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Improved Poststorage Cardiac Function by Poly (ADP-ribose) Polymerase Inhibition: Role of Phosphatidylinositol 3-Kinase Akt Pathway

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Background. Inhibition of poly(ADP-ribose) polymerase 1 (PARP) has been shown to be effective in minimizing cardiac ischemia reperfusion injury. We investigated the cardioprotective effect of the PARP inhibitor, INO-1153, in isolated working rat hearts after 6 hr of hypothermic storage in Celsior.

Methods. Hearts were treated with 1 μM INO-1153 before hypothermic storage, at cardioplegia and storage or after hypothermic storage. Hearts not exposed to INO-1153 served as controls. Another group was pretreated with the phosphatidylinositol 3-kinase inhibitor Wortmannin (0.1 μM) before storage in INO-1153–supplemented Celsior. After baseline measurement of aortic flow, heart rate, coronary flow, and cardiac output were obtained, hearts were arrested and stored in Celsior at 2–3°C for 6 hr. After storage, hearts were reperfused for 15 min before performing work for a further 30 min, at which time poststorage indices of cardiac function were remeasured then heart tissue was stored at −80°C for Western blot analysis.

Results. The presence of INO-1153 during prestorage perfusion or during cardioplegia and storage significantly improved poststorage cardiac function. Functional improvements produced by INO-1153 were completely abolished by Wortmnanin pretreatment. Western blots showed a significant increase in phospho-Akt in presence of INO-1153, which was inhibited by Wortmannin.

Conclusion. Activation of the prosurvival phosphatidylinositol 3-kinase–Akt pathway was involved in the protective action of PARP inhibition in this model of donor heart procurement and hypothermic storage. Importantly for the logistics of clinical organ procurement, maximum protection is observed when the PARP inhibitor is included in the cardioplegic storage solution.

Keywords: Transplantation, Donor heart preservation, Ischemia-reperfusion, PARP inhibition, PI3-kinase/Akt.

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Heart transplantation has become established as an effective therapy for patients with end-stage heart disease. However, success of the procedure is limited by early and late failure of the transplanted heart. It is now recognized that ischemia/reperfusion injury sustained by the allograft during the transplant process is a major determinant of both short- and long-term outcome (*1*). Importantly, of all the factors associated with early allograft failure, ischemia/reperfusion injury is one of the few factors amenable to therapeutic intervention (*2*). Although disparate mechanisms leading to tissue and organ dysfunction after ischemia/reperfusion have been proposed, formation of oxygen and nitrogen centered radicals as well as peroxynitrite at reperfusion may underpin a number of observed pathological sequelae (*3*). Peroxynitrite has been shown to be responsible for formation of single stranded DNA and resultant activation of poly(ADP-ribose) polymerase (PARP) (*4*).

The PARP family of enzymes has many roles in cellular homeostasis, including transcriptional regulation of a number of proteins, detection of DNA strand breaks and initiation of repair to damaged DNA (*5*). In addition to these physiological functions, sustained PARP activation after ischemia reperfusion is thought to play a role in initiation of cell death through apoptosis (*6*) or by profound depletion of NAD^+ and adenosine triphosphate (ATP), resulting in necrosis (*7*).

Proof of principle of the role of PARP activation in ischemia/reperfusion injury has been demonstrated in PARP knockout mice, where deletion of the PARP gene provided significant protection against molecular and functional changes after myocardial ischemia/reperfusion and infarction (*8*, *9*) as well as cerebral ischemia (*10*), lung inflammation (*11*), and shock (*12*).

During the past decade, structure based drug design has facilitated the synthesis of a series of highly potent PARP inhibitors which have verified the protective effects demonstrated in PARP knockout models (*13*). Pharmacological inhibition of PARP has been shown to be beneficial in a number of models of cardiac ischemia reperfusion injury. Improved left ventricular function, decreased oxidative stress and decreased apoptosis was observed in a global ischemia reperfusion model in the rat supplemented with 3-aminobenzidine (*14*). In a Fisher rat model of age-associated heart failure, chronic

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exposure to the PARP inhibitor INO 1001 resulted in improved left ventricular systolic and diastolic left ventricular function and improved acetylcholine-responsive relaxation of aortic rings (*15*). In a porcine model of regional cardiac ischemia produced by ligation of the left anterior descending coronary artery, postreperfusion infarct size and cardiac function was improved when INO1001 was given at the time of reperfusion (*16*). This agent also slowed cardiac rejection and improved allograft function in a rat heterotopic model of acute rejection (*17*). Limited information has been obtained in the area of the application of PARP inhibitor as strategy to optimize donor organ preservation during organ transplantation (*18*).

