

Poly(ADP-ribose) Polymerase Activation and Cell Injury in the Course of Rat Heart Heterotopic Transplantation

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Free radicals and other reactive species generated during reperfusion of ischemic tissues may cause DNA damage and, consequently, the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP). An excessive PARP activation may result in a depletion of intracellular NAD⁺ and ATP, hence cell suffering and, ultimately, cell death. The present study is aimed at clarifying the role of PARP in a heart transplantation procedure and the contribution of myocyte necrosis and/or apoptosis to this process.

In our experimental model, rat heart subjected to heterotopic transplantation, low temperature global ischemia (2 h) was followed by an *in vivo* reperfusion (30 or 60 min). Under these conditions clear signs of oxidative stress, such as lipoperoxidation and DNA strand breaks, were evident. In addition to a marked activation, accompanied by a significant NAD⁺ and ATP depletion, PARP protein levels significantly increased after 60 min of reperfusion. Ultrastructural analysis showed nuclear clearings, intracellular oedema and plasma membrane discontinuity. Other relevant observations were the absence of typical signs of apoptosis like caspase-3 activation and PARP cleavage, random DNA fragmentation, rise in serum levels of heart damage markers. Our results suggest that during heart transplantation, the activation of PARP, causing energy depletion, results in myocardial cell injury whose dominant feature, at least in our experimental model, is necrosis rather than apoptosis.

Keywords: PARP; Heart; Ischemia; Reperfusion; Necrosis; Apoptosis

Abbreviations: LDH, Lactate dehydrogenase; CK, Creatine kinase; cTnI, cardiac troponin I

INTRODUCTION

Reactive oxygen species (ROS), together with other free radicals and reactive compounds, are thought to be responsible for the manifold deleterious effects occurring in ischemic-reperfused (IR) tissues. In fact, during post-ischemic reperfusion many sources concur to an increased ROS production, so that this condition represents one of the main mechanisms leading to the so called oxidative stress.^[1,2]

In addition to other cytotoxic effects ROS and related reactive compounds can induce DNA damage, notably strand breaks, resulting in the activation of poly(ADP-ribose) polymerase (PARP, E.C.2.4.2.30), a 116 kDa nuclear enzyme strictly associated to chromatin.^[3] PARP catalyses the covalent modification of proteins by adding poly(ADP-ribose), using NAD⁺ as a substrate and converting it into nicotinamide.^[4] Poly(ADP-ribosylation) may be induced in several chromatin proteins including PARP, in a self ADP-ribosylation process, histones and many others.^[5] Modified histones can dissociate from DNA allowing the repair enzymes to act, while poly(ADP-ribosylation) of other proteins (topoisomerase I and II, DNA ligase II), can trigger various signalling pathways, for example those involved in the arrest of cell cycle.^[6] With regards to a possible involvement of PARP in the ischemia-reperfusion injury, an excessive activation of this

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enzyme may result in a rapid depletion of intracellular NAD⁺ and ATP, hence in a loss of energy supply which could cause cell suffering and, ultimately, cell death.^[7] The latter has been reported as necrotic ("oncotic" according to Majno and Joris^[8]), apoptotic or a combination of both.^[9,10]

A lot of studies have been reported about the cardiac dysfunction induced by ischemia-reperfusion, a process of great clinical interest since it may arise in a number of circumstances, such as thrombolysis, angioplasty and heart transplantation. As for the contribution of necrosis and apoptosis to cell death occurring in IR myocardium, the reported results are often controversial. This disagreement can be ascribed, at least in part, to the use of different experimental models. In the present study heterotopically transplanted rat heart was used as a model of global ischemia followed by *in vivo* reperfusion, to explore the behaviour of PARP and its possible relationships with myocyte necrosis and/or apoptosis occurring in these conditions.

METHODS

Experimental Model

Wistar rats, weighing 200–250 g, were used in this investigation. The investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). In heart transplantation procedures donor ($n = 14$) and recipient ($n = 14$) rats, fasted from the night before surgery, were anaesthetized with 8% chloral hydrate, given intraperitoneally 0.75 ml/100 g body weight.

Donor Preparation

Anaesthetized animals were heparinized, 3 mg/kg via the inferior vena cava; after 5 min the chest was entered through a bilateral thoracotomy, the inferior vena cava divided, pericardium opened, ascending aorta and pulmonary artery dissected free from the surrounding tissues, the heart rapidly excised and arrested by infusion of 5 ml of St. Thomas solution directly into the aorta. Inferior vena cava was ligated and the heart was stored in ice-cold saline for 2 h.

