Poly(ADP-Ribose) Polymerase Contributes to the Development of Myocardial Infarction in Diabetic Rats and Regulates the Nuclear Translocation of Apoptosis-Inducing Factor

Chun-Yang Xiao, Min Chen, Zsuzsanna Zsengellér, and Csaba Szabó

Inotek Pharmaceuticals Corporation, Beverly, Massachusetts (C.-Y.X., M.C., Z.Z., C.S.); and Department of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary (C.S.)

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ABSTRACT

Activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP)-1 by oxidant-mediated DNA damage is an important pathway of cell dysfunction and tissue injury during myocardial infarction. Because diabetes mellitus can substantially alter cellular signal transduction pathways, we have now investigated whether the PARP pathway also contributes to myocardial ischemia/reperfusion (MI/R) injury in diabetic mellitus in rodents. Myocardial ischemia/reperfusion in control and streptozotocin-diabetic rats was induced by transient ligation of the left anterior descending coronary artery. PARP activation was inhibited by the isoindolinone derivative PARP inhibitor INO-1001. In diabetic rats, a more pronounced degree of myocardial contractile dysfunction developed, which also was associated with a larger infarct size, and significant mortality compared with nondiabetic rats. Inhibition of PARP provided a similar degree of myocardial protective effect in diabetic and nondiabetic animals and reduced infarct size and improved myocardial contractility. In diabetic rats, PARP inhibition reduced mortality during the reperfusion phase. There was marked activation of PARP in the ischemic/reperfused myocardium, which was blocked by INO-1001. In addition, there was a significant degree of mitochondrial-to-nuclear translocation of the cell death effector apoptosis-inducing factor (AIF) in myocardial infarction, which was blocked by pharmacological inhibition of PARP. The role of PARP in regulating AIF translocation in myocytes also was confirmed in an isolated perfused heart preparation. Overall, the current results demonstrate the importance of the PARP pathway in diabetic rats subjected to myocardial infarction and demonstrate the role of PARP in regulating AIF translocation in MI/R.

Poly(ADP ribose) polymerase (PARP), also known as poly(ADP ribose) synthetase, is an abundant nuclear enzyme of eukaryotic cells. When activated by DNA single-strand breaks, PARP initiates an energy-consuming cycle by transferring ADP ribose units from NAD⁺ to nuclear proteins. This process results in rapid depletion of the intracellular NAD⁺ and ATP pools, slowing the rate of glycolysis and mitochondrial respiration, eventually leading to cellular dysfunction and death (Eliasson et al., 1997; Szabo et al., 1997, 1998; Zingarelli et al., 1998; Burkart et al., 1999; Szabó, 2000; Virág and Szabó, 2002). Overactivation of PARP represents an important mechanism of tissue damage in various pathophysiological conditions associated with oxidant stress, including myocardial reperfusion injury (Zingarelli et al., 1998; for review, see Szabó et al., 2004), stroke (Eliasson et al., 1997; for review, see Skaper, 2003), and circulatory shock (Szabó et al., 1997; Oliver et al., 1999; Liaudet et al., 2000). Activation of PARP also contributes to the development of vascular dysfunction, cardiomyopathy, and neuropathy in diabetes (Soriano et al., 2001; Pacher et al., 2002; Obrosova et al., 2004).

Diabetes mellitus is associated with a markedly increased risk of coronary artery disease and myocardial infarction (for review, see Haffner and Cassels, 2003; Hurst and Lee, 2003). The first aim of the current study was to compare the effect of myocardial ischemia and reperfusion injury in normoglycemic and diabetic rats and to determine whether PARP inhibition provides therapeutic benefit against myocardial ischemia/reperfusion injury in diabetes. Because a recent report has implicated the importance of the cell death effector apoptosis-inducing factor (AIF) in oxidant-induced cell...
injury in neurons and implicated the role of PARP in regulating AIF translocation (Yu et al., 2002), we also have investigated herein whether PARP is involved in the regulation of AIF translocation during myocardial infarction in normoglycemic and diabetic animals.

