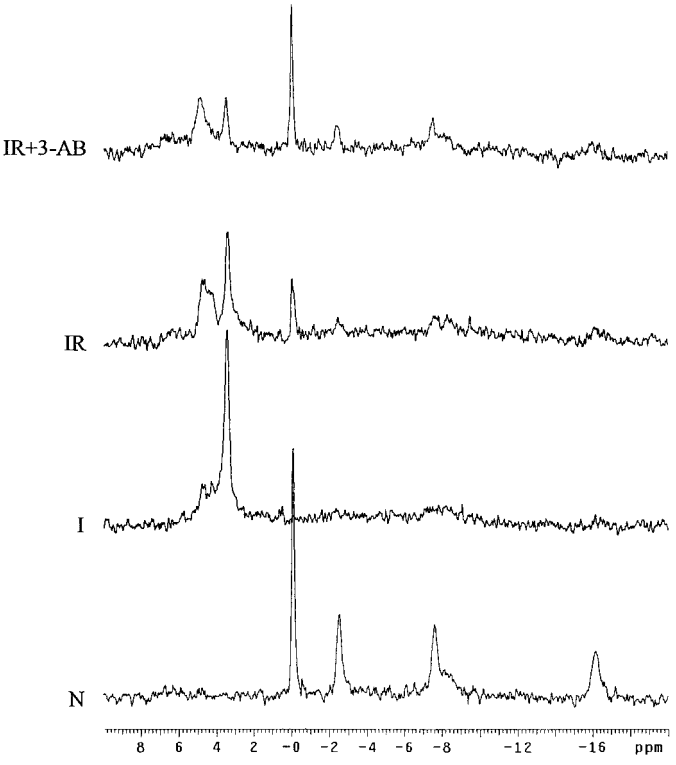

Fig. 6. Effect of 3-aminobenzamide on the recovery of myocardial energy production after ischemia-reperfusion in Langendorff perfused heart as monitored by ^{31}P NMR spectroscopy. Conditions for heart perfusion and NMR measurements were described under *Materials and Methods*. N, normoxic; I, ischemic; IR, ischemia-reperfusion; and IR + 3-AB, ischemia-reperfusion in the presence of 3 mM 3-aminobenzamide.

