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BGP-15, a Nicotinic Amidoxime Derivate Protecting Heart from Ischemia Reperfusion Injury through Modulation of Poly(ADP-ribose) Polymerase

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ABSTRACT. The protective effect of O-(3-piperidino-2-hydroxy-1-propyl)nicotinic amidoxime (BGP-15) against ischemia–reperfusion-induced injury was studied in the Langendorff heart perfusion system. To understand the molecular mechanism of the cardioprotection, the effect of BGP-15 on ischemic–reperfusion-induced reactive oxygen species (ROS) formation, lipid peroxidation single-strand DNA break formation, NAD^+ catabolism, and endogenous ADP-ribosylation reactions were investigated. These studies showed that BGP-15 significantly decreased leakage of lactate dehydrogenase, creatine kinase, and aspartate aminotransferase in reperfused hearts, and reduced the rate of NAD^+ catabolism. In addition, BGP-15 dramatically decreased the ischemia–reperfusion-induced self-ADP-ribosylation of nuclear poly(ADP-ribose) polymerase(PARP) and the mono-ADP-ribosylation of an endoplasmic reticulum chaperone GRP78. These data raise the possibility that BGP-15 may have a direct inhibitory effect on PARP. This hypothesis was tested on isolated enzyme, and kinetic analysis showed a mixed-type (noncompetitive) inhibition with a $K_i = 57 \pm 6$ μM. Furthermore, BGP-15 decreased levels of ROS, lipid peroxidation, and single-strand DNA breaks in reperfused hearts. These data suggest that PARP may be an important molecular target of BGP-15 and that BGP-15 decreases ROS levels and cell injury during ischemia–reperfusion in the heart by inhibiting PARP activity. BIOCHEM PHARMACOL **59**;8: 937–945, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. ischemia–reperfusion; free radicals; reactive oxygen species; cell damage; signal; heart perfusion; BGP-15; poly(ADP-ribose) polymerase; GRP78; lipid peroxidation; chaperone

Ischemia–reperfusion generates ROS‖ such as H_2O_2, superoxide radicals, and hydroxyl radicals formed in the Fenton reaction [1]. ROS in turn can initiate lipid peroxidation [2], protein oxidation [3], and the formation of single-strand DNA breaks [4–6]. These forms of damage are believed to be partly responsible for the myocardial damage associated with infarction and reperfusion [7]. ROS and NO formed under the ischemia–reperfusion cycle induce single-strand DNA breaks which in turn activate nuclear PARP [8, 9]. Upon activation, PARP cleaves NAD^+ to nicotinamide and ADP-ribose, with the latter being coupled to different proteins and protein-bound ADP-ribose residues. Excessive PARP activation leads to cellular NAD^+ depletion and ATP depletion, which eventually cause necrosis [10]. Therefore, inhibition of PARP can partially prevent ROS and NO toxicity [11–13] and ischemia–reperfusion-induced cell death [12]. Mitochondria play a significant role in postischemic cell damage, because ischemia–reperfusion induces a massive influx of Ca^{2+} into the mitochondrial matrix and inhibits electron transport specifically between NADH and ubiquinone [14]. The inhibition of mitochondrial electron transport at complex I induces the excessive formation of ROS in postischemic hearts [15, 16]. Elevated ROS levels can induce mitochondrial permeability transition, which can lead to intramitochondrial NAD^+ loss and the inhibition of mitochondrial NAD^+-linked substrate oxidation [17], further damaging mitochondrial energy production and finally resulting in apoptotic or necrotic cell death [18, 19]. ROS-induced impaired energy metabolism and cell death can be partially reverted by PARP inhibitors, supposingly because these latter significantly decrease the rate of ROS-activated NAD^+ catabolism and reduce ATP utilization for the resynthesis of NAD^+ [20, 21]. The fact that PARP inhibitors decrease the catabolism of cytoplasmic NAD^+ and so possibly decrease ROS-induced mitochondrial NAD^+ loss suggests a connection between oxidative mitochondrial damage and PARP activation. Therefore, it is possible that protection of cells from oxidative damage can be achieved not only by the reduction of ROS levels either with antioxidants [22–25] or with ROS-scavengering enzymes [26], but by the potential of

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‖ *Abbreviations:* ROS, reactive oxygen species; GRP78, glucose-regulated protein; TBARS, thiobarbituric acid reactive substances; DHR, dihydrorhodamine123; MDA, malondialdehyde; LDH, lactate dehydrogenase; CK, creatine kinase; AST, aspartate aminotransferase; NO, nitric oxide; TCA, trichloroacetic acid; IgG, immunoglobuline G; DTT, dithiothreitol; and PARP, poly(ADP-ribose) polymerase.

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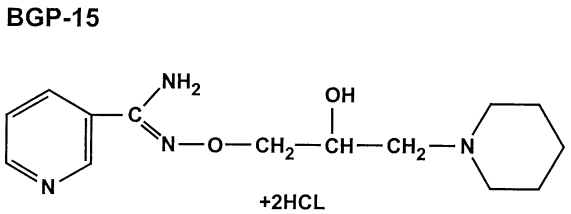

FIG. 1. Chemical structure of BGP-15 (O-(2-hydroxy-3-piperidinepropyl)-pyridine-carbonic acid-amidoxime dihydrochloride).

PARP inhibitors to reduce mitochondrial injury and ROS production.

In this work, we investigate the molecular mechanism by which O-(3-piperidino-2-hydroxy-1-propyl)nicotinic amidoxime (BGP-15), (Fig. 1) protects the heart against ischemia–reperfusion injury in the Langendorff heart perfusion system. Evidence is provided that BGP-15 decreases endogenous ADP-ribosylation in the heart and concomitantly suppresses ROS levels and cell injury.