Although the prevention of severe, acute energy depletion was initially thought to be the prime cause of the protective effect of PARP inhibition (*7*), recent studies have also shown that inhibition of PARP is associated with activation of Akt (*15*, *19*, *20*). Interest in recruitment and activation of pro-survival kinase pathways as an underlying protective principle against ischemia reperfusion injury has been heightened since it has been found that a number of protective physiological and pharmacological pre- and postconditioning strategies involve upregulation of the phosphatidylinositol (PI) 3-kinase/Akt pathway (*21*, *22*).

The aims of the present study, using an isolated working rat heart model of donor heart preservation are to: 1) measure the recovery of poststorage function of hearts treated with the PARP inhibitor INO-1153; 2) to optimize timing of the delivery of INO-1153; and 3) to determine whether the activation of PI 3-kinase/Akt pathway is involved in the protective effects of INO-1153 administration.

MATERIALS AND METHODS

Male Wistar rats weighing 320–380 grams were used in the present study. All procedures were approved by the Animal Ethics Committee of the Garvan Institute of Medical Research, Sydney, Australia (Animal Research Authority no. 03/24 and 06/25). All animals received humane care in compliance with the guidelines set down by the National Health and Medical Research Council (Australia) and the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD).

Krebs-Henseleit solution (37°C) was used for perfusion of the isolated rat heart. Its composition was as follows: NaCl 118.0 mM; KCl 4.7 mM; $MgSO_4$ 1.2 mM; KH_2PO_4 1.2 mM; $NaHCO_3$ 25.0 mM; $CaCl_2$ 2.5 mM, glucose 11.0 mM. The components were dissolved in water purified through a MilliQ water purification system (Millipore, Australia). The resultant solution was bubbled continuously with Carbogen (95% O_2 and 5% CO_2) at 37°C for 1 hour prior to the experiment. The final pH of the Krebs solution was between 7.3 and 7.4. The perfusate was filtered through an inline filter (5-μm pore size) during the course of prestorage perfusion and poststorage reperfusion. Celsior solution (Imtix Sangstat, France) was used for arresting the heart and also as the storage solution for the arrested heart. The PARP inhibitor INO-1153 (previously known as DP264) was a gift from Inotek Pharmaceuticals Corporation (Beverly, MA). The PI3-kinase inhibitor Wortmannin and all components of the Krebs solution (AR grade) were purchased from Sigma Chemical Company (Castle Hill, Australia).

Isolated Working Heart Perfusion

Isolated working hearts were studied by a technique described in our previous publication (*23*). Briefly, rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After bolus injection of 500 IU heparin into the renal vein, the heart was rapidly excised and arrested by immersion in chilled (2–3°C) perfusion buffer. The aorta was cannulated and immediately perfused retrogradely on a Langendorff perfusion apparatus with Krebs buffer at a hydrostatic pressure of 100 cm H_2O. During this time, a small incision was made in the left atrial appendage into which another cannula was inserted and tied off. This nonworking preparation was stabilized for 10 min and then converted to working mode by switching the supply of perfusate from the aorta to the left atrial cannula at a hydrostatic pressure of 20 cm H_2O (preload). The working heart ejected perfusate via the aortic valve into the aortic cannula. The hydrostatic pressure in the aortic cannula was maintained at 100 cm H_2O (after-load) throughout the working phase for all rat hearts.

Aortic pressure was monitored in a side arm of the aortic cannula with a pressure transducer (Ohmeda, Pty Ltd., Singapore). Aortic flow was measured by an in-line flowmeter (Transonics Instruments Inc. Ithaca, NY). Aortic pressure and flow were recorded using MacLab/4e (ADInstruments Pty Ltd, Sydney, Australia) and heart rate was calculated from the flow trace. Coronary flow was measured by timed collection of the effluent draining from the apex of the heart.

