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Beneficial Effects of Poly (ADP-ribose) Polymerase Inhibition Against the Reperfusion Injury in Heart Transplantation

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We investigated the effect of 3-aminobenzamide (3-AB), an inhibitor of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), against early ischemia/reperfusion (IR) injury in heart transplantation. In our experimental model, rat heart subjected to heterotopic transplantation, low temperature global ischemia (2 h) was followed by an *in vivo* reperfusion (60 min). In these conditions, and in the absence of 3-AB treatment, clear signs of oxidative stress, such as lipid peroxidation, increase in protein carbonyls and DNA strand breaks, were evident; PARP was markedly activated in concomitance with a significant NAD^+ and ATP depletion. The results of microscopic observations (nuclear clearings, plasma membrane discontinuity), and the observed rise in the serum levels of heart damage markers, suggested the development of necrotic processes while, conversely, no typical sign of apoptosis was evident.

Compared to the effects observed in untreated IR heart, the administration of 3-AB (10 mg/kg to the donor and to the recipient animal), but not that of its inactive analogue 3-aminobenzoic acid, significantly modified the above parameters: the levels of oxidative stress markers were significantly reduced; PARP activation was markedly inhibited and this matched a significant rise in NAD^+ and ATP levels. PARP inhibition also caused a reduced release of the cardiospecific damage markers and attenuated morphological cardiomyocyte alterations, save that, in this condition, we noted the appearance of typical apoptotic markers: activation of caspase-3, oligonucleosomal DNA fragmentation, ISEL positive nuclei. Possible mechanisms for these effects are discussed, in any case the present results indicate that PARP inhibition has an overall beneficial effect against myocardial reperfusion injury, mainly due to prevention of energy depletion. In this context, the signs of apoptosis observed under 3-AB treatment might be ascribed to the maintenance of sufficient intracellular energy levels. These latter allow irreversible damages triggered during the ischemic phase to proceed towards apoptosis instead of towards necrosis, as it appears to happen when the energetic pools are depleted by high PARP activity.

Keywords: PARP; Heart; Ischemia; Reperfusion; 3-Aminobenzamide

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

INTRODUCTION

Oxidative stress, resulting from an excessive production of reactive oxygen species (ROS) and peroxynitrite, contribute to the ischemia/reperfusion (IR)-induced cell injury. These reactive compounds initiate lipid peroxidation,[1] protein oxidation[2] and DNA damage.[3] This latter mainly consists in single strand DNA breaks which can activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP) that ADP-ribosylates different nuclear proteins on the expense of cleaving NAD^+.[4] Studies *in vitro*, performed with various cell types and in different experimental models suggest that an excessive PARP activation may result in a depletion of intracellular NAD^+ and ATP, hence cell suffering and, ultimately, cell death. Consistently with this view, pharmacological inhibition of PARP

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has been shown to exert beneficial effects against free radicals mediated cell injury.[5,6]

Oxidative stress due to the ischemia/reperfusion mechanism has been reported to occur in several conditions related to cardiac pathology, namely in heart transplantation which may be considered as a kind of global ischemia followed by reperfusion owing to the recipient circulation. With the aim to explore the involvement of PARP in the myocardial injury associated to heart transplantation procedures, a study has been recently performed in our laboratory using heterotopically transplanted rat heart as experimental model.

Under these conditions transplanted hearts showed a significant PARP activation and markedly reduced ATP levels. At the same time, cardiomyocytes exhibited relevant morphological features which suggested a possible development of necrotic processes. As a reasonable interpretation of these findings, we proposed that PARP activation, triggered by the oxidative stress-induced DNA alterations, would result in a severe energy depletion, so that the death mode of the irreversibly damaged cardiomyocytes would be represented by necrosis, a process which does not require an energy supply.

In order to probe more deeply the role of PARP activation in the ischemia/reperfusion injury after heart transplantation, in the present study we used the same experimental model to investigate the effects of the specific PARP inhibitor 3-aminobenzamide (3-AB). More in particular, we intended to test the hypothesis that PARP inhibition attenuates the biochemical alterations and/or changes the mode of cardiomyocyte death in transplanted hearts.