MATERIALS AND METHODS

Chemicals

NAD, DHR, and IgG peroxidase were purchased from Sigma–Aldrich Chemical Co. and Malondialdehyde-bis (diethylacetal) was obtained from Merck. Anti-ADP-ribose antibody was a kind gift from Alexander Buerkle (Heidelberg, Germany) and Masanao Miwa (Tsukuba, Japan). BGP-15 was provided us by N-Gene Research Laboratories, Inc. The low-molecular-weight electrophoresis kit was purchased from Pharmacia. All other reagents were of the highest purity commercially available.

Animals

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

Heart Perfusion

Rats were anesthetized with ketamine (200 mg/kg i.p.) 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 mmHg at 37°, as described previously [27, 28]. 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, and in some experiments the medium contained an additional (40 mg/L (113.9 mM) of 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, non-recirculating period of 15 min, hearts were either perfused under normoxic conditions for the given time, or subjected to a global ischemia of 45 min by closing the aortic influx and reperfused for 5, 15, or 30 min. During ischemia, hearts were submerged in perfusion buffer at 37°. At the end of perfusion, hearts were freeze-clamped.

Assay of NAD^+

The concentration of NAD^+ in the neutralized perchloric acid extract of cardiac muscle was measured by using the alcohol dehydrogenase reaction [29]. The freshly prepared reaction buffer contained 0.1 M Tris, pH 8.4, 1 mM EDTA, 4 mM L-cysteine chloride, and 2% ethanol. Each cuvette contained 300 μL of the tissue extract, 650 μL of the reaction buffer, and 4 units of enzyme. The reaction was initiated by the addition of the enzyme, and the exact tissue NAD^+ concentrations were determined from a calibration curve.

Detection of ROS

ROS formation was detected using the oxidation-sensitive non-fluorescent probe DHR, which can be oxidized by ROS to fluorescent rhodamine123 [30]. ROS formation was first studied in normoxic hearts. After a 15-min washout, DHR (5 μM) was added to the perfusate and perfused for an additional 15 min. In the case of hearts subjected to a 45-min ischemia and a 15-min reperfusion, DHR (5 μM) was added to the perfusate prior to reperfusion. The perfusions were terminated by freeze-clamping the hearts. For the extraction of rhodamine123, 90 mg of heart pieces was homogenized in 2 mL of deoxygenated ice-cold 20 mM Tris buffer pH 7.4 twice for 20 sec with an Ultra Turrax homogenizer and an equal amount of ice-cold 70% ethanol containing 0.1 M HCl was added. The precipitated proteins were removed by centrifugation at 3000 *g* for 15 min. The precipitate was extracted again, and the aliquots of the combined supernatants were neutralized with $NaHCO_3$ and centrifuged at 6000 *g*. Rhodamine123 content in the clear supernatant was determined by a Perkin-Elmer fluorescence spectroscope at an excitation wavelength of 500 nm and an emission wavelength of 536 nm. To correct the extraction procedure, 90 mg of heart pieces (perfused without DHR) was homogenized in 2 mL of 20 mM Tris buffer pH 7.4 containing 5 μM DHR and extracted under the same conditions. The amount of fluorescent rhodamine123 formed under these conditions was in the range of the 3–7% of rhodamine123 formed during perfusion in normoxic heart, whose values were subtracted from those obtained in the different hearts perfused with DHR. To check the effectiveness of rhodamine123 extraction, 90 mg of heart pieces (perfused without DHR) was homogenized in 2 mL of 20 mM Tris buffer pH 7.4 containing 1 μM rhodamine123 and extracted under the same conditions. These data showed that 91–96% of the rhodamide was extracted from the heart homogenate with the procedure described above. Rhodamine123 concentrations were also

determined in the perfusates and turned out to be virtually undetectable, indicating that most of the rhodamine123 was retained by the heart, which is in accord with previous observations [31].

ROS formation was also detected under *in vitro* conditions when DHR was added following a 30-min normoxic perfusion or a 45-min ischemia. In both cases, heart pieces (50 mg) were homogenized in 3 mL of well-oxygenated standard buffer and incubated with 5 μM DHR in the presence or absence of 50 μM BGP-15 for 30 min at 37°. The extraction and movement of rhodamine123 was performed as above. To correct for background heart, tissue samples were incubated under the same conditions without DHR, and the 5-μM DHR was given only at the end of the incubation period.

Determination of DNA Single-Strand Breaks

Single-strand DNA breaks were determined by the alkaline fluorescence analysis of DNA unwinding as described by Birnboim and Jevcak [32]. DNA samples were prepared from normoxic- and ischemic-perfused hearts. To estimate the quantity of undamaged double-stranded DNA, samples were divided into 3 sets of tubes. DNA fluorescence was determined under different conditions. To determine the F (fluorescence) value, DNA was kept at pH 12.4 to permit partial unwinding of DNA. To determinate F_{min}, DNA was kept at pH 12.4, but at the beginning of the incubation period the DNA sample was sonicated for 60 sec. To determinate 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° followed by a 15-min incubation at 15°. 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 on a Perkin-Elmer luminescence spectrometer. Results are expressed as D (percent of double-stranded DNA) = $(F - F_{min})/(F_{max} - F_{min}) \times 100$.

ADP-ribosylation Assay

Hearts were freeze-clamped at the end of perfusion and kept in liquid nitrogen. Cardiac muscle (50 mg) was homogenized by Ultra Turrax in 500 μL of 50 mM Tris pH 7.8, and then 500 μL of 2 × Laemmli sample buffer was added, homogenized with a Potter-Elvehiem homogenizer, and cleared by centrifugation for 5 min at 10,000 *g*. In some experiments, the extraction buffer contained 8 M urea, 20 mM Tris, and 4 mM EDTA. Samples were subjected to SDS–PAGE [33] using a 10% gel and blotted to nitrocellulose membrane for Western blot analysis. ADP-ribosylated proteins were detected by anti-ADP-ribose monoclonal antibody and anti-mouse IgG peroxidase complex and visualized by the enhanced chemiluminescence (ECL) method.