Recipient Preparation

Infrarenal aorta and inferior vena cava, exposed through a median laparotomy, were dissected free for a length of about 1.5 cm, clamped separately and opened with a 7–8 mm incision. The explanted heart was reimplanted with termino-lateral aorta–aorta and pulmonary-cava anastomoses under microscopic magnification and, after 30 or 60 min of reperfusion, was excised. Hearts from sham-oper-

ated animals were used as controls ($n = 7$). Apart from small fragments used for morphological analyses, hearts were immediately frozen in liquid nitrogen and stored at -80°C until use.

Analysis of DNA Fragmentation

Cardiac muscle (200 mg) was incubated in a buffer (50 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% SDS pH 8) containing proteinase K at a final concentration of 1 mg/ml. After incubation at 56°C for 8 h, total genomic DNA was extracted by the phenol/chloroform procedure and precipitated at -20°C with 0.3 M (final concentration) sodium acetate and 2.2 volumes of absolute ethanol. Nucleic acids were collected by centrifugation at 16,000 g for 15 min at 4°C and solubilized in 10 mM Tris pH 7.4 containing 0.1 mM EDTA, at 65°C for 30 min. After treatment with RNase (20 $\mu\text{g}/\text{ml}$) for 1 h at 37°C , the concentration and purity of DNA were estimated by the ratio of absorbances at 260 and 280 nm.

The rate of DNA strand breaks was determined by the alkaline fluorescence analysis of DNA unwinding^[11] and the results were expressed as percentage of undamaged DNA with respect to total DNA.

Agarose Gel Electrophoresis of DNA

Ten μg of purified DNA were loaded on 1.5% agarose gel containing ethidium bromide and electrophoresed at 100 V in 0.04 M Tris, 0.04 M boric acid, 2 mM EDTA, pH 8. DNA was visualized under ultraviolet light and photographed for permanent records.

Determination of Caspase-3 Activity

Heart samples from control and IR hearts were homogenized with a polytron homogenizer kept in ice, sonicated and centrifuged at 22,000 g and 4°C for 10 min. Twenty μl of the supernatant were used for the measurement of the caspase-3 activity by the caspase assay system, colorimetric (Promega, WI, USA). Positive control was obtained by staurosporine-induction of apoptosis in cultured rat cardiomyocytes according to Yue et al.^[12] Activity was expressed as nmol of *p*-nitroaniline (pNA) released/h/mg protein.

Preparation of Homogenates and Purification of Nuclear Fraction

Cardiac sample (100 mg) was incubated in phosphate buffered saline containing 0.1% collagenase type 1, for 1 h at 37°C . The mixture was centrifuged at 100 g for 15 min and the pellet was homogenized (20%, wt/vol with a glass–glass Potter–Elvehjem homogenizer) in ice cold 50 mM Tris–HCl (pH 7.4) buffer containing 1 mM EDTA, 1 mM dithiothreitol,

50 mM NaCl, 0.25 M sucrose, 0.2 mM PMSF and 10 μ g/ml of aprotinin and leupeptin. The homogenate was centrifuged at 600 g for 10 min; the pellet, containing the nuclear fraction, was washed with the homogenising buffer, then suspended in 50 mM Tris-HCl (pH 8.0), 25 mM MgCl₂, 0.1 mM PMSF and finally sonicated on ice. In all the samples protein concentration was measured by the method of Bradford.^[13]

Measurement of Lipid Peroxidation Products

To assess the rate of lipid peroxidation, malonaldehyde (MDA) and 4-hydroxyalkenal (4-HNE) concentrations were determined in the supernatant of the homogenate prepared as above described. Measurements were performed by a colorimetric method based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA or 4-HNE at 45°C.^[14]

PARP Activity and Protein Levels

PARP activity was assessed by Western blot analysis of poly(ADP-ribosylated) proteins. An aliquot of sonicated nuclear suspension, obtained as previously described, was diluted in Laemmli's sample buffer and boiled for 5 min. 10 μ g proteins were separated on 7.5% SDS-PAGE. After blotting, whose completeness was checked by suitable staining, the nitro-cellulose membranes were blocked in 5% bovine serum albumin and then probed using 1:10,000 diluted anti-poly(ADP-ribose) monoclonal antibody (Trevigen Inc., Gaithersburg, MD, USA) for 2 h. Poly(ADP-ribosylated) proteins were detected with the HRP-conjugated secondary antibody and the following ECL procedure. PARP protein levels were determined using the highly specific C2-10 anti-PARP monoclonal antibody (Oncogene Research Products, Boston, MA, USA). The band densities were quantified as densitometric units/10 μ g protein (the constant protein amount applied on SDS-PAGE) using the program for image analysis and densitometry Quantity One (Biorad, Rome, Italy). For each band of interest the control values were considered as 100% and those for IR hearts were calculated as a percentage of the control within the same blot.