Materials and Methods

Animals. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and was performed with approval of the local Animal Care and Use Committee.

STZ-Induced Model of Diabetes in Rats. Diabetes was induced in male Wistar rats weighting 250 ± 20 g by use of a single injection of STZ (65 mg/kg i.v.) into penile vein. STZ was dissolved in the sterilized water (Sigma-Aldrich, St. Louis, MO) and infused through a 0.22-μm filter (Millipore Corporation, Billerica, MA). Two weeks after injection of STZ, blood glucose of diabetic rats was measured using a one-touch blood glucose meter (LifeScan, Johnson & Johnson, Milpitas, CA). Rats with confirmed significant hyperglycemia (>250 mg/dl) were used in the subsequent experiment.

In Vivo Myocardial Ischemia-Reperfusion Injury. Diabetic or nondiabetic rats were anesthetized with pentobarbital (60 mg/kg i.p.) and were placed in a supine position under body temperature control. Each animal was endotracheally intubated and ventilated with a tidal volume of 10 ml/kg at a rate of 80 strokes/min using a rodent respirator (model 683; Harvard Apparatus Inc., Holliston, MA). Myocardial infarction was conducted as described previously (Liaudet et al., 2001; Murthy et al., 2004). After left thoracotomy, a 5-0 silk suture was passed underneath the left anterior descending coronary artery (LAD). Polyethylene tubing was placed along the vessel as a cushion and secured around the tubing to occlude the LAD. Myocardial ischemia was verified by blanching of the left ventricle. The LAD was occluded for 30 min followed by removal of ligation to allow subsequent reperfusion for 2 h. The right femoral vein was cannulated for the administration of drugs. The isoinulinone-based PARP inhibitor INO-1001 (Khan et al., 2003; Shimoda et al., 2003; Murukami et al., 2004; Murthy et al., 2004) was dissolved in 5% dextrose for injection (Abbott Labs, Pomezia, Italy) and was administered intravenously at a dose of 20 mg/kg at 5 min before reperfusion and 1 h after reperfusion. In vehicle group, animals were treated with 5% dextrose injection.

Assessment of Myocardial Contractile Function. A microtip catheter transducer (SPR-524; Millar Instruments Inc., Houston, TX) was passed through right carotid artery into the left ventricle. After stabilization for 20 min, the pressure signal was continuously recorded using a MacLab A/D converter (ADInstruments Pty Ltd., Castle Hill, Australia). The heart rate, left ventricle systolic pressure (LVSP), left ventricle end-diastolic pressure (LVEDP), and positive and negative maximal values of the first derivative of left ventricle pressure (+dP/dt and −dP/dt) were calculated. The cardiac function was continuously monitored during whole course of experiment. At end of the reperfusion, 1 ml of blood was taken from the carotid artery and centrifuged at 3000 rpm for 3 min at 4°C. The plasma was collected to determine activity of creatine kinase (CK) with a CK assay kit (Sigma-Aldrich). A microtip catheter transducer (SPR-524; Millar Instruments Inc.) was inserted directly into the left ventricular cavity. Coronary perfusion pressure, LVSP,"

Histology and Immunohistochemistry. Myocardial sections were fixed for 1 day in paraformaldehyde solution. Adjacent sections were processed for two types of immunochemical labeling as follows.