The ADP-ribosylation of nuclear proteins was detected by immuno-dot blot analysis. Rat heart nuclei were isolated as described in [34]. The purified nuclei were prepared for dot blotting using extraction buffer containing 8 M urea, 20 mM Tris, 4 mM EDTA, and 2× Laemmli sample buffer. The protein-bound ADP-ribose content was determined by anti-ADP-ribose monoclonal antibody and anti-mouse IgG peroxidase complex and visualized by the enhanced chemiluminescence (ECL) method. Dot blot data were quantitated by the Image Tool (Version 1.27) image processing program.

Assay to Test Inhibitory Effects on PARP Enzyme In Vitro

PARP was isolated from rat liver based on a method described previously [35]. The potential inhibitory effect of BGP-15 (over a range of concentrations) was tested in this assay system. PARP activity was determined in a 130-μL reaction mixture containing 100 mM Tris–HCl buffer, pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 1.5 mM DTT, 1 mM [adenine-2,8-^{3}H] NAD^+ (4500 cpm/nmol), 10 μg activated DNA, and 10 μg histones. The incubation time was 5 min, and the reaction was stopped by the addition of TCA (8%). After addition of 0.5 mg albumin, precipitation was allowed to continue for at least 20 min on ice, and the insoluble material was collected on a glass filter washed five times with 5% perchloric acid. Protein-bound radioactivity was determined with an LS-200 Beckman scintillation counter.

Lipid Peroxidation

Lipid peroxidation was estimated from the formation of TBARS. TBARS were determined using a modification of a previously described method [36]. Cardiac tissue was homogenized in 6.5% TCA, and a reagent containing 15% TCA, 0.375% TBA, and 0.25% HCl was added, mixed thoroughly, heated for 15 min in a boiling water bath, cooled, and centrifuged. The absorbance of the supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Using MDA standards, TBARS concentrations were calculated as nmol/g wet tissue.

Myocardial Enzyme Leakage

The release of LDM (EC 1.1.1.27), CK (EC 2.7.3.2), and AST (EC 2.6.1.1) enzymes were measured in the perfusate of Langendorff-perfused hearts under normoxic and ischemic conditions. Enzyme activities were measured by standard methods as described in [37] for LDH, [38] for AST, and [39] for CK.

RESULTS

Releasing Cytoplasmic Enzymes

The release of CK, LDH, and AST from normoxic-perfused rat hearts into the perfusate was extremely low, indicating

TABLE 1. Effect of BGP-15 on intracellular enzyme release during ischemia–reoxygenation in the Langendorff-perfused rat heart system

	AST	LDH	CK
	mU/mL		
Normoxic	2 ± 1	1 ± 1	4 ± 2
Ischemia–reperfusion	96 ± 8*	419 ± 36*	148 ± 17*
Ischemia–reperfusion +40 mg/L of BGP-15	33 ± 6	125 ± 10	61 ± 5

Membrane integrity was followed by enzyme release into the perfusate during heart perfusion in normoxic and postischemic (45-min ischemia and 30-min reperfusion) hearts. For details see Materials and Methods. Values are means ± SEM for five heart preparations.

*Value different from respective normoxic values and ischemia–reperfusion + BGP-15 values at significance $P < 0.01$.

that cell membranes were undamaged under conditions of normoxic perfusion (Table 1). Ischemia–reperfusion induced a significant release of CK, LDH, and AST (Table 1) because of serious damage to plasma membranes of cardiomyocytes [40, 41]. When hearts were perfused for 10 min with 40 mg/L of BGP-15 under normoxic conditions prior to the 45-min ischemia and after the 30-min reperfusion, a significant reduction in enzyme release was observed (Table 1). This finding shows that BGP-15 partially protected plasma membranes of cardiomyocytes from ischemia–reperfusion-related damage. BGP-15 has a low toxicity (LD_{50} = 1203.4 mg/kg orally added to rats) and thus can be safely used in the biologically effective concentration range.

Detection of ROS Formation During Perfusion and In Vitro

ROS oxidize the non-fluorescent DHRI to a fluorescent rhodamine123 [30]. Since DHR is cell-permeable and oxidized rhodamine123 is retained in the cell, this reaction can be used for the detection of ROS formed in the perfused heart. The oxidation of DHR to rhodamine123 was clearly detectable even in normoxic-perfused hearts, and the oxidation rate was significantly increased as a consequence of ischemia–reperfusion (Table 2). The pre-perfusion of hearts with BGP-15 decreased ischemia–reperfusion-induced rhodamine123 formation (Table 2), showing that BGP-15 decreases the steady-state levels of ROS in postischemic hearts. Similar data were obtained under *in vitro* conditions when DHR was added to the heart tissues and ROS were determined by the same method as with normoxic hearts, ischemic hearts, and ischemic hearts having BGP-15 in the incubation medium (Table 2).

BGP-15 did not inhibit the oxidation of DHR to rhodamine123 by chemically generated ROS (H_2O_2, Fe^{2+} ion, and chemically generated $ONOO^-$) (data not shown), indicating that the observed decrease in ROS level was not the consequence of a chemical interaction between ROS and BGP-15.

Effect of BGP-15 on ROS-Induced Lipid Peroxidation and DNA Single-Strand Breaks

Ischemia–reperfusion-induced lipid peroxidation in heart and the extent of lipid peroxidation can be characterized by the formation of TBA-reactive materials [36]. Under our experimental conditions, ischemia–reperfusion increased the amount of TBARS compared to the normoxic cases (Table 2), and the pre-perfusion of hearts with BGP-15 reduced the TBARS formation induced by ischemia–reperfusion (Table 2). Ischemia–reperfusion increased ROS concentrations, which can contribute to the increase in single-strand DNA breaks. In our experimental system, most of the DNA in normoxic-perfused hearts was undamaged, although ischemia–reperfusion induced large amounts of single-strand DNA breaks (undamaged DNA < 20%) (Table 2). As shown in Table 2, 10-min pre-perfusion of hearts with 40 mg/L of BGP-15 decreased the amount of single-strand DNA breaks and increased the quantity of undamaged DNA in postischemic heart.

