SUPPLEMENTAL METHODS

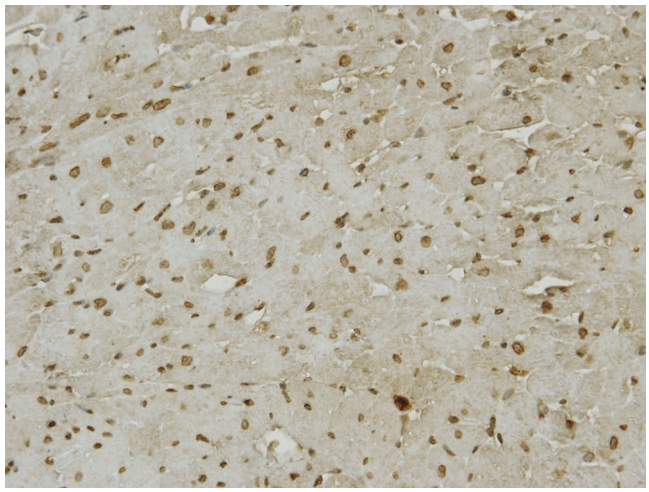
Poly(ADP-ribose) Immunohistochemistry

Accumulation of poly(ADP-ribose), (PAR), the product of poly(ADP-ribose) polymerase, was employed as an indicator of PARP activation. PAR deposition was assessed in hearts arrested and stored in Celsior alone (Group 1) and hearts arrested and stored in Celsior supplemented with the PARP inhibitor INO-1153 (1 μM) (Group 3). Samples of left ventricular free wall were fixed in ice cold 4% paraformaldehyde after postreperfusion functional data were acquired (45 min after reperfusion) then embedded in paraffin. Sections (5 μm) were deparifinised in xylene then rehydrated through a series of decreasing concentrations of ethanol. Antigen was retrieved by incubating sections in 0.1 M sodium citrate (pH 6.0) in a microwave oven for 4 min ("high" setting) then 5 min ("medium" setting). Sections were then cooled for 20 min. Nonspecific binding was blocked by incubating slides in 2% (w/v) skimmed milk/2% (v/v) Triton X-100 in Tris buffered saline (137 mM NaCl, 10 mM Tris, pH 7.4) for 30 min at room temperature. Sections were then treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a monoclonal anti-PAR antibody (isotype mouse immunoglobulin [Ig]G; Alexis Biochemicals, Lausen, Switzerland) at a dilution of 1:200 for 1 hr at room temperature, then treated with a secondary HRP-labeled anti-mouse IgG anti-body for 30 min (also at room temperature; Novolink Polymer Detection Systems, Vision BioSystems Ltd, Mount Waverley, Australia). Color was developed with diaminobenzidine (DAB). Sections were counterstained with hematoxylin, dehydrated and mounted in VECTASHIELD HardSet mounting medium (Vector Laboratories, Burlingame, CA). Photomicrographs were taken using an Olympus BX51 microscope equipped with an Olympus DP70 digital camera.

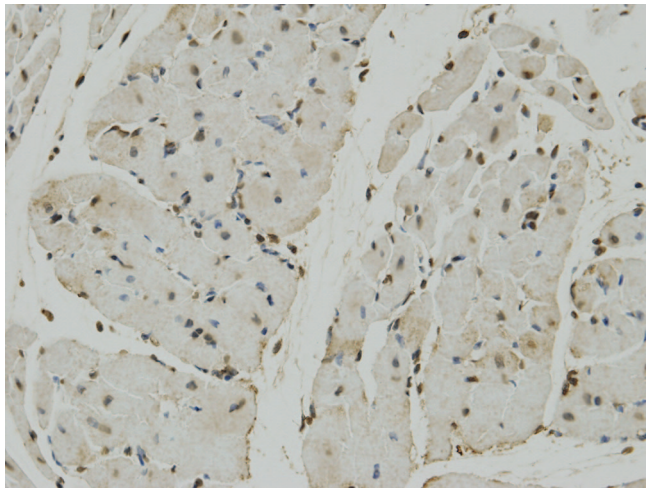
A representative example of PAR deposition in hearts stored in Celsior alone is shown in Supplementary Figure 1. The presence of 1 μM INO-1153 during the storage period (Supplementary Figure 2) results in a substantial reduction in the deposition of PAR positive antibody, indicative of a decrease in PARP activity.