Immunohistochemical detection of poly(ADP-ribose). Paraflin-embedded tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations (100, 95, and 70%) of ethanol followed by a 5-min incubation in PBS. To prevent catabolism of the polymer by poly(ADP-ribose) glycohydrolase, sections were fixed in 10% trichloroacetic acid. Then, sections were treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and rinsed briefly in PBS. Nonspecific binding was blocked by incubating the slides for 2 h in 0.25% Triton/PBS containing 2% goat serum. To detect poly(ADP-ribose), a routine histochemical procedure was applied as described previously (Szabó et al., 2002) with minor modifications as follows. Chicken polyclonal anti-poly(ADP-ribose) antibody (Tulip Biolabs, West Point, PA) and isotype-matched secondary antibody was applied in a dilution of 1:300 for overnight at 4°C. After extensive washing (3 × 10 min) with 0.25% Triton/PBS, immunoreactivity was detected with a biotinylated horse anti-mouse secondary antibody and the avidin-biotin-peroxidase complex both supplied by Vector Laboratories (Burlingame, CA). Color was developed using Ni2+-DAB substrate kit (Vector Laboratories). Sections were then briefly rinsed in Tris/saline (pH 7.6) and incubated in Tris/cobalt (pH 7.2) for 2 min. Sections were then counterstained with nuclear fast red, dehydrated, and mounted.

Immunohistochemical detection of AIF (Komjáti et al., 2004). Detection of AIF was performed using a monoclonal antibody against AIF (2.5 μg/ml), the sections were incubated with the secondary antibody (biotinylated goat anti-rabbit) followed by avidin biotin complex (ABC kit). AIF immunostaining was revealed with Ni2+-enhanced DAB as a chromogen, producing a black stain. Sections were then counterstained with nuclear fast red. At the end of the procedure the sections were dehydrated, cleared and mounted. Quantification of the intensity of the PAR staining was performed as published previously (Pacher et al., 2002). The percentage of PAR-positive nuclei was obtained by conventional microscopy; in total, at least 2000 nuclei profiles were examined in each condition at least in five different sections in each group. The results are expressed as the percentage of PAR or AIF-positive nuclei, relative to the number of total nuclei counted.

Studies in Isolated Perfused Hearts. Male Sprague-Dawley rats (250–300 g) were used. Rats were heparinized with sodium heparin (1000 U/kg i.p.) 10 min before induction of anesthesia. Anesthesia was induced by intraperitoneal administration of sodium pentobarbital (40 mg/kg). Once the animal was anesthetized, the thorax was opened, and the heart was rapidly removed and perfused through ascending aorta. The heart was perfused with Krebs-Ringer buffer consisting of 118 mmol/l NaCl, 4.75 mmol/l KCl, 1.18 mmol/l KH2PO4, 1.18 mmol/l MgSO4, 2.5 mmol/l CaCl2, 25 mmol/l NaHCO3, and 11 mmol/l glucose. The perfusate was bubbled with a mixture of 95% O2 and 5% CO2 at 37°C. The heart was initially perfused at a constant pressure of 70 mm Hg. About 10 min after the constant pressure perfusion, perfusion was switched to constant flow perfusion achieved using a microtube pump. The perfusion pressure was maintained at the same level of constant pressure perfusion by adjusting flow rate. Once the flow rate was determined, it was maintained throughout the experiment. The hearts were stimulated by rectangular pulses at a rate of 5 Hz and 2-ms duration and twice the diastolic threshold, delivered from a stimulus isolation unit (AD- Instruments Pty Ltd.). To assess contractile function, a microtip catheter transducer (Millar Instruments Inc.) was inserted directly into the left ventricular cavity. Coronary perfusion pressure, LVSP,
LVEDP, and $+\frac{dP}{dt_{\text{max}}}$ and $-\frac{dP}{dt_{\text{min}}}$ were calculated. LVDP was calculated as the difference between the systolic and diastolic pressure. Data were collected using a PowerLab data acquisition system (ADInstruments Pty Ltd.) in conjunction with a Macintosh computer, and analyzed using Chart.3 computer package.