TABLE 2. Effect of BGP-15 on free radical formation, single-strand DNA breaks, and lipid peroxidation during the ischemia–reperfusion cycle in the Langendorff-perfused rat heart system

	Rhodamine123 fluorescence in arbitrary units		Non-damaged DNA (%)	TBARS (nmol/g wt)
	in situ	*in vitro*		
Normoxic	21.4 ± 1.7	30.5 ± 3	71 ± 7	59.8 ± 3
Ischemia–reperfusion	28.7 ± 1.8*	54.2 ± 4*	20 ± 6*	78.1 ± 2.5**
Ischemia–reperfusion + 40 mg/L of BGP-15	22.2 ± 2.1	43.4 ± 2	39 ± 5	70.2 ± 2.7

ROS formation was followed by the oxidation of DHR to rhodamine123. Under *in vitro* conditions, hearts were freeze-clamped after 45-min ischemia and 30-min reperfusion and handled as detailed in Materials and Methods. Under *in situ* conditions, the perfusate contained 5 μM DHR during the reperfusion period and was then treated as described in Materials and Methods. DNA single-strand breaks were determined by the alkali unwinding assay. Lipid peroxidation was estimated by the formation of TBARS. Values are means ± SEM for five heart preparations.

*Value different from the other respective normoxic values and ischemia–reoxygenation + BGP-15 values at the significance $P < 0.01$.

**Value different from the respective ischemia–reperfusion + BGP-15 value at significance $P < 0.05$.

TABLE 3. Effect of BGP-15 on ischemia–reoxygenation-induced NAD loss in Langendorff-perfused rat hearts at different reperfusion times

	Reperfusion			
	0 min	5 min	30 min	60 min
Conditions	μmol (NAD^+)/gram wet tissue			
Control	0.45 ± 0.03			
Ischemia	0.38 ± 0.04**			
Ischemia–reoxygenation		0.29 ± 0.03	0.26 ± 0.02	0.24 ± 0.02
Ischemia–reoxygenation + 40 mg/L of BGP-15		0.39* ± 0.03	0.31* ± 0.01	0.30* ± 0.02

Experimental conditions and NAD^+ determinations were performed as described under Materials and Methods. Values are means ± SEM for five heart preparations.
*Value different from values without BGP-15 at significance $P < 0.02$.
**Because a significant fraction of NAD^+ was reduced to NADH, we determined the sum of NAD^+ and NADH under ischemic conditions.

Ischemia–Reperfusion-Induced NAD^+ Catabolism in Perfused Rat Hearts

Ischemia–reperfusion activates PARP which, in turn, stimulates intracellular NAD^+ catabolism so that the decreased NAD^+ content can compromise energy metabolism. Under our experimental conditions, ischemia caused only a slight decrease in the NAD^+ pool (Table 3), but ischemia followed by 5-, 30-, and 60-min reperfusion induced a significant loss of intracellular NAD^+ (Table 3). The 10-min pre-perfusion of hearts with 40 mg/L of BGP-15 partially protected the heart against ischemia–reperfusion-induced NAD^+ depletion (Table 3).

Ischemia–Reperfusion-Induced ADP-ribosylation of GRP78

Previous reports indicated the occurrence of mono-ADP-ribosylated proteins (78 and 52 kDa) in rat heart [3, 8, 42, 43]. These ADP-ribosylated proteins were detectable under our experimental conditions using anti-ADP-ribose antibodies in Western blotting (Fig. 2). Western blot analysis of ADP-ribosylated heart proteins revealed that ischemia–reperfusion increased the ADP-ribosylation of the 78 kDa protein. However, the ADP-ribosylation of the 52 kDa protein did not change significantly. On the basis of previous work, we hypothesized that the 78 kDa ADP-ribosylated protein would be the glucose-regulated protein called GRP78 [44] and belonging to the HSP70 family. Ischemia–reperfusion significantly increased the ADP-ribosylation of GRP78 (Fig. 2). The perfusion of hearts with BGP-15 prevented the ischemia–reperfusion-induced ADP-ribosylation of GRP78. In fact, the level of the ADP-ribosylation of GRP78 affected by BGP-15 was under the normoxic values (Fig. 2).

Regulation of Nuclear PARP by BGP-15

The activation of endogenous ADP-ribosylation reactions is the main cause of ischemia–reperfusion-induced NAD^+ catabolism. Therefore, it was worthwhile to study how the ADP-ribosylation of cardiac proteins changed as a consequence of ischemia–reperfusion. The self-poly-ADP-ribosylation of PARP can be detected by Western blot, as described previously [42]. In hearts undergoing ischemia–reperfusion, the self-poly-ADP-ribosylation of PARP increased (~116 kDa) when compared to the normoxic hearts (Fig. 3). The pre-perfusion of hearts with BGP-15 showed a clear inhibitory effect on the self-ADP-ribosylation of the nuclear poly(ADP-ribose) polymerase (Fig. 3). The total amount of protein-bound ADP-ribose in nuclei was determined by immuno-dot blot analysis (Fig. 3). These data indicate that ischemia–reperfusion caused a 481 ± 12% increase in the ADP-ribosylation of nuclear proteins, while the presence of BGP-15 decreased the activation of PARP to 192 ± 8% of the control value.