EXPERIMENTAL PROCEDURES

Materials

All reagents were of analytical grade or the highest purity available. Unless otherwise stated, chemicals were purchased from Sigma, Italy.

Experimental Model

The experimental model was described elsewhere.[7] Briefly, donor hearts were explanted from Wistar rats. After 2 h of ischemic preservation at 4°C, the hearts were implanted intraabdominally anastomosing the aorta and the pulmonary artery of the donor heart with the abdominal aorta or the vena cava of the recipient rat, respectively. After 1 h of reperfusion the hearts were rapidly excised and apart from small fragments used for morphological analyses, were immediately frozen in liquid nitrogen and stored at −80°C until use. In treated animals 3-AB (10 mg/kg) was administered to the donor rat before heart excision and to the recipient rat at the beginning of reperfusion. In one group of animals 3-aminobenzoic acid (3-ABA 10 mg/kg) was used instead of 3-AB. Hearts from sham-operated animals were used as controls. For each set of experiments $n = 5$ animals were used. No animals were excluded from the statistical analysis. The investigation conforms with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Measurement of DNA Strand Breaks

Two hundred milligram samples were incubated in a buffer (50 mM Tris pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS) 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 μg/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[8] and the results were expressed as percent of undamaged DNA with respect to total DNA.

Preparation of Homogenates and Purification of Nuclear Fraction

One hundred milligram samples were incubated in phosphate buffered saline containing 0.1% collagenase type 1, for 1 h 15 min at 37°C. The mixture was centrifuged at 100*g* for 15 min and the pellet was homogenised (20% wt/vol with a glass–glass Potter–Elvejhem 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 μg/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 homogenizing buffer, then suspended in 50 mM Tris–HCl (pH 8.0), 25 mM $MgCl_2$, 0.1 mM PMSF and finally sonicated on ice. In all the samples protein concentration was measured by the method of Bradford.[9]

Measurement of Lipid Peroxidation Products

To assess the rate of lipid peroxidation, malonaldehyde (MDA) and 4-hydroxyalkenals (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.[10]

Determination of Protein Carbonyls

The protein carbonyl content was determined by using the 2,4-dinitrophenylhydrazine method by Levine *et al.*[11] In brief cytosolic samples (200 μg protein) were dried in a vacuum centrifuge, after to each tube was added 500 μl of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl and then the described procedure was followed. Spectrophotometric measurement was performed at 375 nm considering 22,000 M^{-1} cm^{-1} as the molar absorption coefficient.

Western Blot for Poly(ADP-ribosylated) Proteins, PARP Protein Levels and Caspase-3 Active Fragment

PARP activity and protein levels were assessed by Western blot. Briefly, an aliquot of sonicated nuclear suspension, obtained as previously described, was diluted in Laemmly's sample buffer and incubated at 65°C for 15 min. Ten microgram proteins were separated on 10% SDS-PAGE. For caspase-3 activity determination, total homogenate, obtained as previously described, was sonicated in ice and centrifuged at 14,000*g*. The obtained supernatant was diluted in Laemmly's sample buffer, boiled for 5 min and separated on 15% SDS-PAGE.

After blotting, whose completeness was checked by suitable staining, the nitrocellulose membranes were blocked in 5% bovine serum albumin and then probed using anti-poly(ADP-ribose) monoclonal antibody (Trevigen, Inc., MD, USA), C2-10 anti-PARP monoclonal antibody (Oncogene Research Products, MA, USA), anti caspase-3/CPP32 polyclonal antibody (Biosource International, CA, USA) for 2 h. Incubation with the HRP-conjugated secondary antibody and ECL procedure followed. The band densities were quantified as densitometric units/10 μg protein (the constant protein amount applied on SDS-PAGE) using the program for image analysis and densitometry Quantity One (Biorad, 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 pulverization, were added to ice-cold 0.6 M perchloric acid (1:5 wt/vol) and homogenized 3 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 K_2CO_3 and centrifuged again as above indicated. The supernatant was adjusted to pH 7 and filtered through a 0.45 μm filter. The clear obtained solution was used for HPLC analysis which was performed using a 5-μm Supelcosil LC-18 reversed-phase column (25 cm × 4.6 mm i.d.), (Supelco, Belefonte, PA). The chromatographic conditions were as described by Stocchi *et al.*[12] 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.