After a period of 15-min stabilization, the hearts were subjected to global ischemia for 30 min by turning off the perfusion system. After 30 min of ischemia, the perfusion system was restarted, and the hearts were reperfused for the indicated time. The pacer was turned off during ischemia and turned on again during reperfusion. INO-1001 was applied by infusing from a syringe to the heart via a cannula connected to the sidearm of the aortic cannula using a syringe microinfusion pump. In one subgroup of the studies, INO-1001 infusion started 5 min before ischemia and continued throughout the studies. In another subgroup of experiments, the start of INO-1001 infusion was at the time of reperfusion. The final perfused concentration of INO-1001 was 1 μM. Coronary effluent was collected during reperfusion. CK activity in coronary effluent was determined using a kit according to the manufacturer’s instructions (Sigma-Aldrich). Immunohistochemical analysis of PAR and AIF of the perfused hearts was conducted as described above.

**Statistical Analysis.** All values are expressed as means ± S.E.M. of n independent experiments. Statistical analysis was performed with unpaired t test for two groups. Survival rates were compared by the chi square test. A difference was considered statistically significant at $P < 0.05$.

Under basal conditions, the values of heart rate, LVSP, $+\frac{dP}{dt}$, and $-\frac{dP}{dt}$ in diabetic rat were $333.7 ± 25.2$, $119.4 ± 3.6$, $5.636 ± 0.1$, and $-4.302 ± 0.2$, respectively, which were significantly lower than in nondiabetic rat ($419 ± 10.8$, $129.5 ± 2.5$, $6.086 ± 0.21$, and $-5.165 ± 0.12$, respectively; $P < 0.05$), confirming that diabetes impairs in vivo cardiac mechanical function (Fig. 1). There were no differences in heart rate during ischemia and reperfusion between the INO-1001-treated diabetic or nondiabetic group and their respective vehicle controls (data not shown).

LAD occlusion caused a marked decrease in LVSP, $+\frac{dP}{dt}$, and $-\frac{dP}{dt}$ in both the diabetic and the nondiabetic groups. When perfusion was restored, LVSP, $+\frac{dP}{dt}$, and $-\frac{dP}{dt}$ exhibited a rebound, followed by a gradual decrease. In the PARP inhibitor-treated group, the recovery of LVSP, $+\frac{dP}{dt}$, and $-\frac{dP}{dt}$ during reperfusion were significantly improved in both nondiabetic and diabetic rats (Fig. 1).

In the reperfusion period, no animals died in the normoglycemic group. In contrast, in the diabetic group of animals five of 12 rats did not survive until the end of the reperfusion. In the PARP inhibitor-treated diabetic animals subjected to myocardial infarction, one of the 12 animals died during the

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Fig. 1. Inhibition of PARP with INO-1001 improves myocardial mechanical function in both nondiabetic (A) and diabetic (B) rat after ischemia/reperfusion. LVSP, $dP/dt$, and $-dP/dt$ were monitored continuously during whole course of the experiment. Values are means ± S.E.M. control ($n = 7–12$); INO-1001 ($n = 7–10$). * $P < 0.05$ versus their respective control. † $P < 0.05$ versus nondiabetic rat in baseline conditions.
Myocardial ischemia–reperfusion induced a marked increase in the poly(ADP-ribose) polymerase staining (the product of the PARP enzyme and a marker of cellular PARP activation) in the cardiac myocytes and vascular cells as detected by immunohistochemical methods (Fig. 3). The PARP inhibitor INO-1001 markedly reduced PAR staining in the reperfused myocardium. The percentage of PAR-positive nuclei increased from 17 ± 3 to 59 ± 3% and was reduced to 28 ± 6% in the presence of the PARP inhibitor. Consistently with previous observations (Pacher et al., 2002; Szabó et al., 2002; Obrosova et al., 2004), we have detected significantly higher “basal” poly(ADP-ribose) staining in the hearts of the diabetic animals than in the hearts of nondiabetic animals before the initiation of the LAD occlusion (Fig. 3), but ischemia–reperfusion induced an increase in PAR staining, which was reduced by the PARP inhibitor. The respective percentages of the nuclei exhibiting PAR positivity were 44 ± 9, 68 ± 2, and 45 ± 3%.