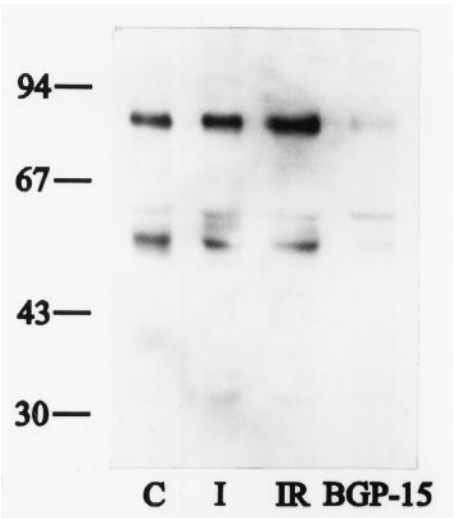

FIG. 2. Effect of BGP-15 on the ischemia–reperfusion-induced ADP-ribosylation of cytoplasmic proteins in cardiomyocytes. ADP-ribosylation of cardiomyocyte proteins was detected by Western blot using anti-ADP-ribose antibodies as described in Materials and Methods. Lane 1, normoxic heart (20 μg protein). Lane 2, ischemic heart (20 μg protein). Lane 3, postischemic heart (45-min ischemia and 30-min reperfusion) in the absence of BGP-15 (20 μg protein). Lane 4, postischemic heart (45-min ischemia and 30-min reperfusion) in the presence of 40 mg/L of BGP-15 (20 μg protein).

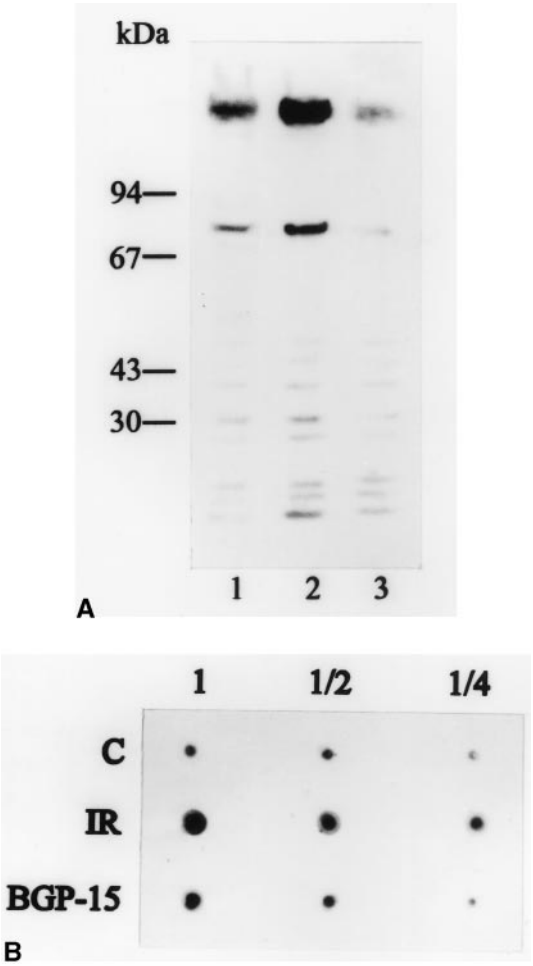

FIG. 3. Ischemia–reperfusion-induced self-ADP-ribosylation of nuclear poly(ADP-ribose) polymerase. Extraction of heart proteins occurred in the presence of 8 M urea (see Materials and Methods). Proteins were separated with SDS polyacrylamide (8%) gel electrophoresis, and the levels of ADP-ribosylation were determined by Western blot using antibodies developed against ADP-ribose. (A) Lane 1, normoxic heart (10 μg protein). Lane 2, postischemic heart (45-min ischemia and 30-min reperfusion) (10 μg protein). Lane 3, postischemic heart (45-min ischemia and 30-min reperfusion) in the presence of 40 mg/L of BGP-15 (10 μg protein). (B) Dot blot analysis of the ADP-ribosylation of nuclear proteins. From control (C), ischemia-reperfused (IR), and ischemia-reperfused in the presence of 40 mg/L of BGP-15 (IR + BGP-15) heart tissues, 250, 500, and 1000 ng protein were bound to nitrocellulose filter. Blocking and immunological detection were carried out as described under Materials and Methods. Data were analyzed by the Image Tool (Version 1.27) image processing program.

Direct Inhibition of Nuclear Poly(ADP-ribose) Polymerase

The self-ADP-ribosylation of PARP was inhibited by BGP-15 in perfused hearts under our experimental conditions (Table 2). This effect of BGP-15 on PARP could be either a direct or indirect inhibitory effect. To differentiate between these two alternatives, we investigated BGP-15 inhibition on purified PARP under *in vitro* conditions, attempting to determine whether BGP-15 inhibited the

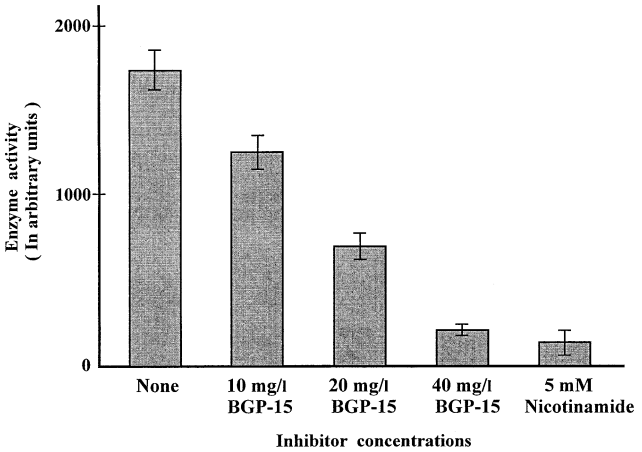

FIG. 4. Effect of BGP-15 on the activity of isolated poly(ADP-ribose) polymerase. PARP activity was determined in a 130-μl reaction mixture containing 100 mM Tris–HCl buffer, pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 1.5 mM DTT, 1 mM [adenine-2,8-^{3}H]NAD^+ (4500 cpm/nmol), 10 μg activated DNA, and 10 μg histones. Concentration of BGP-15 and nicotinamide as indicated in the figure. The incubation time was 5 min, and the reaction was stopped by the addition of trichloroacetic acid (8%). Values are means ± SEM for four experiments.

self-ADP-ribosylation of PARP under *in vitro* conditions as well (Fig. 4). The IC_{50} for BGP-15 was found to be 120 μM on purified PARP. Kinetic analysis showed a mixed-type (noncompetitive) inhibition with a $K_i = 57 \pm 6$ μM calculated from the changes in slope (Fig. 5). These data also show that the actually determined $I_{0.5}$ depended on the NAD^+ concentration used (Fig. 5).