Determination of Adenine Nucleotides and Related Compounds

Frozen heart samples, after pulverisation, were added to ice cold 0.6 M perchloric acid (1:5 wt/vol) and homogenized three times for 15 s with an Ultra-Turrax homogenizer. Homogenates were centrifuged at 6000 g and 4°C for 10 min, the supernatant was separated, neutralized with 5 M K₂CO₃ and

centrifuged again as above indicated. The supernatant was adjusted to pH 7 and filtered through a 0.45 μ m filter. The clear obtained solution was used for HPLC analysis which was performed using a 5 μ m Supelcosil LC-18 reversed-phase column (25 cm \times 4.6 mm i.d.), (Supelco, Bellefonte, PA). The chromatographic conditions were as described by Stocchi et al.^[15] with slight modifications. The flow rate was 1.3 ml/min and detection was at 254 nm. Integration of peak areas was performed by the Turbochrom Navigator system, version 4.0 (Perkin Elmer).

cTnI, CK and LDH Measurements

cTnI, CK and LDH were measured in the plasma of recipient rats before surgery (control values) and after 60 min of reperfusion. cTnI was assayed by a fluorogenic sandwich ELISA test (OPUS Troponin I, Dade Behring, IL, USA) which uses monoclonal antibodies conjugated with alkaline phosphatase. CK and LDH were assayed by routine laboratory spectrophotometric methods at 340 nm.

Ultrastructural Analyses

Fragments of myocardium, taken from the anterior wall of the left ventricle of control and IR rat hearts, were immediately fixed in 4% cold glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature and post fixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.4, at 4°C. The samples were then dehydrated in graded acetone series and embedded in Epon 812. Semithin sections were cut and stained with Toluidine blue-sodium tetraborate. Ultrathin sections were also obtained from the same specimens, stained with uranyl acetate and alkaline bismuth subnitrate, and examined under a Jeol 1010 transmission electron microscope.

Statistical Analysis

Student's t-test was used to assess statistical significance of the differences between control and IR hearts. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

With reperfusion, transplanted hearts rapidly recovered normal colour and, in the first few minutes, resumed sinus rhythm after a brief ventricular fibrillation.

Lipid Peroxidation and DNA Fragmentation

To determine the presence of oxidative stress under our experimental conditions we measured the markers of two effects typically induced by this condition: lipid peroxidation and DNA damage. As for lipid peroxidation, the concentrations of MDA and 4-HNE, stable and distinctive products of this process, were significantly higher in transplanted hearts compared to the control, even after 30 min of reperfusion (Table I). A further significant increase was observed after 60 min of reperfusion, when the mean level of these compounds was about 66% over the control value.

DNA damage, as judged by the rate of strand breaks, assessed with the analysis of DNA unwinding, appeared to progress with the duration of the reperfusion (Table I). In fact, the fraction of undamaged DNA was significantly reduced under the control level after 30 min of reperfusion, and a more marked decrease was found after 60 min.

Agarose gel electrophoresis of DNA from 60 min reperused and control myocardium revealed a "smear" or "diffusion" pattern attributable to lower size fragments, which has been suggested to indicate a random DNA fragmentation.^[16] No laddering was evident (Fig. 1).

Caspase-3 Activity

Mean values of caspase-3 activity (nmol pNA/h per mg protein) were 2.75 in the control hearts, 2.77 and 2.71 in the IR after, respectively, 30 and 60 min of reperfusion. These differences were not statistically significant. Values ten fold higher than those measured in control hearts were found in rat cardiomyocytes as described above (see "Methods" section) and used as a positive control.

PARP Activity and Protein Levels

PARP activity, determined on the basis of its auto-poly(ADP-ribosylation) level, was revealed by a 116 kDa band, in Western blot analysis performed with anti-poly(ADP-ribose) antibodies (Fig. 2). Densitometric analysis of this band showed that

PARP activity significantly increased in IR hearts, at an extent which, on average, was about 22 and 78% over the control value after, respectively, 30 and 60 min of reperfusion.