Reverse-Transcription Polymerase Chain Reaction Analysis of mRNA Expression in NF-κB and Related Genes

Left ventricle sample of each heart was taken out of liquid nitrogen and thawed in TRIzol reagent (Invitrogen). After homogenization with Polytron PT 1200 homogenizer (Kinematica AG, Littau, Switzerland) in presence of TRIzol reagent, total RNA was isolated according to the manufacturer's instructions. Redissolved RNA was further purified using Micro Bio-Spin Chromatography Columns with Bio-Gel P-6 in tris buffer (Bio-Rad). Total RNA from each heart was quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop). RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies) or by gel electrophoresis. One microgram of total RNA from each sample was used for first strand cDNA synthesis using an Oligo$(dT)_{20}$ primer and SuperScript II Reverse Transcriptase (Invitrogen). Aliquots of the resulting cDNA were used for quantitative reverse transcriptase polymerase chain reaction with Lightcycler FastStart DNA Master^{Plus} SYBR green I (Roche, Mannheim, Germany). All primers (listed in Supplemental Table 1) were designed with OligoPerfect Designer at www.invitrogen.com and synthesized by Sigma-Genosys (Australia). Quantitative polymerase chain reaction was performed using the Rotor-Gene thermocycler (Corbett Research, NSW, Australia). Cycling conditions were as follows: 95°C for 10 min (predenaturation), 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and



SUPPLEMENTAL FIGURE 1. Immunohistochemical detection of PAR in a representative myocardial section of a heart arrested and stored in Celsior alone 45 min after reperfusion. The brown staining of all nuclei is indicative of PAR accumulation (from poly(ADP-ribose) polymerase activation). Magnification: x400. These results are consistent with the essentially nuclear distribution of PARP and PAR first described by Ikai and Ueda (*1*).



SUPPLEMENTAL FIGURE 2. Immunohistochemical detection of PAR in a representative myocardial section of a heart arrested and stored in Celsior supplemented with the PARP inhibitor, INO-1153 (1 μM) 45 min after reperfusion. In contrast to Supplemental Figure 1, decrease in the number of brown staining nuclei and the presence of a large number of bluish (counterstained) nuclei indicated that the addition of INO-1153 has markedly attenuated PAR deposition and decreased PARP activity. Magnification: $\times$400. Similar decreases in PAR deposition have been previously demonstrated by other PARP inhibitors including 3-aminobenzidine (*2*), PJ-34 (*3*) and INO-1001 (*4*) in other models of cardiac ischemia reperfusion injury.

SUPPLEMENTAL TABLE 1. Oligonucleotide primer sequences for RT-PCR analysis of gene expression of NF-κB related proteins

Gene description	Gene name	Primer sequence (forward; reverse)
NF-κB inhibitor	IκBα	AAGGACGAGGATTACGAGCA; GTCTCCCTTCACCTGACCAA
NF-κB	NF-κB1	GAAGAGGATGTGGGGTTTCA; ACACTGTCCCCGTTCTCATC
	NF-κB2	TGACTGTGGAGCTGAAGTGG; GGTGTGTTTCCAGCAAAGGT
	RelA	AACACTGCCGAGCTCAAGAT; CATCGGCTTGAGAAAAGGAG
NF-κB target	Birc2	GGCCATCGAGTGTTCTTGTT; CTCCTGACCCTTCATCCGTA
	Birc3	ACCGAACAGGAGTACGATGC; AGCTGCAATGTCATCTGTGG
	Birc4	CGCAGGATGAGTCAAGTCAG; CTGCTTCCGCACACTGTTTA
	Bcl2	AGTACCTGAACCGGCCATCTG; CAGGTATGCACCCAGAGTGA
	Bcl-XL	ACCGGAGAGCATTCAGTGAT; TGCAATCCGACTCACCAATA

72°C for 40 sec with fluorescent signal acquisition after each cycle. Melting curves were recorded between 72°C and 95°C. A threshold was set in the linear part of the amplification curve and the number of cycles needed to reach it was calculated for each gene. Relative quantitation was done using the standard curve method (*5*). Four serial 1:5 dilutions of each gene served as a standard curve that was assayed together with the corresponding unknown samples in each batch. Each gene was normalized to reference gene GAPDH before comparison between different experimental groups. Results are shown in Supplemental Table 2.

SUPPLEMENTAL TABLE 2. Quantitative reverse transcriptase polymerase chain reaction analysis of NF-κB–related mRNA

Gene description	Gene name	Normalized mRNA level (mean±SD) Group 1 (n=3)	Group 3 (n=4)	*P* value
NF-κB inhibitor	IκBα	1.57±0.32	1.16±0.13	0.15
NF-κB	NF-κB1	1.00±0.20	1.00±0.14	0.98
	NF-κB2	1.24±0.31	1.35±0.06	0.59
	RelA	1.04±0.26	0.99±0.21	0.81
NF-κB target	Birc2	0.95±0.13	0.85±0.06	0.32
	Birc3	2.03±1.11	2.44±0.76	0.62
	Birc4	0.89±0.23	1.00±0.26	0.56
	Bcl2	0.65±0.05	0.71±0.12	0.38
	Bcl-XL	0.71±0.03	0.64±0.10	0.27

SUPPLEMENTAL REFERENCES

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