Detection of DNA Fragmentation by ELISA

Cellular DNA fragmentation was determined using the cell death detection ELISA reagent (Boehringer Mannheim, Germany) following the manufacturer's instructions. The DNA fragmentation was expressed as the enrichment of histone-associated mono- and oligonucleosomes released into the cytoplasm. The enrichment factor (EF) was calculated according to absorption at 405 nm, which represented the enrichment of histone-associated DNA fragmentation and accounted for apoptosis of cardiomyocytes.

Evaluation of Apoptosis

In situ end labeling of nicked DNA (ISEL assay) was performed on paraffin-embedded sections from control and ischemic/reperfused myocardium as previously reported[13] with minor modifications. Briefly, after a treatment with 2 mg/ml proteinase K to remove the excess protein from nuclei, the tissue sections were incubated with the Klenow fragment of DNA polymerase I and biotinylated deoxynucleotides (FRAGEL-Klenow, DNA fragmentation kit, Calbiochem, CA) in a humified chamber at 37°C for 90 min. After the sections were incubated with streptoavidin-peroxidase for 10 min and stained with diaminobenzidine tetrahydrochloride (DAB). Counterstaining was performed with methyl green. Apoptotic nuclei were easily recognized by the presence of a dark brown staining in contrast with that of necrotic and mitotic cells which instead appeared weakly stained. Viable cells appeared

TABLE I Oxidative stress markers in heterotopical transplanted rat heart after 60 min of reperfusion in absence and in presence of 3-AB (10 mg/kg) or 3-ABA (10 mg/kg) administration

	MDA + 4-HNE (nmol/mg protein)	Undamaged DNA (%)	Protein carbonyls (nmol/mg protein)
Control	5.8 ± 0.35	75 ± 5.0	1.81 ± 0.11
Ischemic-reperfused	9.3 ± 0.39*	41 ± 4.7*	4.10 ± 0.53*
Ischemic-reperfused +3-AB	6.8 ± 0.35°	65 ± 6.2°	2.80 ± 0.21°
Ischemic-reperfused +3-ABA	8.7 ± 0.60*	45 ± 5.0*	3.81 ± 0.29*

MDA and 4-HNE were determined by the colorimetric procedure described under Experimental Procedures in the control and transplanted hearts. DNA fragmentation was determined by the alkali unwinding assay. The method described by Levine *et al.* was used to assess protein carbonyl content. Values are means ± S.E.M of determinations performed on 5 transplanted-reperfused hearts and relative controls. *Significantly different *vs* control at the $P < 0.05$ level. $^{\circ}$Significantly different vs ischemic-reperfused heart at the $P < 0.05$ level.

green. The number of ISEL-positive cardiomyocytes were counted in at least five different microscopic fields on each section containing approximately 200 cardiomyocytes.

Statistical Analysis

The results were examined by one-way analysis of variance and individual group means were then compared with Bonferroni's test. A p value less than 0.05 was considered significant.

RESULTS

Lipid Peroxidation, DNA Fragmentation and Protein Carbonyl Content

Lipid peroxidation, DNA damage and protein carbonyl content were measured as markers of oxidative stress. Ischemia/reperfusion-induced lipid peroxidation is characterised by the formation of MDA and 4-HNE, stable and distinctive products of this process.[14] Under our experimental conditions, these lipid peroxidation markers significantly raised in transplanted hearts (60% increase with respect to control values). The treatment with 3-AB reduced MDA and 4-HNE formation to only about 17% over the control values (Table I).