As for PARP protein levels, densitometry of the 116 kDa band, detected with the C2-10 antibody, indicated that these were unchanged in transplanted hearts subjected to 30 min of reperfusion, but a marked and significant increase with respect to control values was found after 60 min (Fig. 3).

Concentrations of Adenine Nucleotides and Related Compounds

As shown in Fig. 4, NAD⁺ levels (which in our assay conditions also included NADH+H⁺) decreased significantly in IR heart, both after 30 min and, more markedly, after 60 min of reperfusion, when they were about 40% of the control value. Similar changes were observed for ATP and ADP whose mean concentrations at 60 min were 32 and 23% of the control values, respectively. Other significant differences from the control hearts were an increase in inosine and xanthine concentrations.

Plasma Levels of cTnI, CK and LDH

After transplantation, the plasma concentration of cTnI, a highly specific marker of myocardial injury, increased reaching a level about three fold over the basal value after 60 min of reperfusion. Even higher increases were found for LDH and CK, whose mean plasma levels, at the same reperfusion times, were about four fold over control values (Fig. 5).

Ultrastructural Analyses

The main abnormal features observed in the IR cardiomyocytes included, substantial nuclear clearings with highly dispersed chromatin (Fig. 6A, B), mitochondrial swelling with loss of cristae (Fig. 6B, C), marked increase in non-myofilament spaces (an indication of intracellular oedema) (Fig. 6C), accumulation of intramitochondrial electron-dense amorphous bodies (Fig. 6D) and several discontinuities at the plasma membrane (Fig. 6D).

TABLE I Signs of oxidative stress in heterotopical transplanted rat heart after 30 and 60 min of reperfusion. MDA and 4-HNE were determined by the colorimetric procedure described under experimental procedures in the control and ischemic-reperused hearts. DNA fragmentation was determined by the alkali-unwinding assay. Values are means \pm SEM of determinations performed in seven control and seven transplanted-reperused hearts. Significantly different from the control at the $P < 0.01$ level*, or at the $P < 0.05$ level**. Significantly different from ischemic-reperused (30 min) at the $P < 0.05$ level***.

	MDA+4-HNE (nmol/mg protein)	Undamaged DNA (%)
Control	7.21 \pm 0.32	75 \pm 5.0
Ischemia and 30 min of reperfusion	9.31 \pm 0.39*	60 \pm 4.5**
Ischemia and 60 min of reperfusion	12.04 \pm 1.20***	41 \pm 4.7***

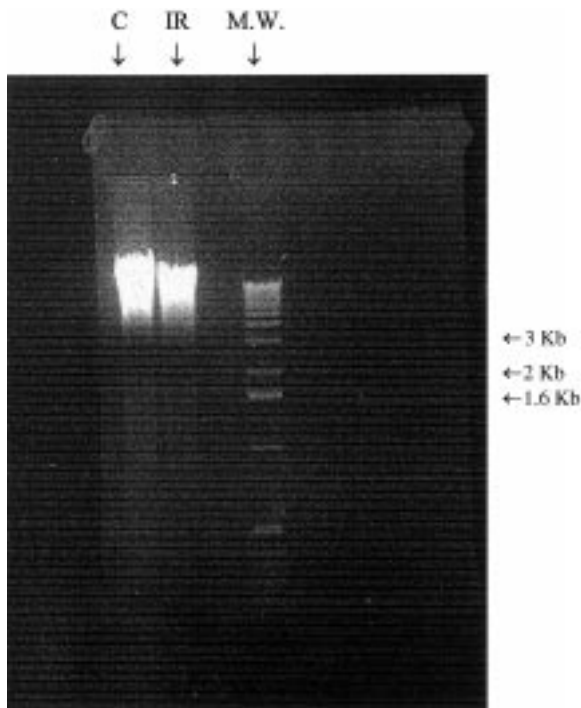


FIGURE 1 Agarose gel electrophoresis of DNA extracted from control (C) and ischemic-reperfused (IR) rat heart. A diffusion pattern suggestive of random DNA fragmentation was observed in IR heart; DNA laddering was not noted. MW indicates molecular weight markers.

These modifications were already evident after 30 min of reperfusion but they were more pronounced after 60 min. At 30 min sporadic nuclei showed condensed chromatin trending to segregate into sharply defined bodies within an intact nuclear envelope.