Experimental Protocol

Hearts remained in working mode for 15 min prior to storage. Measurements of heart rate (HR), aortic flow (AF), coronary flow (CF), and cardiac output (CO) were made at 10 min after conversion to working mode and used as prestorage baseline. Any hearts having a baseline aortic flow less than 35 ml/min, heart rate less than 200 beats/min, or coronary flow less than 10 mL/min were excluded at this stage. After collection of baseline hemodynamic data, the heart was arrested by infusion of Celsior solution (at 2–3°C) into the coronary circulation for 3 min from a reservoir 60 cm above the heart. All hearts were stored on ice (2–3°C) in 100 mL of the same solution for 6 hr. Hearts were then remounted on the perfusion apparatus and reperfused in Langendorff mode for 15 min. Hearts were then switched to working mode then stabilized for 30 min. The indices of cardiac function measured at baseline were then rerecorded. Recovery of each parameter was expressed as a percentage of its prestorage baseline. After the 30-min functional observations were taken, the left ventricular free wall of each heart was rapidly frozen by immersion in liquid nitrogen then stored at −80 °C for Western blot analysis.

Experimental Groups

Rat hearts were divided into the five groups according to the treatment they received (Fig. 1): Group 1, control group (n=9) in which rat hearts received no interventional treatment and were stored in Celsior solution for 6 hr; Group 2, prestorage INO-1153 (n=8) in which 1 μM INO-1153 added to Krebs prestorage perfusate only; Group 3, storage INO-1153 (n=9) in which 1 μM INO-1153 was present in the

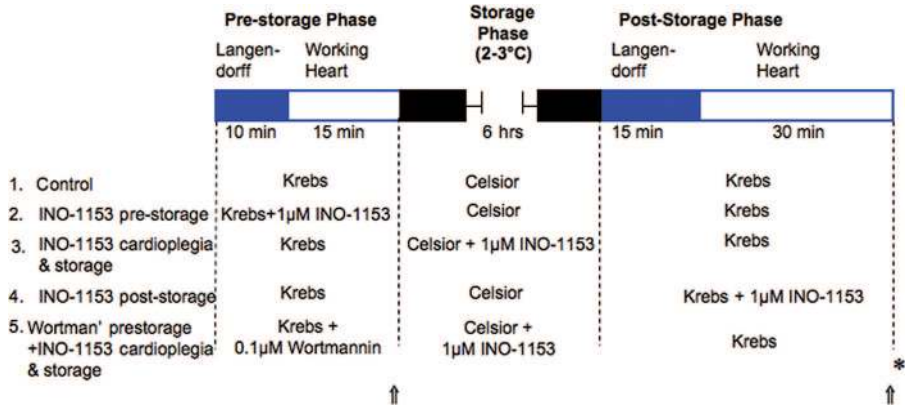

FIGURE 1. Protocol timeline and experimental groups. Arrows indicate times at which measurements of cardiac function were made. Asterisk indicates the point where heart tissue was harvested for Western blot analysis.

Celsior storage solution during cardioplegia and 6 hr cold storage; Group 4, poststorage INO-1153 (n=8) in which 1 μM INO-1153 was added into Krebs perfusion buffer during poststorage reperfusion; and Group 5, wortmannin/INO-1153 (n=6) in which hearts were exposed to 0.1 μM Wortmannin (an inhibitor of PI 3-kinase) during prestorage perfusion then arrested and stored in Celsior supplemented with 1 μM INO-1153. Supplements (INO-1153 or Wortmannin) were dissolved in 0.5 ml dimethyl sulfoxide (DMSO) to facilitate dissolution in Celsior or Krebs solution.

Poly(ADP-ribose) Immunohistochemistry

To assess the extent of PARP activation in control and INO-1153 treated hearts, paraformaldehyde-fixed sections were stained with a monoclonal antibody to poly(ADP-ribose) to detect the product of the PARP reaction (see Supplemental Methods for details; available at www.transplantjournal.com).