It is well known that ischemia-reperfusion enhances ROS formation[15] contributing to the increase in DNA single strand breaks. In our experimental system, most of the DNA was undamaged (75% ± 5.0) in control hearts; ischemia/reperfusion induced a marked DNA fragmentation, as judged by the rate of strand breaks, assessed with the analysis of DNA unwinding, so that the percent of undamaged DNA was strongly reduced (41% ± 4.7) (Table I). 3-AB treatment decreased the amount of DNA breaks and increased the fraction of undamaged DNA in reperfused hearts (65% ± 6.2).

ROS formation in ischemia/reperfusion can induce the oxidation of cellular proteins which can be characterised by the quantity of protein-bound carbonyl groups.[2] Table I shows that ischemia/reperfusion significantly raised protein carbonyl content (126% over the control value); this change was prevented by 3-AB administration which reduced this increase in carbonyl groups to about 55% over the control values. All the above effects of 3-AB treatment were not reproduced by the administration of its inactive analogue 3-ABA.

PARP Activity and Protein Levels

PARP activity, assessed on the basis of its auto-poly(ADP-ribosylation) level, was determined evaluating the density of a 116 kDa band, in Western blot analysis performed with anti-poly(ADP-ribose) antibodies (Fig. 1). Densitometric analysis of this band showed that PARP activity significantly increased in transplanted hearts, at an extent which, on average, was about doubled with respect to control value. In the presence of 3-AB treatment, PARP activity resulted only 30% over the control value.

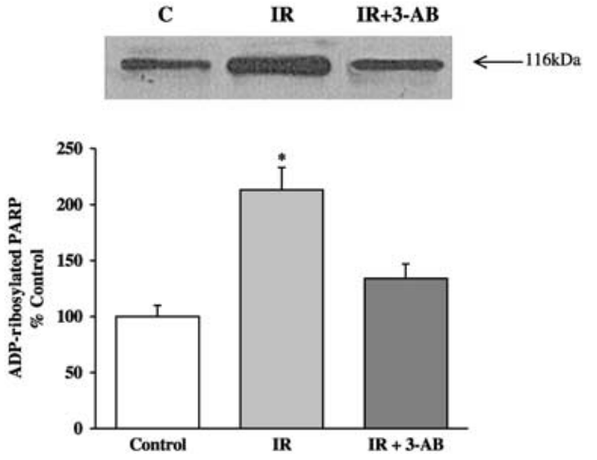

FIGURE 1 Top: representative Western blot showing poly(ADP-ribosylation) (PAR) level of PARP in control (C), untreated (IR) and 3-AB treated (IR + 3-AB) transplanted rat heart. Bottom: quantitative data, obtained by densitometric analysis (Means ± S.E.M) are expressed as percentage of the control value (see Methods for details). *Significantly different ($P < 0.05$) vs. control. $^{\circ}$Significantly different ($P < 0.05$) vs. IR.

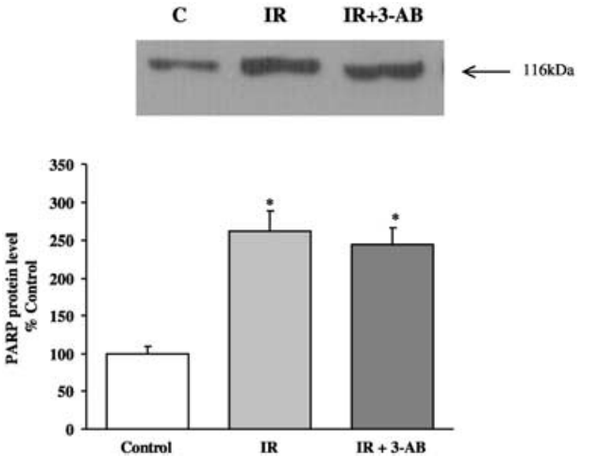

FIGURE 2 Top: representative Western blot showing PARP protein level in control (C), untreated (IR) and 3-AB treated (IR + 3-AB) transplanted rat heart. Bottom: quantitative data, obtained by densitometric analysis (Means ± S.E.M) are expressed as percentage of the control values (see Methods for details). *Significantly different ($P < 0.05$) vs. control. Statistical analysis of data from treated and untreated hearts did not show any significant difference.