DISCUSSION

In the present investigation a model of global ischemia (already used in a previous study from this laboratory^[17]) was chosen for two main reasons: (1) heart is completely and uniformly affected; and (2) there are little if no differences in the severity of ischemia in the used myocardial samples since the intervention of possible inter-coronary anastomoses is excluded. Another consideration that prompted us to use the present model is that heart transplantation, as stated in the "Introduction" section, represents one of the major circumstances occurring in medical practice in which myocardial damage may be induced by oxidative stress arising from ischemia-reperfusion mechanisms.^[18] We thought it was of interest to perform this study with an animal model which would mimic this condition; moreover, to our knowledge, scientific literature lacks reports about PARP modifications in transplanted hearts. The chosen time periods for ischemia and reperfusion

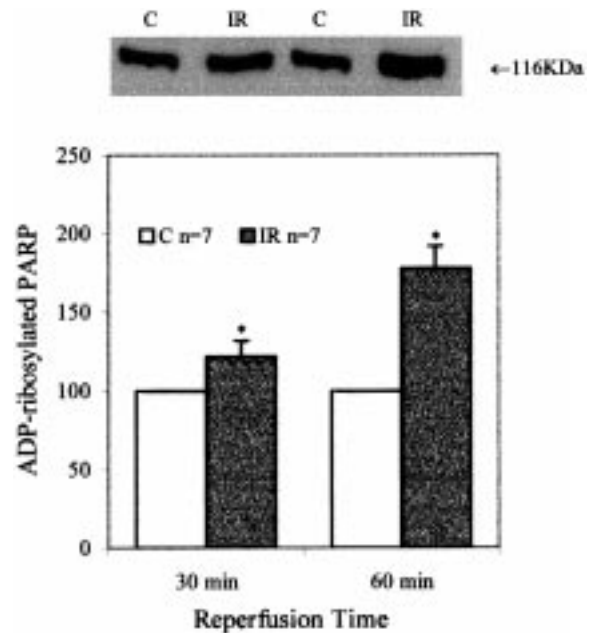


FIGURE 2 Top: representative Western blot showing the poly(ADP-ribosylation) (PAR) level of PARP in control (C) and ischemic-reperfused (IR) rat heart after 30 and 60 min of reflow. Bottom: quantitative data, obtained by densitometric analysis (means \pm SEM) are expressed as percentage of the control values (see "Methods" for details). *Significantly different ($P < 0.01$) from the control.

were similar to those used by other authors in similar heart transplantation procedures on small-sized animals.^[19,20] Both lipid peroxidation and DNA damage, typical effects of oxidative stress, were clearly evident under our experimental conditions, as judged from the levels of lipid peroxidation markers (MDA and 4-HNE) and from the analysis of DNA unwinding. DNA damage in the IR compared to the control hearts was confirmed by agarose gel electrophoresis, which showed a "diffusion" pattern, suggestive of a random DNA fragmentation, as it generally occurs in free radical-induced DNA damage. Although the signs of lipid peroxidation and of DNA damage were present after 30 min of reperfusion, both appeared to be increased after 60 min. This is consistent with the results reported by other authors,^[21] and a possible explanation for this observations is that prolonged reperfusion results in an enhanced infiltration of the ischemic tissue by inflammatory cells, whose recruitment is enhanced by PARP activation.^[22] These cells represent an important source of ROS, and other cytotoxic compounds such as peroxynitrite.^[23]

Our results also indicate that in IR hearts the rate of DNA fragmentation paralleled with PARP activation, in agreement with the view that this enzyme is directly activated upon DNA damage, notably by strand breaks, functioning as a "nick sensor".^[6] PARP, in fact, was extensively and significantly auto-poly(ADP-ribosylated), compared