There was a diffuse low-level cytoplasmic staining for AIF in the control hearts, consistent with the mitochondrial localization of this factor under baseline conditions. In response to myocardial ischemia–reperfusion, there was a reduction in the intensity of the diffuse cytoplasmic AIF staining, and an increase in nuclear staining became apparent.

Fig. 2. Inhibition of PARP with INO-1001 reduces the ischemia/reperfusion-induced infarct size in both nondiabetic (A) and diabetic (B) rat. INO-1001 or vehicle (5% dextrose) was given 5 min before ischemia and 1 h after reperfusion as described under Materials and Methods. Values are means ± S.E.M. control (n = 7–8); INO-1001 (n = 7–8). *, P < 0.05 versus their respective control. †, P < 0.05 versus nondiabetic rat in baseline conditions.

Fig. 3. Inhibition of PARP with INO-1001 reduces the ischemia/reperfusion-induced increase in poly(ADP-ribose) immunostaining in nondiabetic (left) and diabetic (right) rats. Figures show representative immunohistochemical stainings from four to five animals.
ent, most notably in vascular and infiltrating mononuclear cells, but also in some cardiac myocytes (Fig. 4). These patterns of AIF staining and translocation were essentially identical in normoglycemic and diabetic heart samples (Fig. 4), although in the diabetic animals there seemed to be a higher baseline nuclear AIF staining. Respective percentages of AIF staining in normoglycemic animals under baseline, after ischemia/reperfusion and in the PARP inhibitor-treated animals subjected to ischemia-reperfusion amounted to 18 ± 3, 56 ± 4, and 24 ± 3%. The respective values in the diabetic animals were 37 ± 4, 69 ± 4, and 33 ± 3%.

To explore whether the AIF translocation depends on the presence of circulating cells or blood-borne mediators during myocardial ischemia-reperfusion, we have conducted a series of additional experiments in Langendorff perfused hearts, in the presence of buffer perfusion (i.e., in the absence of circulating blood cells), with or without PARP inhibition (Figs. 5 and 6). Ischemia-reperfusion of the isolated perfused hearts resulted in an increase in AIF translocation, which was primarily localized to the cardiac myocytes in this experimental setting (Fig. 6). Inhibition of PARP with INO-1001 suppressed the translocation of AIF and improved the function of the perfused hearts (Figs. 5 and 6).

**AIF staining**

- Non-diabetic hearts
  - shum
  - I/R
  - INO+I/R

- Diabetic hearts
  - shum
  - I/R
  - INO+I/R

**Fig. 4.** Inhibition of PARP with INO-1001 reduces the ischemia/reperfusion-induced cytoplasmic-to-nuclear translocation of AIF in nondiabetic (left) and diabetic (right) rats. Figures show representative immunohistochemical stainings from four to five animals.

**Discussion**

The importance of the PARP pathway is well documented in various experimental models of myocardial ischemia-reperfusion injury (Zingarelli et al., 1997; 1998; Thiemermann et al., 1997; Grupp et al., 1999; Pieper et al., 2000; Yang et al., 2000). The current results are consistent with these data and demonstrate the cardioprotective effect of PARP inhibition in the current model of myocardial infarction. The cardioprotective effects of the PARP inhibitor were evidenced by 1) improved myocardial contractility; 2) reduction in infarct size; and 3) reduction in the release of creatine kinase, a marker of myocardial necrosis.

The results of the current study also demonstrate that there is a depressed baseline myocardial function in diabetic rats and a relative increase in the size of the myocardial infarct in response to coronary ischemia-reperfusion. These observations are consistent with the findings of a recent report (Marfella et al., 2004), which demonstrated that in diabetic hearts subjected to myocardial ischemia and reperfusion, there is an increased production of reactive species, such as peroxynitrite. Because peroxynitrite (among other species) is an endogenous trigger of DNA strand breakage, which, in turn, triggers PARP activation, the current results are consistent with other recent experimental reports. The current results also may be interesting in the context of the clinical data, demonstrating that diabetic patients develop myocardial ischemic events with a higher incidence and a poorer outcome than nondiabetic age-matched controls (see Introduction).