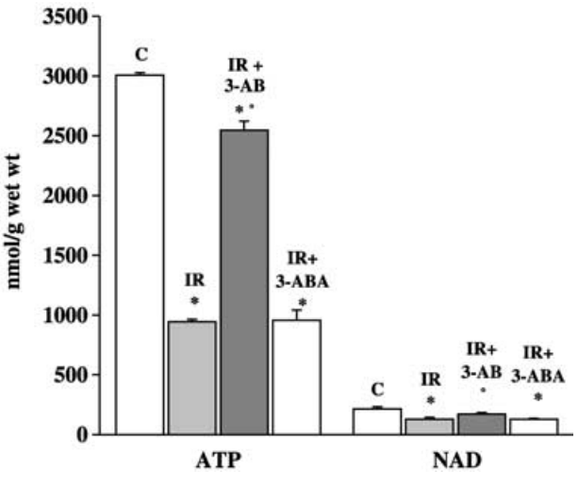

FIGURE 3 ATP and NAD levels in control (C), untreated (IR) and 3-AB (IR + 3-AB) or 3-ABA (IR + 3-ABA) treated transplanted rat heart. Each bar represents the Mean ± S.E.M of determinations performed on 5 transplanted-reperfused hearts and relative controls. *Statistically significant differences vs. control ($P < 0.05$). °Statistically significant differences vs. IR. ($P < 0.05$).

In IR hearts also PARP protein levels, assessed by densitometry of the 116 kDa band detected with the C2-10 antibody, were markedly and significantly increased with respect to control values (Fig. 2). 3-AB slightly reduced PARP expression which however did not significantly differ compared to the untreated hearts.

Concentrations of Adenine Nucleotides and Related Compounds

As shown in Fig. 3, NAD levels (which in our assay conditions included NAD^+ and $NADH + H^+$) decreased significantly in IR heart reaching about 40% of the control value. A similar and more marked reduction was observed for ATP whose mean concentration was about 30% of the control value. Under 3-AB treatment both NAD and ATP levels significantly increased with respect to the untreated hearts being respectively 80 and 85% of the control value. Administration of 3-ABA did not affect the nucleotide concentrations in the IR hearts.

Plasma Levels of cTnI, CK and LDH

After 60 min reperfusion of transplanted hearts, the concentration of cTnI, a highly specific marker of myocardial injury, increased in the plasma of recipient animals reaching a level which was about 4 fold over the control value. Similar increases were found for LDH and CK (Table II). 3-AB treatment strongly reduced the levels of the considered myocardial injury markers which resulted not significantly different from control values. The administration of 3-ABA did not modify the plasmatic concentrations of these enzymes with respect to the levels observed in the untreated recipient animals.

Determination of Caspase-3 Active Fragment

Western blots performed with monoclonal anti-caspase-3 antibodies, and the relative densitometric analyses, revealed that in control and untreated IR hearts the 20 kDa bands corresponding to the active fragment of this enzyme did not significantly differ (Fig. 4). Under 3-AB treatment we found an increase of this band whose densitometric analysis gave a value which was more than doubled compared to control.

Oligonucleosomal DNA Fragmentation

The cell death detection ELISA kit was used to analyze and quantify apoptotic DNA degradation. The anti-histone monoclonal antibodies detect the histone-associated DNA fragmentation (mono- and oligonucleosomes). As shown in Fig. 5 in the 3-AB treated transplanted hearts the EF values raised significantly with respect to control (about 8 fold increase) and also to untreated hearts (about 4 fold increase). 3-ABA treatment hearts did not result in a similar effect.