DISCUSSION

ROS and peroxynitrite are considered major mediators of tissue injury under various pathological conditions including neuronal damage in stroke [45], myocardial infarction [11], cytokine or oxidant damage to pancreatic islet cells and hepatocytes [12, 46], and oxidant damage to pulmonary epithelium, macrophages, and smooth muscle cells [13, 47]. ROS and peroxynitrite generate single-strand DNA breaks which in turn induce activation of PARP [8, 9] and rapid depletion of intracellular NAD^+ and ATP [10], which are important for the energy metabolism of the cells and the maintenance of healthy mitochondrial function. As the disruption of the PARP gene increases tolerance against oxidative damage [6, 47], PARP inhibitors are widely used to protect cells against this type of damage [11, 45, 47, 48].

In the present work, we have investigated the effect of BGP-15 (Fig. 1) on ischemia–reperfusion injury of cardiac muscle in the Langendorff heart perfusion system.

BGP-15 significantly reduced the release of intracellular enzymes (CK, AST, and LDH) from postischemic hearts (Table 1), indicating a protection against cell membrane damage. *In vitro* studies showed that BGP-15 inhibits isolated PARP with $IC_{50} = 120$ μM at a 1-mM NAD^+ concentration. The inhibition kinetic pattern is mixed-

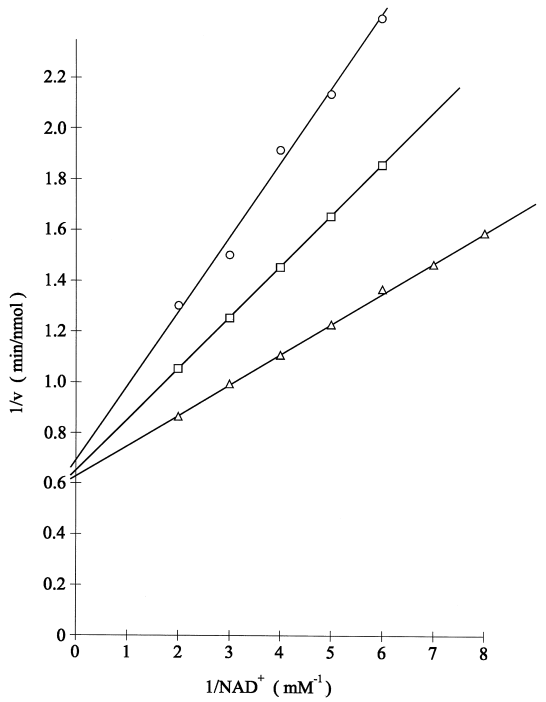

FIG. 5. Kinetic analysis of BGP-15 inhibition on poly(ADP-ribose) polymerase. The reaction mixture (130 μL) contained 100 mM Tris–HCl buffer, pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 1.5 mM DTT, 10 μg activated DNA, 10 μg histones, and [adenine-2,8-^{3}H]NAD^+ (4500 cpm/nmol) as indicated in the figure. BGP-15 concentrations were (▲) 0 mg/L, (■) 20 mg/L, and (●) 40 mg/L.

type noncompetitive with a $K_i = 57 \pm 6$ μM calculated from the changes in slope, showing that IC_{50} depends on the NAD^+ concentration. These data indicate that the protective effect of BGP-15 in postischemic heart may be the consequence of PARP inhibition. In agreement with the *in vitro* data, BGP-15 inhibited mono- and poly-ADP-ribosylation reactions *in situ* in Langendorff-perfused hearts (Figs. 2 and 3). Although there are no data showing that mono-ADP-ribose polymerases are involved in ischemia–reperfusion-induced oxidative damage, the involvement of PARP in oxidative cell damage is well documented [11, 45, 47, 48]. Generally, PARP activation is considered the consequence of oxidative cell damage mediated by single-strand DNA break formation [8]. In addition, there are data suggesting that PARP inhibitors partially protect mitochondrial respiration from externally added oxidant [25, 49–51], but these inhibitors failed to protect the genome from single-strand DNA breaks [49–51]. In these systems, the diffusion rate of the externally added oxidant primarily determines the amount of single-strand DNA breaks. Therefore, we cannot expect any protective effect from PARP inhibitors. On the other hand, ROS are generated predominantly by mitochondrial respiration in postischemic heart [15, 16], suggesting that any compounds that moderate mitochondrial ROS formation are likely to eventually reduce lipid peroxidation and single-strand DNA breaks in postischemic Langendorff heart. Our data (Table 2) show that BGP-15 decreased the ischemia–reperfusion-induced ROS level in hearts, and indeed reduced lipid peroxidation and single-strand DNA breaks. Since BGP-15 did not decrease the steady-state level of chemically generated ROS, it is likely that BGP-15 does not chemically react with ROS but interferes—directly or indirectly—with the cellular ROS-generating processes. Therefore, BGP-15 either directly inhibits the ischemia–reperfusion-induced mitochondrial ROS formation or interferes with the cellular processes at some point, which leads to an excessive mitochondrial ROS production in postischemic hearts. Very active Ca^{2+} uptake, hydroxyapatite crystal formation, and mitochondrial permeability transitions are considered to be major factors in ischemia–reperfusion injury of mitochondria, leading to mitochondrial NAD^+ loss, the inhibition of NAD^+-dependent substrate utilization, and an activation of mitochondrial ROS formation [14, 17]. PARP inhibitions via the moderation of cytoplasmic NAD^+ loss can help retain mitochondrial NAD^+ and may prevent the decrease in mitochondrial NAD^+-linked substrate oxidation and ROS formation. It is very likely that the protective effect of BGP-15 against oxidative damage in postischemic heart is related to PARP inhibition and relies on a similar molecular mechanism as the protective effect of other PARP inhibitors against mitochondrial damage induced by externally added oxidants [25, 49–51].