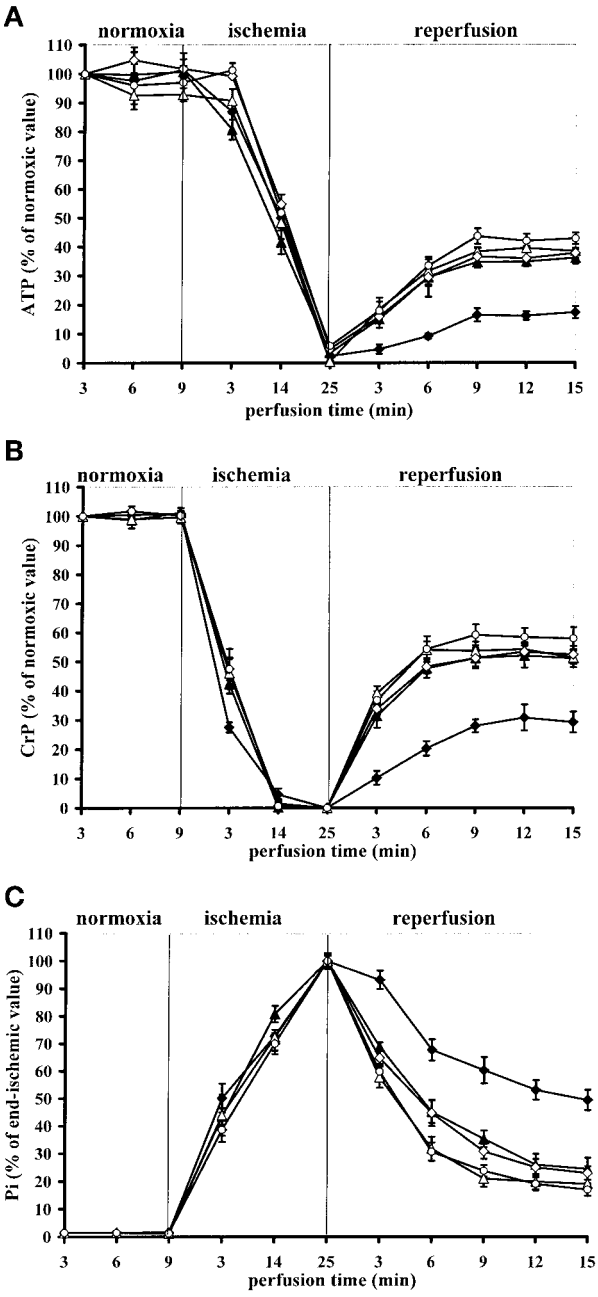

Fig. 7. Effect of PARP inhibitors on the recovery of ATP (A), creatine phosphate (B), and inorganic phosphate (C) in Langendorff perfused hearts. Conditions for heart perfusion and NMR measurements were described under *Materials and Methods*. IR, ischemia-reperfusion (♦); IR + NA, ischemia-reperfusion in the presence of 3 mM nicotinamide (▲); IR + 3-AB, ischemia-reperfusion in the presence of 3 mM 3-aminobenzamide (△); IR + BGP-15, ischemia-reperfusion in the presence of 40 mg/l (0.1139 mM) BGP-15 (◇); and IR + 4HQ, ischemia-reperfusion in the presence of 0.1 mM 4-hydroxyquinazoline (○) are given as mean ± S.E.M. for five experiments. A, significant changes were seen: IR versus IR+ NA, $p < 0.05$ at all time points of reperfusion phase; IR versus IR+ 3-AB, $p < 0.05$ at all time points of reperfusion phase; IR versus IR+ BGP-15, $p < 0.05$ at all time points of reperfusion phase; IR versus IR + 4HQ, $p < 0.05$ at all time points of reperfusion phase. B, IR versus IR+ NA, $p < 0.01$ at 3, 6, 9, and 15 min and $p < 0.05$ at 12 min; IR versus IR + 3-AB, $p < 0.01$ at all time points of reperfusion phase; IR versus IR + BGP-15, $p < 0.01$ at all time points of reperfusion phase; IR versus IR + 4HQ, $p < 0.01$ at all time points of reperfusion phase. C, IR versus IR + NA, $p < 0.05$ at all time points of reperfusion phase; IR versus IR + 3-AB, $p < 0.05$ at all time points of reperfusion phase; IR versus IR + BGP-15, $p < 0.01$ at all time points of reperfusion phase; IR versus IR + 4HQ, $p < 0.01$ at all time points of reperfusion phase.

Effect of PARP Inhibitors on Oxidative Inactivation of Cytochrome Oxidase. Hydrogen peroxide (0.5 mM) induced a relatively fast inactivation of cytochrome oxidase in isolated mitochondria (Fig. 9), showing that ROS can indeed inactivate respiratory complexes. In the same system, PARP inhibitors (3-aminobenzamide, nicotinamide, BGP-15, and 4-hydroxyquinazoline) could almost completely protect cytochrome oxidase from H_2O_2-induced inactivation, but the chemical analog of 3-aminobenzamide, 3-aminobenzoic acid, failed to do so (Fig. 9). At the same time, PARP inhibitors did not affect the amount of H_2O_2-induced ROS production as determined by the oxidation of dihydrorhodamine123 to rhodamine123 (data not shown), so the protection was not due to a decreased amount of ROS in the presence of PARP inhibitors. Dose response of PARP inhibitors in the protection of respiratory complexes against H_2O_2-induced inactivation is shown on Fig. 9. The IC_{50} values were 394, 238, 38, and 14 μM for 3-aminobenzamide, nicotinamide, BGP-15, and 4-hydroxyquinazoline, respectively.