Detection of Apoptotic Cells

To further characterize apoptosis in ischemic myocardium, we used the ISEL method to detect

TABLE II Plasma concentration of cTnI, CK and LDH in control and IR after 60 min of reperfusion in absence and in presence of 3-AB (10 mg/kg) and 3-ABA (10 mg/kg) administration

	cTnI (ng/l)	CK (U/l)	LDH (U/l)
Control	72 ± 10	258 ± 28	433 ± 44
Ischemic-reperfused heart	310 ± 25*	1099 ± 92*	1623 ± 152*
Ischemic-reperfused heart +3-AB	98 ± 15°	350 ± 42°	612 ± 71°
Ischemic-reperfused heart +3-ABA	293 ± 32*	1207 ± 133*	1598 ± 74*

cTnI, CK and LDH were determined by routine laboratory methods, as described under Experimental Procedures, in the plasma of recipient rats before surgery (control values) and after 60 min of reperfusion. *Significantly different *vs* control at the $P < 0.05$ level. $^{\circ}$Significantly different vs. ischemic-reperfused heart at the $P < 0.05$ level.

cardiomyocytes showing apoptotic DNA fragmentation. As reported in Fig. 6, ISEL-positive cardiomyocytes were practically not detectable in control hearts (0.12 ± 0.08% of total number of cells); ischemia-reperfusion did not significantly raise the number of those cells (1.03 ± 0.25%). In contrast, 3-AB administration induced a marked and significant increase in ISEL-positive cardiomyocytes (21.27 ± 1.83%).

DISCUSSION

As above stated, in the section Introduction, heart transplantation is one of the major clinical circumstances in which oxidative stress, due to the ischemia/reperfusion mechanism, has a relevant role in inducing myocardial damage.[16] In order to define more precisely the role of PARP in this condition and to verify possible benefits resulting from its inhibition, the present paper reports a study about the effects of 3-AB in an experimental model represented by rat heart subjected to heterotopic transplantation after a hypothermic ischemic phase. This model, already used in a previous study from this laboratory, was used as one of the most suitable to simulate the clinical conditions of whole blood reperfusion and has some advantages: heart is completely and uniformly affected; there are little if no differences in the severity of ischemia in the used myocardial samples since the intervention of possible intercoronary anasthomoses is excluded. The chosen time periods for ischemia and reperfusion were similar to those used by other authors in similar heart transplantation procedures on small-sized animals.[17,18] To ensure that the observed effects of 3-AB were specific to PARP inhibition, parallel determinations were performed with a chemical analogue of this compound, notably 3-aminobenzoic acid (3-ABA), devoid of inhibitory effect on PARP activity.

The results of the present study clearly indicate that in our experimental conditions, and in absence of 3-AB treatment, oxidative stress is an early event in the reperfusion of transplanted heart, as indicated by the significant increase in all the considered markers of this condition: lipid peroxidation pro-

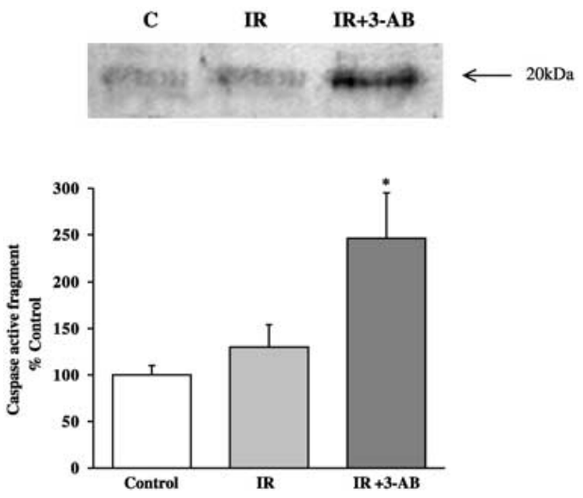

FIGURE 4 Top: representative Western blot showing the active caspase-3 level in control (C), untreated (IR) and 3-AB treated (IR + 3-AB) transplanted rat heart. Bottom: quantitative data, obtained by densitometric analysis (Means ± S.E.M) are expressed as percentage of the control value (see Methods for details).*Significantly different ($P < 0.05$) vs. control.