However, we cannot exclude the possibility that BGP-15 may have another cellular target to modulate ROS formation in postischemic hearts. The data presented here are consistent with those reported earlier [25, 49–51], suggesting that inhibition of PARP reduces the rate of NAD^+ catabolism and mitochondrial permeability transition-induced metabolic abnormalities, and decreases the ischemia–reperfusion-induced mitochondrial ROS formation and oxidative cell damage (lipid peroxidation and single-strand DNA breaks). In conclusion, PARP inhibitors through the above-described pathways behave similarly to antioxidants since they reduce ischemia–reperfusion-induced oxidative damage of myocardium.

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References

1. Halliwell B and Gutteridge JM, Role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol* **186:** 1–85, 1990.
2. Pan N and Hori H, The interaction of acteoside with mitochondrial lipid peroxidation as an ischemia/reperfusion injury model. *Adv Exp Med Biol* **361:** 319–325, 1994.
3. Packer L, Valenza M, Serbinova E, Starke-Reed P, Frost K and Kagan V, Free radical scavenging is involved in the protective effect of L-propionyl-carnitine against ischemia–

reperfusion injury of heart. *Arch Biochem Biophys* **288:** 533–537, 1991.
4. Takeda N, Ota Y, Tanaka Y, Shikata C, Hayashi Y, Nemoto S, Tanamura A, Iwai T and Nakamura I. Myocardial adaptive changes and damage in ischemic heart disease. *Ann N Y Acad Sci* **793:** 282–288, 1996.
5. Kajstura J, Cheng W, Reiss K, Clark WA, Sonenblick EH, Krajewski S, Reed JC, Olivetti G and Anversa P, Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* **74:** 86–107, 1996.
6. Heller B, Wang ZQ, Wagner EF, Radons J, Burkle A, Fehsel F, Burkart V and Kolb H, Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J Biol Chem* **270:** 11176–11180, 1995.
7. Simpson PJ and Lucchesi BR, Free radicals and myocardial ischemia and reperfusion injury. *J Lab Clin Med* **110:** 13–30, 1987.
8. Lindahl T, Satoh MS, Poirier GG and Klungland A, Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem Sci* **20:** 405–411, 1995.
9. Radons J, Heller B, Burkle A, Hartmann B, Rodriguez ML, Kroncke KD, Burkart V and Kolb H, Nitric oxide toxicity in islet cells involves poly(ADP-ribose) polymerase activation and concomitant NAD^+ depletion. *Biochem Biophys Res Commun* **199:** 1270–1277, 1994.
10. Janero DR, Hreniuk D, Sharif HM and Prout KC, Hydroperoxide-induced oxidative stress alters pyridine nucleotide metabolism in neonatal heart muscle cells. *Am J Physiol* **264:** 1401–1410, 1993.
11. Thiemermann C, Bowes J, Myint FP and Vane JR, Inhibition of the activity of poly(ADP ribose) synthetase reduces ischemia–reperfusion injury in the heart and skeletal muscle. *Proc Natl Acad Sci USA* **94:** 679–683, 1997.
12. Mizumoto K, Glascott PA Jr and Farber JL, Roles for oxidative stress and poly(ADP-ribosyl)ation in the killing of cultured hepatocytes by methyl methanesulfonate. *Biochem Pharmacol* **46:** 1811–1818, 1993.
13. Said SI, Berisha HI and Pakbaz H, Excitotoxicity in the lung: *N*-Methyl-D-asparate-induced, nitric oxide-dependent, pulmonary edema is attenuated by vasoactive intestinal peptide and by inhibitors of poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA* **93:** 4688–4692, 1996.
14. Turrens JF, Beconi M, Barilla J, Chavez UB and McCord JM, Mitochondrial generation of oxygen radicals during reoxygenation of ischemic tissues. *Free Radic Res Commun* **12–13:** 681–689, 1991.
15. Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M and Chiariello M, Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem* **268:** 18532–18541, 1993.
16. Vanden Hoek TL, Becker LB, Shao Z, Li C and Schumacker PT, Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* **273:** 18092–18098, 1998.
17. Takeyama N, Matsuo N and Tanaka T, Oxidative damage to mitochondria is mediated by the Ca^{2+}-dependent inner-membrane permeability transition. *Biochem J* **294:** 719–725, 1993.
18. Halestrap AP, Kerr PM, Javadov S and Woodfield KY, Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim Biophys Acta* **1366:** 79–94, 1998.
19. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA and Herman B, The mitochondrial permeability transition in cell death: A common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta* **1366:** 177–196, 1998.
20. Cuzzocrea S, Zingarelli B and Caputi AP, Peroxynitrate-mediated DNA strand breakage activates poly(ADP-ribose) synthetase and causes cellular energy depletion in a nonseptic shock model induced by zymosan in the rat. *Shock* **9:** 336–340, 1998.
21. Szabo C, Virag L, Cuzzocrea S, Scott GS, Hake P, O'Connor MP, Zingarelli B, Salzman A and Kun E, Protection against peroxynitrite-induced fibroblast injury and arthritis development by inhibition of poly(ADP-ribose) synthase. *Proc Natl Acad Sci USA* **95:** 3867–3872, 1998.
22. Horwitz LD, Fennessey PV, Shikes RH and Kong Y, Marked reduction in myocardial infarct size due to prolonged infusion of an antioxidant during reperfusion. *Circulation* **89:** 1792–1801, 1994.
23. Abadie C, Ben Baouali A, Maupoil V and Rochette L, An alpha-tocopherol analogue with antioxidant activity improves myocardial function during ischemia reperfusion in isolated working rat hearts. *Free Radic Biol Med* **15:** 209–215, 1993.
24. Serbinova E, Khwaja S, Reznick AZ and Packer L, Thioctic acid protects against ischemia–reperfusion injury in the isolated perfused Langendorff heart. *Free Radic Res Commun* **17:** 49–58, 1992.
25. Virag L, Salzman AL and Szabo C, Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidant-induced cell death. *J Immunol* **161:** 3753–3759, 1998.
26. Fu LX, Kirkeboen KA, Liang QM, Sjogren KG, Hjalmarson A and Ilebekk A, Free radical scavenging enzymes and G protein mediated receptor signalling systems in ischaemically preconditioned porcine myocardium. *Cardiovasc Res* **27:** 612–616, 1993.
27. Sumegi B, Butwell NB, Malloy CR and Sherry AD, Lipoamide influences substrate selection in post-ischaemic perfused rat hearts. *Biochem J* **297:** 109–113, 1994.
28. Sumegi B, Podanyi B, Forgo P and Kover K, Metabolism of [3-^{13}C]pyruvate and [3-^{13}C]propionate in normal and ischaemic rat heart *in vivo*: 1H- and 13C-NMR studies. *Biochem J* **312:** 75–81, 1995.
29. Brent E, Bergmeyer HU and Mollering H, Creatine. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), pp. 1772–1776. Academic Press, New York, 1974.
30. Miles AM, Bohlt DS, Glassbrenner PH, Hansert B, Winks DA and Grisham MB, Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J Biol Chem* **271:** 40–47, 1996.
31. Summerhayes IC, Lampidis TJ, Bernal SD, Nadakavukaren JJ, Nadakavukaren KK, Shepherd EL and Chen LB, Unusual retention of rhodamine123 by mitochondria in muscle and carcinoma cells. *Proc Natl Acad Sci USA* **79:** 5292–5296, 1982.
32. Birnboim HC and Jevcak JJ, Fluorimetric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation. *Cancer Res* **41:** 1889–1892, 1981.
33. Sumegi B, Melegh B, Adamovich K and Trombitas K, Cytochrome oxidase deficiency affecting the structure of the myofibre and the shape of mitochondrial cristae membrane. *Clin Chim Acta* **192:** 9–18, 1990.
34. Inoue A, Yamamoto N, Morisawa Y, Uchimoto T, Yukioka M and Morisawa S, Unesterified long-chain fatty acids inhibit thyroid hormone binding to the nuclear receptor. Solubilized receptor and the receptor in cultured cells. *Eur J Biochem* **183:** 565–572, 1989.