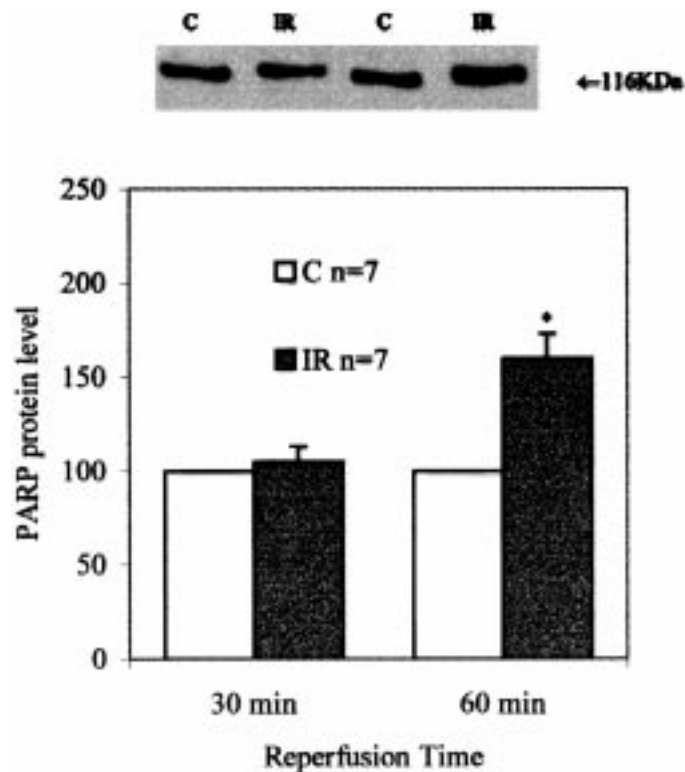


FIGURE 3 Top: representative Western blot showing the PARP protein level in control (C) and ischemic-reperfused (IR) rat heart after 30 and 60 min of reflow. Bottom: quantitative data obtained by densitometric analysis (means \pm SEM), are expressed as percentage of the control values (see "Methods" for details). *Significantly different ($P < 0.01$) from the control.

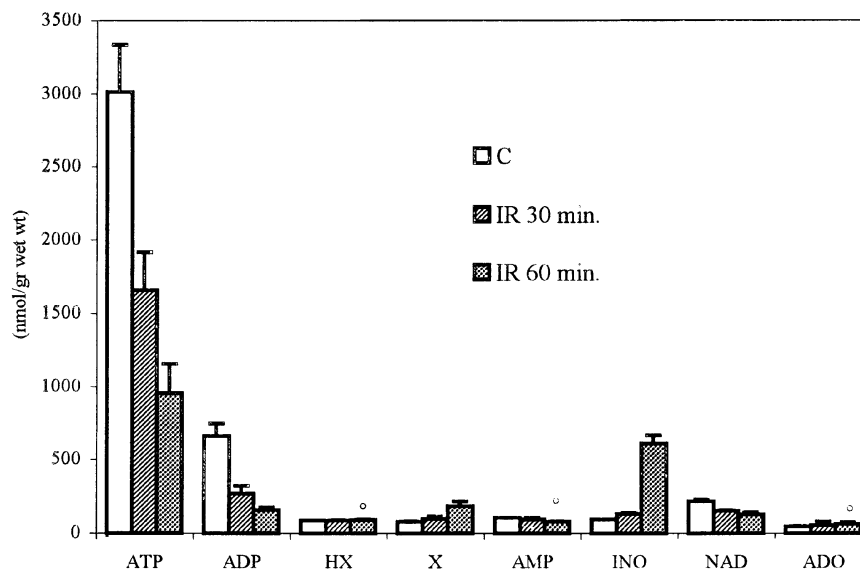


FIGURE 4 Adenine nucleotides and related compounds after 30 and 60 min of reflow. Each bar represents the Mean \pm SEM of determinations performed on seven transplanted-reperfused hearts (for 30 and 60 min) and relative controls. For each analysis all the differences from the control value were statistically significant ($P < 0.01$) except for those indicated with $^{\circ}$. When not shown, SEM was less than 5% of the mean. ADO: adenosine; HX: hypoxanthine; INO: inosine; X: xanthine.

to the control level, after 60 min and also, at a lesser extent, after 30 min of reperfusion. It is generally accepted that PARP is constitutively expressed at high levels, so that increases in its activity are thought to reflect an enhanced catalytic power instead of a rise in number of its molecules.^[3]

Nevertheless, we found that not only PARP activity but also PARP protein concentration was higher than the control level after 60 min of reperfusion. In any case, increased PARP activity matched a significant decrease in NAD^+ and ATP levels; also ADP and AMP were reduced, although the decrease in AMP