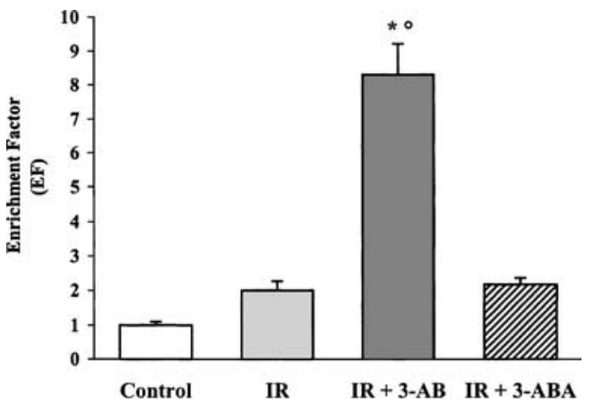

FIGURE 5 Enrichment factor (EF) of apoptotic DNA fragmentation in control (C), untreated (IR) and 3-AB (or 3-ABA) treated (IR + 3-AB, IR + 3-ABA) transplanted rat heart.

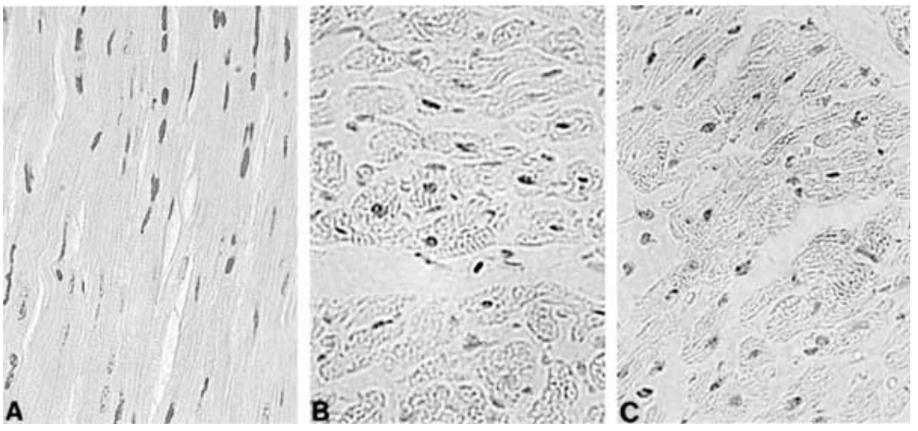

FIGURE 6 Detection of apoptotic cells by in situ end labeling (ISEL) assay performed in transplanted rat heart. (A) Representative light microscopic micrograph of control myocardium, no cardiomyocyte stain is positive for apoptotic nuclear fragmentation. (B) Light microscopic micrograph of 3-AB treated ischemic-reperfused rat heart, note the presence of cardiomyocytes exhibiting dark brown-stained ISEL positive nuclei. (C) Light microscopic micrograph of ischemic-reperfused rat heart, note that a few scattered ISEL-positive nuclei are evident.

ducts, protein carbonyl content and DNA damage. Along with these changes we also found a significant enhancement of PARP activity, in agreement with the view that this enzyme is activated by DNA structural alterations, namely by strand breaks, induced by oxidative stress. In this connection, it is current opinion that PARP is a constitutive enzyme, so its activation is thought to reflect an increased catalytic power rather than a rise in protein expression;[19] nevertheless, we found that not only activity but also PARP protein concentration was higher in the IR hearts compared to the control hearts. In any case, enhanced PARP activity was associated to a significant decrease in the levels of NAD and, especially, of ATP whose concentration in IR hearts was less than one third with respect to the control value. These findings, indicative of a marked energy depletion, suggested a severe cardiomyocyte suffering, a conclusion that was consistent with other data emerging from the present study: the conspicuous leakage of typical myocardial injury markers and the microscopic (TEM) observations that pointed out several morphologic alterations in IR cardiomyocytes, some of which (especially nuclear clearings with highly dispersed chromatin and plasma membrane discontinuities) are indicative for the establishment of necrotic processes[7].