35. Shah GM, Poirier D, Duchaine C, Brochu G, Desnoyers S, Lagueux J, Verreault A, Hoflack JC, Kirkland JB and Poirier GG, Methods for biochemical study of poly(ADP-ribose) metabolism *in vitro* and *in vivo*. *Anal Biochem* **227:** 1–13, 1995.
36. Tzeng WF, Lee JL and Chiou TJ, The role of lipid peroxidation in menadione-mediated toxicity in cardiomyocytes. *J Mol Cell Cardiol* **27:** 1999–2008, 1995.
37. Bergmeyer HU and Bernt E, Lactate dehydrogenase. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), pp. 574–579. Academic Press, New York, 1974.
38. Bergmeyer HU and Bernt E, Glutamate-oxaloacetate transaminase. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), pp. 727–733. Academic Press, New York, 1974.
39. Forster G, Bernt E and Bergmeyer HU, Creatine kinase. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), pp. 784–793. Academic Press, New York, 1974.
40. Haramaki N, Stewart DB, Aggarwal S, Kawabata T and Packer L, Role of ascorbate in protection by nitecapone against cardiac ischemia–reperfusion injury. *Biochem Pharmacol* **50:** 839–843, 1995.
41. Latocha G and Bernauer W, Effect of antiarrhythmic drugs on the postischaemic metabolic recovery of isolated rat hearts. *Arch Int Pharmacodyn Ther* **312:** 39–54, 1991.
42. Ledford BE and Leno GH, ADP-ribosylation of the molecular chaperone GRP78/BiP. *Mol Cell Biochem* **138:** 141–148, 1994.
43. Piron KJ and McMahon KK, Localization and partial characterization of ADP-ribosylation products in hearts from adult and neonatal rats. *Biochem J* **270:** 591–597, 1990.
44. Leno GH and Ledford BE, ADP-ribosylation of the 78 kDa glucose-regulated protein. *FEBS Lett* **276:** 29–33, 1990.
45. Eliasson MJ, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH and Dawson VL, Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischaemia. *Nat Med* **3:** 1089–1095, 1997.
46. Radons J, Heller B, Burkle A, Hartmann B, Rodriguez ML, Kroncke KD, Burkart V and Kolb H, Nitric oxide toxicity in islet cells involves poly(ADP-ribose) polymerase activation and concomitant NAD^+ depletion. *Biochem Biophys Res Commun* **199:** 1270–1277, 1994.
47. Szabo C, Zingarelli B, O'Connor M and Salzman AL, DNA strand breakage activation of poly(ADP-ribose) synthetase and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc Natl Acad Sci USA* **93:** 1753–1758, 1996.
48. Wang ZQ, Auer B, Stingl L, Berghammer H, Haidacher D, Schweiger M and Wagner EF, Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Dev* **9:** 509–520, 1995.
49. Szabo C and Dawson VL, Role of poly(ADP-ribose) synthetase in inflammation and ischaemia–reperfusion. *Trends Pharmacol Sci* **19:** 287–298, 1998.
50. Szabo C, Zingarelli B and Salzman AL, Role of poly-ADP ribosyltransferase activation in the vascular contractile and energetic failure elicited by exogenous and endogenous nitric oxide and peroxynitrite. *Circ Res* **78:** 1051–1063, 1996.
51. Zingarelli B, Cuzzocrea S, Zsengeller Z, Salzman AL and Szabo C, Protection against myocardial ischemia and reperfusion injury by 3-aminobenzamide, an inhibitor of poly(ADP-ribose) synthetase. *Cardiovasc Res* **36:** 205–215, 1997.