Western Blot Analysis

A subgroup of three hearts was chosen from each experimental group for Western blot analysis. Sixty milligrams of tissue from each heart were homogenized in ice cold lysis buffer (150 mM NaCl, 50 mM Tris HCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 5 mM dithiothreitol, 15% Roche cocktail protease inhibitors, pH 7.4). Samples were then centrifuged at 10,000 rpm for 5 min at 4°C. Protein concentration of each lysate was measured using a Bradford assay kit (Pierce Biotechnology Inc, Rockford, IL), with bovine serum albumin used as standard. Protein samples were boiled in sample loading buffer for 5 min before loading onto 8% SDS-polyacrylamide gel (30 μg per lane). After electrophoretic separation on a Protean III system (BioRad Laboratories, Regents Park, Australia), proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Australia). Membranes were blocked for 1 hr in Tris buffered saline, (pH 7.4) which contained 1% bovine serum albumin and 0.1% Tween 20. Membranes were probed overnight with rabbit polyclonal antibodies raised against total Akt (1: 2500 dilution), phospho-Akt (Ser 473; 1:2500 dilution), or β-actin (Cell Signaling Technology, Danvers, MA). The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham Life Sciences). The protein bands were visualized using enhanced chemiluminescence (Amersham Life Sciences). Band intensities were digitised and normalized against a β-actin loading control.

Statistical Analysis

All functional data and densitometric antibody intensities were expressed as means±SE unless otherwise specified. Data were analyzed by the StatView 4.5 statistical software package. Differences among groups were compared by one-way analysis of variance followed by Fisher's paired least significant difference posthoc test. *P* values less than 0.05 were considered significant.

RESULTS

Improved Poststorage Cardiac Function by PARP Onhibition With INO-1153 Treatment

The prestorage baseline measures of cardiac function for control and all treatment groups are shown in Table 1. Pilot studies were performed to ensure that the chosen con-

TABLE 1. Prestorage baseline values

Groups	N	Aortic flow (mL/min)	Coronary flow (mL/min)	Cardiac output (mL/min)	Heart rate (bpm)
Control	9	45±9.1	21±2.9	66±7.6	256±29.0
INO-1153 prestorage	8	51±6.2	20±1.6	71±6.9	260±30.0
INO-1153 storage	9	46±4.9	20±1.3	65±5.1	262±18.7
INO-1153 poststorage	8	42±5.6	19±1.8	61±5.5	266±29.0
Wortmannin+INO-1153	6	44±8.5	19±1.9	63±9.6	255±19.7

Data are expressed as means±SD.

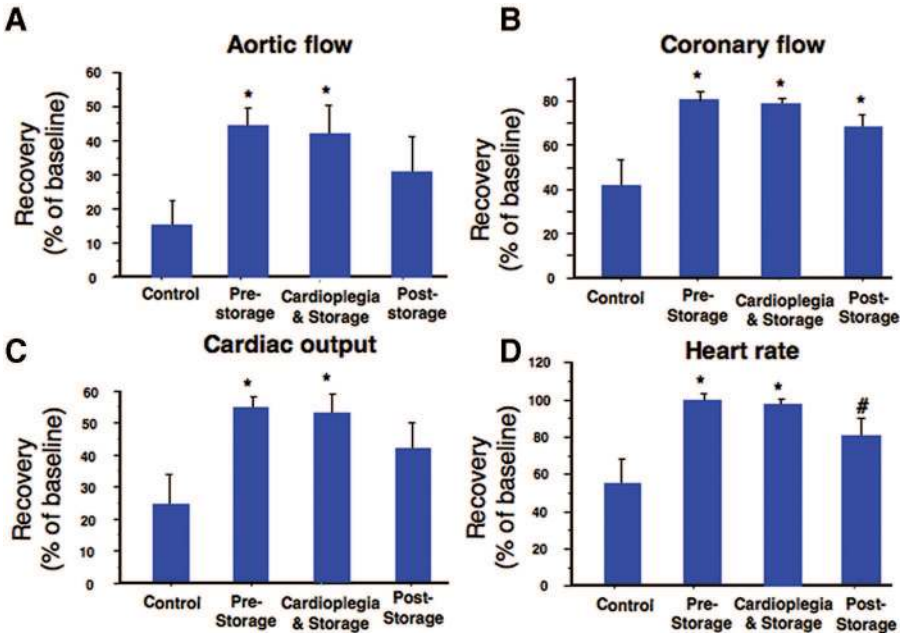

FIGURE 2. Comparison of the effect of treatment on indices of poststorage cardiac function after 45 min poststorage reperfusion (15 min nonworking and 30 min working mode) after 6 hr hypothermic storage. Graphs show recovery of aortic flow (A), coronary flow (B), cardiac output (C), and heart rate (D). Data are expressed as means±SE as a percentage of their baseline values. *$P<0.05$ vs. control, #$P=0.05$ vs. control.

centrations of INO-1153 (1.0 μM) and Wortmannin (0.1 μM) had no direct effect on any the measured indices of cardiac function. No significant differences between the experimental groups for any of the parameters were found.