A rather different picture was observed when heart heterotopic transplantation was performed in 3-AB treated animals. In the first place we found that under 3-AB treatment the transplanted hearts did not undergo increases in oxidative stress markers, whose levels, on the contrary, did not significantly differ from the control values. Since there is no evidence that 3-AB can function as free radical scavenger,[20] other mechanisms must be invoked to explain the attenuation of oxidative stress induced by this drug. In this connection, it has been suggested that activated PARP can trigger polymorphonuclear leukocytes (PMNs) recruitment and activation in the IR tissues, hence a vicious circle leading to an enhancement of oxidative stress.[21,22] Thus, 3-AB, by inhibiting PARP activity, could also prevent PMNs infiltration and the related ROS production. Consistently with this view, when 3-ABA was administered instead of 3-AB oxidative markers levels in transplanted heart were similar to those observed in the absence of pharmacological treatment.

Under 3-AB treatment PARP activation was markedly attenuated in transplanted hearts, an effect which matched a parallel reduction in energy depletion, as indicated by the levels of ATP whose concentrations were more than doubled with respect to those observed in untreated transplanted hearts. Compared to the results obtained with the untreated transplanted hearts, also the morphological cardiomyocyte alterations and the release of cardiac damage markers were significantly attenuated by 3-AB treatment. None of these effects was observed when the inactive analogue 3-ABA was administered instead of 3-AB; this clearly indicates that a cardioprotective action of 3-AB which can manifest itself at various level, but in any case depends from its ability to inhibit PARP activity.

A novel finding that emerges from the present study regards the possibility that PARP inhibition changes the mode of cell death of irreversibly damaged IR cardiomyocytes. It is still matter of debate whether the reperfusion of ischemic myocardium induce prevalently necrotic or apoptotic cell

death.[23,24] However, these two processes, although quite different in their morphological and biochemical aspects, may often coexist in many pathological conditions.[25] In this connection, several reports suggest that cell death may be necrotic or apoptotic according to the rate of energy depletion;[26,27] apoptosis, in fact, requires an energy supply and a severe lack of ATP may prevent the completion of the apoptotic program. In this study, we found that transplanted hearts exhibited typical signs of apoptosis only under 3-AB treatment. Apoptosis was assessed on the basis of ISEL positive cardiomyocyte nuclei and confirmed by the presence of two specific hallmark of this process: caspase-3 activation and oligonucleosomal DNA fragmentation. It is well known that, in addition to these findings, apoptosis is characterised by a particular mode of PARP cleavage. In our 3-AB treated hearts we never detected any increase in the typical 85 kDa cleavage product, but we clearly observed a decrease in the 116 kDa band—corresponding to the entire protein—which in the untreated transplanted hearts was significantly increased with respect to controls. Thus we suppose that the lack of the typical apoptotic PARP fragment may be ascribed to additional pathways activated during reperfusion and resulting in further degradation of the 85 kDa cleavage product. In our knowledge these results represent the first evidence about the possibility that apoptosis is triggered by PARP inhibition in transplanted heart. Taken together the present findings indicate that 3-AB administration has an overall beneficial effect against the myocardial injury that may occur in heart transplantation, when a more or less extended time of ischemia is followed by reperfusion. The protective effect of 3-AB appears to be specifically linked to its ability in preventing PARP activation and the consequent energy depletion. In our opinion also the appearance of apoptotic hallmarks under 3-AB treatment may fit in with this picture. In fact, the maintenance of sufficient energy could allow irreversible cardiomyocyte damages, triggered during the ischemic phase, to proceed towards apoptosis instead of necrosis, as it appears to happen when energy stores are depleted by high PARP activity. Considering that apoptosis, besides being less damaging for the affected tissue owing to the absence of inflammatory reaction, is a potential preventable form of cell death, also this action of 3-AB may be looked as a beneficial effect. In any case, this work represents an initial approach aimed to assess the usefulness of PARP inhibition for the prevention of IR injury in the context of cardiac transplantation, a field that till now has been poorly investigated. Further studies with the present experimental model would be of interest to assess the severity and the type of heart damage according to the duration of both ischemia and reperfusion, as well as to optimize the treatment with 3-AB or other PARP inhibitors.

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