The current results demonstrate that both in diabetic hearts and in nondiabetic hearts, PARP inhibition affords a significant cardioprotective effect. This is important, because there are significant changes in inflammatory and signal transduction pathways in the diabetic heart, and the cardioprotective effect of certain approaches, which can be seen in control (nondiabetic) animals, disappears or reverses in diabetes. A recent example for this type of reversal is the role of inducible nitric-oxide synthase in myocardial infarction: whereas inhibition of the inducible nitric-oxide synthase pathway is protective in normoglycemia, the protective effect disappears in diabetic animals (Marfella et al., 2004) (by comparison, some other approaches, for instance peroxisome proliferator-activated receptor-γ-agonists, are equally effective in control and diabetic animals subjected to myocardial infarction; Khandoudi et al., 2002). Because PARP inhibition is approaching clinical efficacy trials (Southan and Szabó, 2003; Szabó et al., 2004), it is important to confirm that the cardioprotective effect of PARP inhibition is maintained both in normoglycemia and in diabetes. In fact, in diabetic rats inhibition of PARP with INO-1001 even provided a survival benefit. The more pronounced decrease in myocardial contractile function in the diabetic animals subjected to myocardial infarction may have contributed to the poor survival rate of this group; improvement of the myocardial function by the PARP inhibitor may, therefore, be responsible for the observed survival benefit.

The dose of the PARP inhibitor used in the present study was sufficient to block poly(ADP-ribose) accumulation (a marker of tissue PARP activation) in the hearts subjected to myocardial ischemia and reperfusion. This finding is consistent with prior work using this compound in murine and large animal models of myocardial infarction and other mod-
els of acute diseases (Khan et al., 2003; Shimoda et al., 2003). In a recent study, we have reported that there is an increase in PARP activity in circulating blood cells of rats subjected to myocardial ischemia-reperfusion (Murthy et al., 2004). In this study, too, INO-1001, at a dose similar to the one used in the current study, was able to prevent the increase poly(ADP-ribose) accumulation in circulating leukocytes.

Multiple reports indicate the importance of PARP activation in the development of mitochondrial dysfunction under conditions of oxidative stress (Vira\'g et al., 1998; Yu et al., 2002; Du et al., 2003a). Even though the major isoform of the PARP family, PARP-1, is widely considered as a nuclear enzyme, there is apparently a nuclear-to-mitochondrial signaling process, which initiates early mitochondrial alterations, as demonstrated in thymocytes (Vira\'g et al., 1998) and in neurons (Yu et al., 2002). Recent work implicates the role of the mitochondrial cell death factor AIF in oxidant-induced neuronal death (Cregan et al., 2002; Yu et al., 2002). We have recently demonstrated that there is mitochondrial-to-nuclear translocation of AIF in stroke in vivo (Komjati et al., 2004), and recent studies have demonstrated AIF translocation in perfused hearts subjected to hypoxia/reoxygenation in vitro (Kim et al., 2003; Varbiro et al., 2003). The present study demonstrates that myocardial ischemia and reperfusion in vivo also is associated with the nuclear translocation of AIF. Furthermore, the current data also demonstrate that PARP regulates the translocation of AIF in the ischemia/reperfused hearts; in the PARP inhibitor-treated hearts, the release of the mitochondrial cell death factors AIF was attenuated. The cells where our studies demonstrated AIF translocation (regulated by PARP) include vascular cells, as well as the cardiac myocytes. A reduction in AIF translocation may constitute an additional mode of myocardial pro-


Address correspondence to: Dr. Csaba Szabo, Inotek Pharmaceuticals Corporation, Suite 419E, 100 Cummings Center, Beverly, MA 01915. E-mail:CsabaSzabo@isil.com