The recovery of function of control hearts (no treatment) after 45 min reperfusion was uniformly poor (Fig. 2A–D). Previous work in our laboratory with an identical experimental model showed DMSO had no significant effect on heart recovery (*24*).

After 6 hr of cold storage, poststorage function of hearts was better preserved by INO-1153 treatment. Figure 2A shows the recovery of aortic flow after 45 min reperfusion of rat hearts. Rat hearts receiving INO-1153 either before or during cardioplegia and storage demonstrated significantly improved recovery of aortic flow compared to control group (43.6±5.6% and 41.3±8.6% vs. 14.9±7.7%; $P<0.05$). Coronary flow was also significantly improved in all treatment groups compared with control (79.9±4.3%, 77.6±2.7%, and 67.4±6.1% vs. 41.4±11.8%; $P<0.05$; Fig. 2B). Similarly, significantly increased cardiac output was observed with INO-1153 treatment either before or during storage (54.2±4.3% and 52.4±6.4% vs. 24.1±9.5%; $P<0.05$; Fig. 2C). Finally, significantly improved poststorage recoveries of heart rate were observed in all treatment groups, with the most significant improvements observed with INO-1153 treatment before or during cardioplegia and storage (99.1±4.3% and 96.5±3.9% vs. 54.2±14.2%; Fig. 2D).

Poly(ADP-Ribose) Immunohistochemistry

Control hearts that were not exposed to INO-1153 at any stage during the study (group 1), showed enhanced nuclear immunoreactivity for the poly(ADP-ribose) antibody consistent with increased PARP activation after hypothermic storage and reperfusion. Hearts that had been arrested and stored in Celsior supplemented with 1 μM INO-1153 (group 3) showed markedly attenuated nuclear poly(ADP-ribose) deposition after hypothermic storage and reperfusion (Supplemental Figures 1 and 2).

Wortmannin Pretreatment Inhibited Cardioprotective Effect of INO-1153

When rat hearts were pretreated with the phosphatidylinositol 3-kinase inhibitor Wortmannin before cardioplegia and storage in INO-1153–supplemented Celsior, all cardioprotective effects observed with INO-1153 treatment alone were completely abolished (Fig. 3). There were no significant differences between Wortmannin treatment group and control group for recovery of any parameter after 45 min poststorage reperfusion.

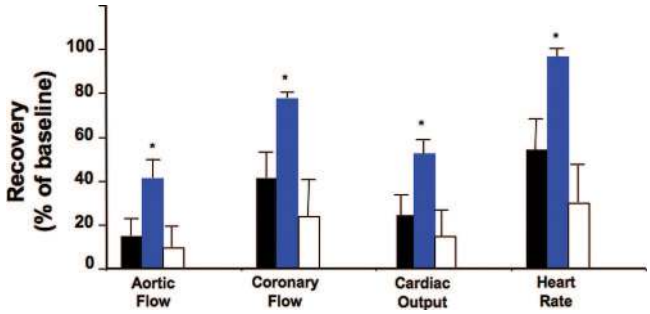

FIGURE 3. Effect of PI3-k inhibition on recovery of cardiac function induced by INO-1153. Treatments were: control (black bar; n=9); 1 μM INO-1153 present at cardioplegia and during hypothermic storage (blue bar; n=9); and hearts treated with 0.1 μM Wortmannin during prestorage perfusion then arrested and stored in 1 μM INO-1153 (white bar; n=6). AF, aortic flow; CF, coronary flow; CO, cardiac output; HR, heart rate. Data are expressed as means±SEM. *$P<0.05$ vs. control and INO-1153+Wortmannin.