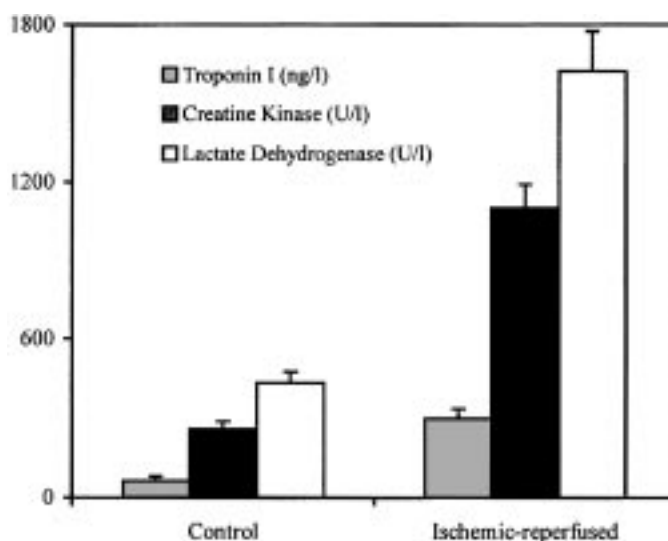


FIGURE 5 Plasma concentration of LDH, CK and TnI in control and IR rats after 60 min of reperfusion. All the differences were statistically significant ($P < 0.01$).

was not statistically significant. The entire adenine nucleotide pool exhibited a depletion, which was not stoichiometrically rescued by the increases of inosine and xanthine levels. These findings can be explained by the activity of adenylate kinase, an impaired recharge of the adenylic system and an enhanced leakage from membranes, permeabilized by the lack of ATP. In any case, our results on adenine nucleotides agree with those reported by other authors^[24] and suggest a development of a marked energy depletion due to PARP activation.

A loss of energy content can result, owing to its severity, in a reversible or in an irreversible cell injury. In the second case, several reports^[9,10] suggest that the mode of cell death may be necrotic or apoptotic according to the degree of the energy depletion. Apoptosis, in fact, is an active process requiring energy and a lack of ATP prevents the completion of the apoptotic program. Myocyte death occurring in cardiac ischemia-reperfusion has been considered as prevalently necrotic for a long time. Some authors, however, have recently stressed the contribution of apoptosis in this condition.^[18,25]

In order to establish the occurrence, and the relative contribution of necrosis and apoptosis in our experimental model, we referred to several well established biochemical markers and morphological analyses based on TEM observations. Among the biochemical aspects, caspase-3 activation and PARP cleavage into a 85 kDa fragment are considered, by general consent, as important hallmarks of apoptosis. However, as stated in the "Results" section, the activation of caspase-3, an early event in the apoptotic cascade, was not observed either after 30 or 60 min of reperfusion. As for PARP cleavage, the use of C2-10 antibodies, able to react with both the native and cleaved form

of PARP, never revealed an immunoreaction with the 85 kDa band in any of our Western blot analysis. Although not absolutely specific, another method often used to distinguish between apoptotic and necrotic cell death is DNA agarose gel electrophoresis showing characteristic ladders of double strand fragments (apoptosis) or a diffuse random DNA breakage (necrosis). In our case, the electrophoretic pattern of DNA in IR hearts, far from resembling an apoptotic ladder, appeared to indicate a random fragmentation probably due to a prevalence of single strand breaks. Taken together, these findings suggest that in our experimental model the contribution of apoptosis was scarce or even absent while, conversely, necrosis seems to represent the dominant pattern of cell injury. This interpretation is supported by the increased serum levels of the biochemical markers typical of myocardial damage, notably the cardiospecific cTnI, and by the results of morphological analysis. As for the last aspect, the observed alterations, especially nuclear clearings with highly dispersed chromatin, plasma membrane discontinuities and intracellular oedema are much more indicative for a necrotic than for an apoptotic injury. The only findings that might be suggestive for apoptosis were the above described alterations (see "Results" section: ultrastructural analysis) that were sporadically observed nuclei after 30 min but not after 60 min of reperfusion. It is possible that at this time some myocytes had sufficient ATP to enter apoptosis, but the subsequent increase in energy depletion due to a further PARP activation, caused the arrest of apoptotic cascade and switched the pathway of cell death towards the necrotic mode.

