

Research Article

Protective effects of the PARP-1 inhibitor PJ34 in hypoxic-reoxygenated cardiomyoblasts

C. Fiorillo^{a,*}, V. Ponziani^a, L. Giannini^a, C. Cecchi^a, A. Celli^a, N. Nassi^b, L. Lanzilao^a, R. Caporale^c and P. Nassi^a

^aDipartimento di Scienze Biochimiche, Università di Firenze, Viale Morgagni 50, 50134, Firenze (Italy),
Fax: +39 055 4598905, e-mail: claudia.fiorillo@unifi.it

^bDipartimento di Pediatria, Università di Firenze, Via L. Giordano 13, 50132, Firenze (Italy)

^cLaboratorio Centrale, Azienda Ospedaliera Universitaria Careggi, Viale Morgagni 85, 50134, Firenze (Italy)

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Abstract. To clarify the role of poly(ADP-ribose)polymerase-1 (PARP-1) in myocardial ischemia-reperfusion injury, we explored some effects of PJ34, a highly specific inhibitor of this enzyme, in hypoxic-reoxygenated (HR) H9c2 cardiomyoblasts. Compared to the control, HR cells showed signs of oxidative stress, marked PARP-1 activation, NAD⁺ and ATP depletion and impaired mitochondrial activity. HR cardiomyoblasts were affected by both necrosis and apoptosis, the latter involving the nuclear translocation of apoptosis-inducing factor. In HR cardio-

myoblasts treated with PJ34, oxidative stress and PARP-1 activity were decreased, and NAD⁺ and ATP depletion, as well as mitochondrial impairment, were attenuated. Above all, PJ34 treatment improved the survival of HR cells; not only was necrosis significantly diminished, but apoptosis was also reduced and shifted from a caspase-independent to a caspase-dependent pathway. These results suggest that PARP-1 modulation by a selective inhibitor such as PJ34 may represent a promising approach to limit myocardial damage due to post-ischemic reperfusion.

Keywords. Cardiomyoblasts, PARP, hypoxia-reoxygenation, apoptosis, necrosis.

Introduction

Reactive oxygen species (ROS) are believed to play a key role in the myocardial ischemia-reperfusion (IR) injury. In this condition, ROS production, which may initiate during the ischemic phase but is exacerbated by reperfusion, often overcomes the antioxidant defenses and induces oxidative stress [1]. Similar effects are observed in hypoxic-reoxygenated (HR) cells and these may, therefore, represent a suitable experimental model for studies about the cardiac damages due to post-ischemic reperfusion [2, 3].

Myocardial cell death in IR is mediated mainly by necrosis [4], but recent evidence suggests that cardiomyocyte apoptosis could also play a significant role in these condi-

tions [5]. A mechanism that is involved in both myocyte necrosis and apoptosis induced by ischemia-reperfusion is based on the oxidative stress-induced activation of the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1), whose activity consists in the transfer of ADP-ribose units from oxidized nicotinamide dinucleotide (NAD⁺) to nuclear proteins [6, 7].

The covalent modification of proteins by poly(ADP-ribosyl)ation is an immediate and dramatic biochemical response to DNA damage induced by oxidation and other agents such as alkylation, and ionizing radiation. Binding to damaged DNA potentially activates the enzymatic activity of PARP-1, which can, therefore, function as a DNA damage sensor. PARP-1 has been implicated in multiple DNA repair pathways, including those involved in the repair of single-strand breaks (SSB), double-strand breaks (DSB), and base excision (BE) [8]. As expected, PARP-1

* Corresponding author.

physically and functionally interacts with various proteins involved in these pathways and may recruit the repair proteins to the sites of DNA damage (*e.g.* XRCC-1 in BE, DNA-dependent protein kinase in DSB repair) [9].

It is generally accepted that PARP-1 activation results in NAD⁺ and ATP depletion; hence, cell dysfunction and cell death occur mainly via the necrotic route. However, in addition to this well-established mechanism, recent studies conducted on various cell types have suggested a potential role of PARP-1 in apoptosis through the translocation of apoptosis-inducing factor (AIF), from the mitochondria to the nucleus [10].

Based on this background, the aim of the present study was to investigate if the rate of PARP-1 activation could affect the extent and the mode of death of hypoxic (H) and HR cardiac cells. Necrosis and apoptosis were evaluated in H and HR cardiomyoblasts in the absence and in the presence of PJ34, a potent and specific PARP-1 inhibitor.