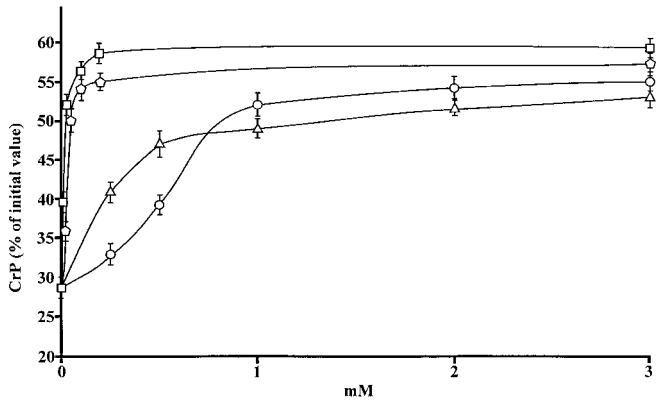

Fig. 8. Dose response of PARP inhibitors on the ischemia-reperfusion-induced creatine phosphate recovery in Langendorff perfused rat hearts. Conditions for heart perfusion and NMR measurements were described under *Materials and Methods*. Concentrations of PARP inhibitors are as indicated in the figure. Values are mean ± S.E.M. for five experiments. A significant increase was seen in creatine phosphate level: 3-aminobenzamide (○) at 0.5 mM, $p < 0.05$ and at higher concentrations, $p < 0.01$; nicotinamide (△) at 0.25 mM, $p < 0.05$ and at higher concentrations, $p < 0.01$; BGP-15 (⬠) at 0.025 mM, $p < 0.05$ and at higher concentrations, $p < 0.01$; and 4-hydroxyquinazoline (□) at 0.01 mM, $p < 0.05$ and at higher concentrations, $p < 0.01$.

TABLE 3

Effect of PARP inhibitors on the ischemia-reperfusion-induced inactivation of respiratory complexes

Conditions for heart perfusion and the measurement of mitochondrial enzyme activities were detailed under *Materials and Methods*. Concentration of the applied chemicals were 3 mM for 3-aminobenzamide (3-AB), 3-aminobenzoic acid (3-ABA) and nicotinamide, 0.114 for mM BGP-15, and 0.1 for mM 4-hydroxyquinazoline (4-HQ). Values are given as mean ± S.E.M. for five experiments.

Treatment	Cytochrome oxidase	NADH:Cytochrome *c* Oxidoreductase
	% of normoxic value	
Normoxic	100 ± 3.2	100 ± 2.9
IR	69.1 ± 2.3	68.3 ± 2.4
IR + 3-AB	99.3 ± 2.8*	93.6 ± 2.9*
IR + 3-ABA	65.7 ± 3.1	67.4 ± 2.1
IR + nicotinamide	97.5 ± 2.4*	93.5 ± 3.1*
IR + BGP-15	97.8 ± 3.2*	94.4 ± 3.2*
IR + 4HQ	95.2 ± 4.1*	97.2 ± 3.8*

Difference from ischemia-reperfusion group * $p < 0.01$.

Discussion

The Langendorff heart perfusion system has been successfully used for studying ischemia-reperfusion-induced cardiac damages. In this model, PARP inhibitors were reported to improve the recovery of heart function and ATP level, as well as decrease the infarct size (Bowes et al., 1999; Docherty et al., 1999). Similar protective effects of PARP inhibitors were also observed in local cardiac ischemia in living animals (Zingarelli et al., 1997; Bowes et al., 1998). It is known that ROS are mainly produced by mitochondrial respiratory complexes in postischemic heart (Ambrosio et al., 1993; Vanden Hoek et al., 1998), and that ROS-induced oxidative damages represent an important mechanism of the pathological processes in postischemic heart. Our previous data indicated that a new PARP inhibitor (BGP-15) decreased the ischemia-reperfusion-induced oxidative damages in Langendorff perfused heart model system without having any obvious antioxidant property (Szabados et al., 2000). These experiments raise an interesting question whether this is a special property of the compound BGP-15 or other PARP inhibitors have similar characteristics. To answer this question we investigated the effect of well-established PARP inhibitors (3-aminobenzamide and nicotinamide) together with BGP-15 on the oxidative cell damage induced by either ischemia-reperfusion or hydrogen peroxide in Langendorff heart perfusion system.