Enhanced Phosphorylation of Akt Upon INO-1153 Treatment During Storage

Figure 4A shows typical phosphorylation status of Akt in untreated controls, (lane 1), hearts exposed to INO-1153 during cardioplegia and storage (lane 2), and hearts pretreated with Wortmannin before cardioplegia and storage in INO-1153 (lane 3). Quantitation of band intensity is shown in Figure 4B. Any differences in lane loadings were accounted for by dividing band intensities for phospho and total Akt by β-actin intensities for each blot (i.e., $I_{(\text{phospho-Akt})}/I_{(\beta\text{-actin})}$ and $I_{(\text{total Akt})}/I_{(\beta\text{-actin})}$). A ratio of normalized phospho-Akt/total Akt was then calculated for a random subset of three hearts per experimental treatment. The presence of INO-1153 during cardioplegia and storage resulted in a 4.3 fold increase in phospho-Akt compared with untreated control (1.16 ± 0.37 vs. 0.27 ± 0.07; $P<0.05$). This increase was completely inhibited by the presence of the PI 3-kinase/Akt pathway inhibitor, Wortmannin.

DISCUSSION

Three salient findings arose from this study. First, significant improvement in all poststorage functional parameters was observed when hearts were exposed to the PARP inhibitor INO-1153 either before hypothermic storage, or when the PARP inhibitor was included in the cardioplegic/storage solution. These findings are congruent with previous studies demonstrating efficacy of several different chemical classes of PARP inhibitor in improving a range of endpoints of normothermic cardiac ischemia reperfusion injury (*4*, *7*). Second, exposure of the heart to the phosphatidylinositol 3-kinase inhibitor, Wortmannin, completely blocked the protective effect of INO-1153. Finally, in line with these functional results, immunoblotting studies showed a significant increase in phospho-Akt in the presence of PARP inhibition, which was abolished by Wortmannin.

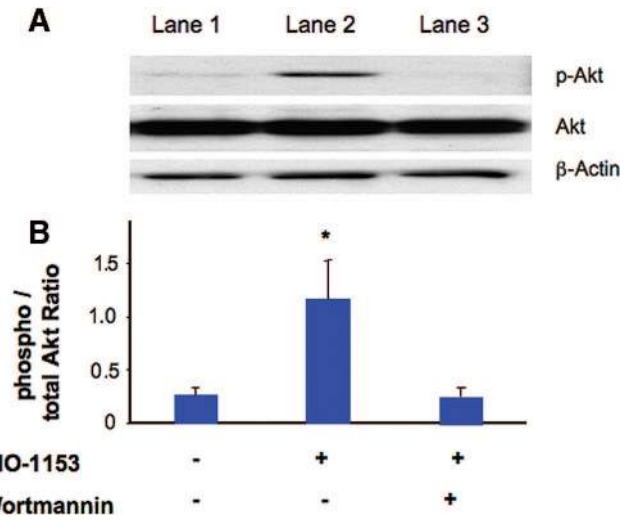

FIGURE 4. (A) Representative Western blots showing Akt phosphorylation status and β-actin in rat hearts after 45 min poststorage reperfusion after 6 hr cold storage. Lane 1, Group 1: untreated control hearts; Lane 2, Group 3: hearts treated with 1 μM INO-1153 during storage period; Lane 3, Group 5: hearts pretreated with 0.1 μM Wortmannin during prestorage perfusion, then arrested and stored in Celsior supplemented with 1 μM INO-1153. (B) Histogram showing ratio of phospho Akt/total Akt (n=3 each). Values are means$\pm$SE. *$P<0.05$ vs. control.

While the initial rationale for pharmacological inhibition of PARP in the context of prevention of ischemia reperfusion injury was the prevention of the profound ATP and NAD depletion resulting from "hyperactivation" of PARP at reperfusion (*25*), recent studies have demonstrated that recruitment of the highly conserved PI 3-kinase/Akt survival kinase pathway also accompanies PARP inhibition (*19*, *20*, *26*, *27*). Our studies confirm this mechanism. Figure 4 shows significant phosphorylation (activation) of Akt in the presence of INO-1153, which was returned to control levels by pretreatment of the heart with the PI 3-kinase inhibitor Wortmannin.