Materials and methods

Chemicals and reagents. All chemicals were of reagent grade and were obtained from Sigma (Milan, Italy) unless otherwise indicated; PJ34 was from Calbiochem (La Jolla, CA, USA).

Cell cultures. Rat heart myoblasts [line H9c2 (2-1), no. 88092904; European Collection of Cell Cultures, ECACC] were plated at a density of 5×10^5 /100-mm plate and cultured at 37 °C in a 5% CO₂ humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin, 1% streptomycin and 1% L-glutamine. Cells were passaged regularly and subcultured to ~90% confluence before experimental procedures.

Hypoxia/reoxygenation treatment protocol. Hypoxia was achieved using a modular incubator chamber (Bil-lups-Rothenberg Inc., CA, USA) gassed with 95% N₂, 5% CO₂. A flow meter was used to measure the quantity of gas mixture introduced into the chamber (25 l/min) according to Namiki et al. [11]. After incubating in hypoxic conditions for 6 h, the cells were reoxygenated by immediate replacement of fresh culture medium and were returned to a normoxic environment at 37 °C for 120 min. Cell treatment was started at the moment of reoxygenation by adding PJ34 (0.5 µM final concentration) [12]. Normoxic control H9c2 cells with and without PJ34 treatment were cultured concomitantly to check possible toxic effects of this compound. No significant differences in lactate dehydrogenase (LDH) release or in cell viability was observed when comparing the untreated with the treated cells (data not shown).

Preparation of cell homogenates and isolation of nuclear, cytosolic and mitochondrial fractions. H9c2 cells (1×10^6) were washed twice with phosphate buffered saline (pBS), trypsinized, collected by centrifugation and then resuspended in 120 µl lysis buffer [1% Triton X-100, 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA and 6 M urea supplemented with 0.2 mM PMSF, as well as 10 µg/ml leupeptin and aprotinin]. Except for particular assays to obtain cell homogenate (see below), samples, after three freeze-thaw cycles, were sonicated twice in ice for 5 s and centrifuged at 14 000 g for 10 min at 4 °C. The supernatant was then collected. Nuclear, cytosolic and mitochondrial fractions were achieved using a cytosol/mitochondria fractionation kit according to the manufacturer's instructions (Oncogene Research Products, San Diego, CA, USA). The protein concentration was determined in total homogenates and in subcellular fractions according to the BCA assay [13] with BSA as standard.

Assays for oxidative-stress markers: Lipid peroxidation products, protein carbonyls, 8-hydroxy-2'-deoxyguanosine, total antioxidant capacity. These determinations were all performed using total homogenate samples. Aliquots of 200 µl were used to assess the rate of lipid peroxidation by measuring malonaldehyde (MDA) and 4-hydroxyalkenals (4-HNE) using a colorimetric method (LPO-586, Oxis International, Inc., OR, USA) based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA or 4-HNE at 45 °C [14].

The protein carbonyl content was determined according to the method of Levine et al. [15] after the removal of nucleic acids from the samples. Spectrophotometric measurement was performed at 375 nm considering $22\,000\text{ M}^{-1}\text{ cm}^{-1}$ as the molar absorption coefficient.

The oxidative DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) was measured after DNA extraction (Quantum Prep AquaPure Genomic DNA kit; Bio-Rad, Rome, Italy) and digestion (DNase I, Sigma, Milano, Italy) with a competitive enzyme-linked immunosorbent assay (8-OHdG Check Assay by JAICA, Shizuoka, Japan). The absorbance of the chromogenic product was measured at 450 nm and the results were calculated from a standard curve based on a 8-OHdG solution.

Total antioxidant capacity (TAC) was determined by a competition-based chemiluminescent assay (ABEL Antioxidant Test Kit, Knight Scientific Limited, Plymouth, UK) using the photoprotein Pholasin. The results were calculated from a standard curve based on the soluble antioxidant L-ascorbic acid.

PARP-1 activity measurement. PARP-1 activity was assessed on nuclear extracts (5 µg protein) by an immunodot blot for poly(ADP-ribosylated) proteins according to Affar et al. [16] using the highly specific LP 98-10

anti-poly(ADP-ribose) antibody (Alexis Biochemicals, Lausen, Switzerland). Image analysis of the dot blot was performed with Quantity One software (Bio-Rad Laboratories, Milan, Italy).