Under our experimental conditions, PARP inhibitors abrogated the ischemia-reperfusion-induced lipid peroxidation (Fig. 2; Table 1) and protein oxidation (Fig. 3; Table 2), and significantly decreased ssDNA break formation (Fig. 4), suggesting that PARP inhibitors somehow reduced the ischemia-reperfusion-induced mitochondrial ROS production and ROS-related oxidative damages. When the oxidant was given to the heart externally (1 mM H_2O_2), it induced lipid peroxidation and protein oxidation, but PARP inhibitors only partially protected heart tissue from the lipid peroxidation and protein oxidation (Figs. 1 and 2). These observations could not be explained by a simple antioxidant effect because the studied compounds could not entrap ROS generated chemically as shown in this article and by Szabados et al. (2000). In perfused heart, most of the ROS is produced in the mitochondria during ischemia-reperfusion cycle (Ambrosio et al., 1993; Vanden Hoek et al., 1998); therefore, our data (Figs. 1–3) indicate that PARP inhibitors probably decrease the ischemia-reperfusion-induced increase of mitochondrial ROS production, and so all oxidative damages related to enhanced ROS production. Because the blocking of normal electron flow generally activates the formation of partially reduced reactive oxygen intermediates (Turrens et al., 1991), the observation that PARP inhibitors prevent the ischemia-reperfusion-induced inactivation of respiratory complexes (Fig. 9) supports this argument.