The PARP inhibitor INO 1001 has recently been shown to be effective in models of cardiac transplantation. In the Brown-Norway to Lewis rat heterotopic model of donor heart rejection, INO 1001 prolonged graft survival (*28*), provided suppression of the immune response (*29*), and improved contractile and vasomotor deficits accompanying rejection (*17*). In a canine model of orthotopic heart transplantation, INO 1001 significantly improved posttransplant cardiac contractility when the drug was given at reperfusion of the donor heart (*18*). Although we have shown some benefit of addition of INO-1153 at reperfusion, maximum benefit was only observed when INO-1153 was administered prior to or during cardioplegia and cardiac storage (Fig. 2). Efficacy of PARP inhibition at cardioplegia and during storage is logistically attractive in clinical heart transplantation. At multiorgan procurement, the pharmacological PARP inhibitor needs only to be added to the cardiac preservation solution at the time of cardioplegia, while the other organs are isolated from exposure to the agent. This may be an important point as little information has been published on the effect of PARP inhibitors on lungs or abdominal organs. Indeed, the PARP inhibitors 3-aminobenzamide and PJ34 were shown to increase poststorage injury in a model of hypothermic renal storage using University of Wisconsin solution as the preservation solution (*30*).

Recruitment and activation of the PI 3-kinase/Akt pathway has many potential beneficial effects for the donor heart postreperfusion. Endothelial nitric oxide synthase, expressed in the endothelium and cardiac myocytes (*31*), has been shown to be phosphorylated and activated by an Akt dependent process (*32*). The resultant production of nitric oxide at reperfusion can rapidly normalize endothelial function and cardiac perfusion. Previous studies in our laboratory, mimicking this process, have demonstrated improved poststorage cardiac function by supplementation of storage solution with nitric oxide donors such as glyceryl trinitrate or diethylamine NONOate (*23*, *33*).

In the cardiac myocyte, pharmacological activation of Akt pathway with insulin protected these cells from hypercontracture at reperfusion by activation of the sarcoplasmic reticulum Ca-ATPase (*34*). This protection was prevented by inhibition of either Akt, endothelial nitric oxide synthase or the sarcoplasmic Ca-ATPase. In another study, adenoviral gene transfer of Akt to rat hearts enhanced myocardial contractility and intracellular calcium handling (*35*). Akt activation has been demonstrated to phosphorylate and inactivate a

number of key regulatory proteins involved in the apoptotic cascade including BAD (*36*), Mdm2 (*37*), and FoxO (previously referred to as forkhead transcription factors) (*38*). Phosphorylation of I kappa B kinase by Akt leads to NF-κB dependent induction of expression of a number of antiapoptotic genes (*39*). Using quantitative reverse transcriptase polymerase chain reaction techniques, we measured the expression of several NF-κB target genes thought to be responsible for inhibition of apoptosis (*40*, *41*), rat nomenclature Birc-2, -3, -4, Bcl-2 (*42*), and Bcl-XL (*43*, *44*), as well as some genes encoding members of the pathway itself (IκB, NF-κB1, -2, and RelA) in hearts stored in Celsior alone and Celsior supplemented with 1 μM INO-1153 after 45 min reperfusion. There was no significant difference between mRNA levels from either of these treatments. This argues against a prominent role for the NF-κB pathway in the cardioprotective effects of INO-1153. Quantitative reverse transcriptase-polymerase chain reaction methodology and results are displayed in the Supplemental Methods and Supplemental Tables 1 and 2. In ongoing studies, we are employing a microarray approach to obtain comprehensive data on any transcriptional changes in this experimental system.

Recruitment and activation of the PI3-k/Akt pathway (termed the "RISK" pathway) has been shown to be a mechanism common to ischemic preconditioning and postconditioning, two powerful protective strategies against ischemia reperfusion injury (*22*). Although the RISK pathway was initially thought to be focused on maintaining mitochondrial integrity, Piper et al. have recently postulated that the sarcoplasmic reticulum and the calcium handling elements therein may also be the target of this pathway (*45*). Trophic factors and pharmacological agents such as insulin, erythropoietin, and statins have been shown to activate this pathway. PARP inhibitors may be another group of pharmacological agents to add to this list.

In conclusion, the present study demonstrates that cardioprotective effects of PARP inhibition and Akt activation previously observed in a range of normothermic models of cardiac ischemia reperfusion injury also holds true for ischemia reperfusion in the context of donor heart procurement and hypothermic storage. Importantly for the logistics of the potential use of PARP inhibition in the context of clinical donor heart retrieval, maximum efficacy is obtained when the inhibitor is present at a time prior to reperfusion at the time of cardioplegia and hypothermic storage. The success of this strategy must be investigated in a more clinically relevant setting, a task currently being undertaken by us in a braindead porcine model of organ transplantation developed in our laboratory.

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