In conclusion, the results here reported suggest that, under our experimental conditions, oxidative stress induced by reperfusion of ischemic myocar-

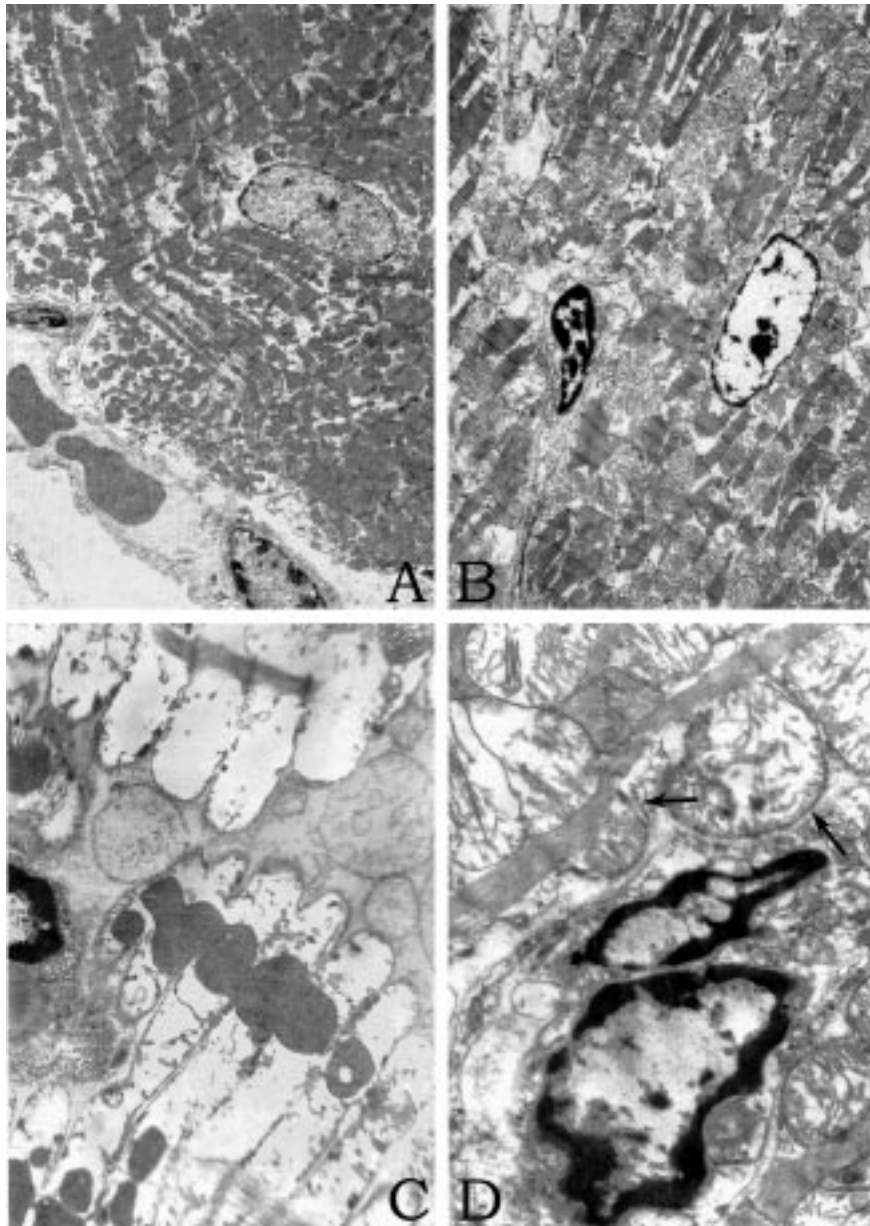


FIGURE 6 (A) Control myocardium. A cardiomyocyte shows a dense nucleus and a cytoplasm filled with regularly arranged myofilaments, mitochondria with intact cristae and glycogen particles. TEM \times 3000. (B) Rat myocardium subjected to 60 min of reperfusion. A cardiomyocyte exhibits an enlarged nucleus showing clearing of its matrix and a cytoplasm containing numerous swollen mitochondria with fragmentation of cristae. TEM \times 4000. (C) Rat myocardium subjected to 60 min of reperfusion. A remarkable intercellular oedema associated with loss of glycogen particles is evident in two adjacent cardiomyocytes. TEM \times 7000. (D) Rat myocardium subjected to 60 min of reperfusion. A cardiomyocyte shows dissolution of the plasma membrane lining and injured mitochondria (arrows) containing electron-dense amorphous bodies in their matrix. TEM \times 14,000.

dium can elicit PARP activation leading to a significant decrease of cellular energy content and to a myocardial injury consisting in the development of necrotic rather than apoptotic processes. Clearly, the present work is preliminary and further investigations are needed to verify the proposed correlation between heart oxidative damage and PARP activation. In this connection, we think that further studies with the present experimental model would be useful to explore how the duration of ischemia and reperfusion may affect the severity and the type of heart injury, as well as to assess possible

favourable effects of pharmacological PARP inactivation.

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