In the case of externally added H_2O_2, PARP inhibitors

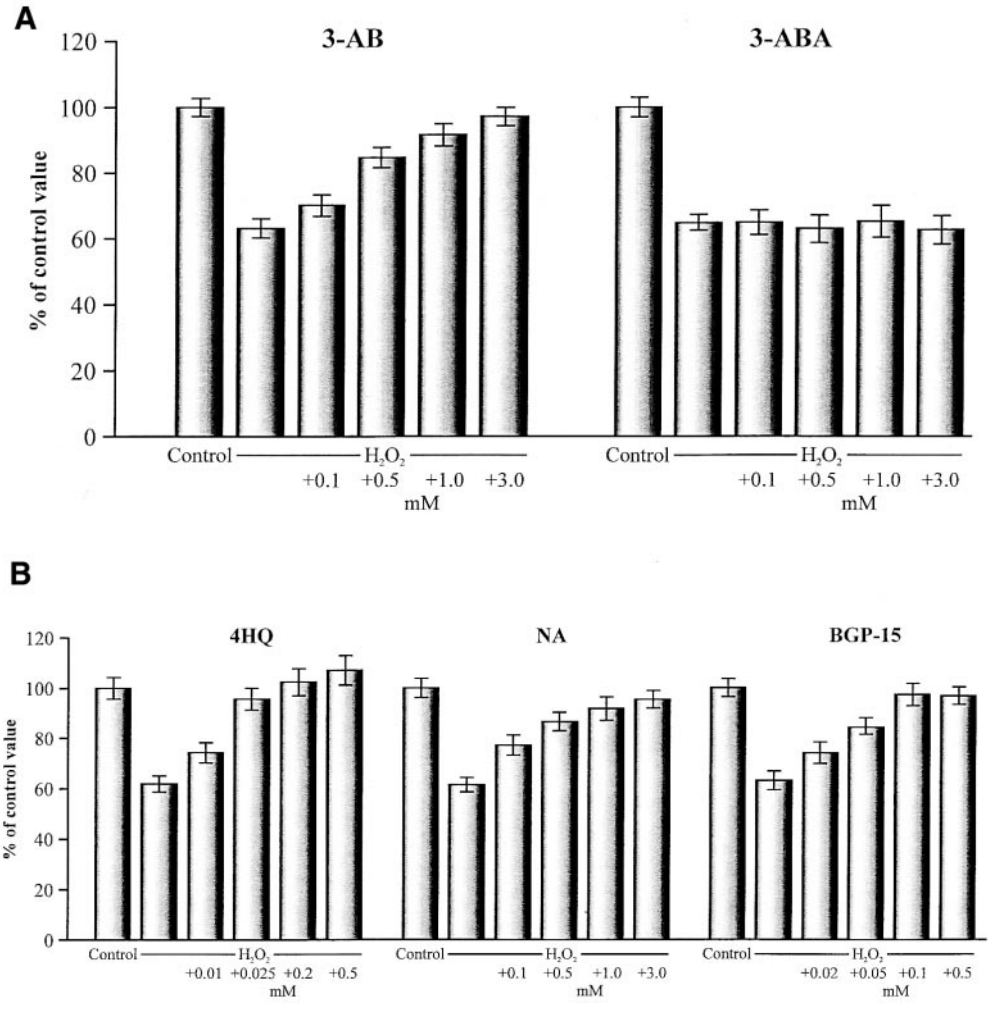

Fig. 9. Effect of PARP inhibitors on the H_2O_2-induced inactivation of cytochrome oxidase. Experimental conditions and cytochrome oxidase activity measurement are detailed under *Materials and Methods*. Concentrations of the applied chemicals are as indicated in the figure. 3-AB, 3-aminobenzamide; 3-ABA, 3-aminobenzoic acid; 4-HQ, 4-hyroxyquinazoline; NA, nicotinamide; and BGP-15, BGP-15. Values are mean ± S.E.M. for five experiments. Significant increase was seen: 3-AB at 0.5 mM and higher concentrations, $p < 0.05$; NA at 0.1 and 0.5 mM, $p < 0.05$ and at higher concentrations, $p < 0.01$; BGP-15 at 0.05 mM, $p < 0.05$ and higher concentrations, $p < 0.01$; and 4HQ at 0.025 mM and higher concentrations, $p < 0.05$.

could not prevent the direct oxidative damage caused by the H_2O_2 (Tables 1 and 2) but could decrease the H_2O_2-induced inactivation to the components of the mitochondrial respiratory chain (Fig. 9). The endogenous ROS production could thereby decrease the total amount of oxidative damages seen in Figs. 2 and 3 and Tables 1 and 2. Biochemical effects of H_2O_2 can be different depending on the concentration applied. High concentration (higher than 1 mM) of H_2O_2 induces DNA breaks, PARP activation, NAD^+ depletion, ATP depletion, cell membrane damage, and necrotic cell death, processes that can be attenuated by PARP inhibitors (Gardner et al., 1997; Virag et al., 1998; Filipovic et al., 1999). H_2O_2 can also induce apoptic cell death in several cell types, which can also be prevented under certain conditions by PARP inhibitors (Hivert et al., 1998). In contrast to these, very low concentrations of H_2O_2 can be regarded as a life signal, and help proliferation and protect against apoptosis possibly by preventing caspase activation and PARP cleavage (delBello et al., 1999).

Under our experimental conditions, PARP inhibitors decreased the ischemia-reperfusion-induced NAD^+ depletion. This obviously PARP-dependent nuclear process through the modulation of NAD^+ level (normoxic 0.45 mg/g of wet tissue, ischemia-reperfusion 0.24 mg/g of wet tissue, and ischemia-reperfusion in the presence of PARP inhibitors 0.35–0.41 mg/g wet tissue) (Fig. 5) could contribute to the better recovery of the high-energy phosphate intermediates (Figs. 6–8). The PARP inhibitors decreased the rate of NAD^+ cleavage, and so reduced the ATP consumption for the resynthesis of NAD^+ in postischemic hearts. Our data show that PARP inhibitors uniformly promoted the recovery of ATP and creatine phosphate levels and resulted in not only higher percentage of recovery but also the a significantly faster rate of recovery (Fig. 7). In addition, the consumption rate of inorganic phosphate was increased during the recovery period by PARP inhibitors. Because Ca^{2+} and inorganic phosphate can induce mitochondrial permeability transition (Javadov et al., 2000), the faster and more complete utilization of inorganic phosphate can be advantageous for the preservation of metabolically active, coupled mitochondria in postischemic heart. Hearts predominantly produce energy by mitochondrial oxidation, therefore the faster recovery seen in the presence of PARP inhibitors (Fig. 7) can be, at least in part, the consequence of protected mitochondrial energy metabolism. It is known that ROS can inactivate respiratory complexes (Turrens et al., 1991; Fig. 9), therefore, protection against ischemia-reperfusion-induced inactivation of cytochrome oxidase and NADH:ubiquinone oxidoreductase can also be a factor in addition to the inhibition of nuclear PARP in the faster and more complete recovery of high-energy phosphate intermediates.

According to one possible argument, PARP inhibition by moderating cytoplasmic NAD^+ loss can help to retain mitochondrial NAD^+, and may prevent the decrease of the mitochondrial NAD^+-linked substrate oxidation and ROS formation between NADH-dehydrogenase and ubiquinone. Therefore, the protective effect of PARP inhibitors against oxidative damages (lipid peroxidation, protein oxidation, single-strand DNA breaks, and the inactivation of respiratory complexes) in postischemic heart may not rely exclusively on the inhibition of PARP. It required further confirmation that the inhibition of a nuclear enzyme (PARP) can result in such a good protection against mitochondrial damages. Therefore, we investigated whether PARP inhibitors can have a protective effect against H_2O_2-induced inactivation of cytochrome oxidase in isolated rat heart mitochondria (Fig. 9). Our data showed the H_2O_2-induced inactivation of cytochrome oxidase was almost completely protected by PARP inhibitors in isolated mitochondria (Fig. 9). In the same experimental system, 3-aminobenzoic acid, an inactive chemical homolog of the PARP inhibitor 3-aminobenzamide, did not protect cytochrome oxidase against H_2O_2-induced inactivation (Fig. 9). In isolated mitochondria, nuclear PARP cannot play any possible role (PARP activity was not detectable in our isolated mitochondria with autoradiography, data not shown); therefore, we have to assume that PARP inhibitors bind to another mitochondrial protein that plays a role in the oxidative damage of mitochondria. It is known that several PARP inhibitors can also inhibit mono-ADP-ribose transferase or NAD^+ glycohydrolase, which are present in the mitochondria (Ziegler et al., 1997; Jorcke et al., 1998); therefore, it is possible that PARP inhibitors by decreasing the activity of any of these enzymes prevent the inactivation of respiratory complexes.

These data do not conflict with the previous observation using PARP-1-deleted cells or PARP-1 knockout mice (Endres et al., 1998; Zingarelli et al., 1998; Grupp et al., 1999; Yang et al., 2000), but indicate that the PARP inhibitors besides their inhibitory effect on nuclear PARP, which results in significant protection against oxidative damage, can have an additional mitochondrial target (binding site for these molecules) and this direct mitochondrial effect can play a protecting role in oxidative mitochondria damage.

In conclusion, the studied PARP inhibitors, besides their primary effect of decreasing the activity of nuclear PARP and therefore decreasing NAD^+ consumption and ATP consumption, protect mitochondrial energy metabolism (Figs. 5–8), decrease the ischemia-reperfusion-induced mitochondrial ROS formation (Figs. 2 and 3), and protect the respiratory complexes from ROS-induced inactivation (Fig. 9). The mitochondrial protective effect of PARP inhibitors, at least in partially, is independent of nuclear PARP activity because it can be observed in isolated mitochondria. Therefore, it represents a novel mechanism for the mitochondrial protective effects of PARP inhibitors.

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