Another approach that was used in the present study to quantify PARP activity comprised an enzymatic assay (Universal Colorimetric PARP assay kit, Trevigen Inc, Helgerman Ct., Gaithersburg, MD, USA). This method measures the incorporation of biotinylated poly(ADP-ribose) onto histone proteins in cell homogenates according to manufacturer instructions. The amount of PARP-1 activity, assayed on cellular samples (50 µg) was determined by comparison to a standard curve obtained with an high specific activity (HSA) PARP [13 793 U/mg: 1 U is defined as the amount of PARP that incorporates 100 pmol poly (ADP-ribose) from NAD into acid-insoluble form in 1 min at 22 °C].

Intracellular ROS measurement. This was performed using the ROS-sensitive fluorescent probe dichlorodihydrofluorescein diacetate (DCFH-DA; final concentration 10 µM) as described previously [17]. Fluorescence was monitored on a Perkin Elmer LS55 Spectrofluorimeter with λ_{ex} 488 nm and λ_{em} 520 nm. The results were expressed as percent of DCF fluorescence relative to control cells.

MTT reduction and trypan blue exclusion cell viability assays. 3-(4,5-Dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction ability was determined as an index of the metabolic viability, notably of the mitochondrial function of our cardiomyoblasts using the method of Denizot [18]. The absorbance of solubilized MTT formazan products was measured at 570 nm and the results were expressed as percentages, assuming the absorbance of control cells as 100%.

For trypan blue staining, control, H and HR cells were gently harvested, and mixed with 0.4% trypan blue solution (Sigma). The resulting cell suspension was counted with a hemocytometer; non-viable cells are stained and viable cells exclude the stain. The percentage of viable cells in the H and HR groups was determined from cell counts in H and HR groups divided by cell counts of controls. Thus, a reduction in the number of viable cells reflects decreased cell viability.

Determination of intracellular NAD⁺ and ATP levels. Cellular NAD⁺ levels were measured using a method based on its conversion to NADH by alcohol dehydrogenase [19]. Cells were homogenized in 0.05 M potassium phosphate buffer containing 0.1 M nicotinamide (pH 6.0), frozen rapidly, placed in a boiling water bath for 5 min, then cooled in an ice bath for 5 min. Samples were centrifuged for 10 min at 100 g at 4 °C. The supernatant was then added to the reaction mixture containing

glycylglycine buffer (0.065 M glycylglycine, 0.1 M nicotinamide, 0.5 M ethanol, pH 7.4), alcohol dehydrogenase, MTT and phenazine methosulfate (PMS).

ATP measurement was performed by a highly sensitive bioluminescence assay (HS II, Roche Diagnostics, Mannheim, Germany), which is based on the ATP dependency of the light emitting oxidation of luciferin catalyzed by luciferase [20]. ATP measurement was achieved using a luminometer Lumat LB 9507 (EG & G Berthold). Both NAD⁺ and ATP levels were normalized to total protein content.

LDH measurement. LDH activity was assessed spectrophotometrically in the culture medium and in adherent cells (to obtain total LDH content) using the LDH assay kit (Roche Diagnostics). LDH release was calculated as a percentage of total LDH content.

DNA fragmentation analysis. DNA fragmentation, accounting for cell apoptosis, was determined using an immunometric method (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics) according to the manufacturer's instructions. DNA fragmentation was expressed as the enrichment of histone-associated mono- and oligonucleosomes released into the cytoplasm by measuring the absorption at 405 nm. The enrichment factor is considered to be proportional to the number of apoptotic cells present in the population. DNA fragmentation was also determined in the presence of the caspase inhibitor Z-VAD-fmk (100 µM) to assess the caspase dependence of this process.

Annexin V-FITC and PI labeling. FITC-conjugated Annexin V and PI labeling (Bender MedSystems, Vienna, Austria) were used to detect the externalization of phosphatidylserine during the apoptotic progression in cells exposed to hypoxia-reoxygenation [21]. After detachment, cells were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a density of 5×10^5 cells/ml. Then, 25 µl/ml annexin V-FITC and 50 µl/ml PI were added. Within 30 min the labeled cells were determined using a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy).

PARP-1, caspase-3, cytochrome c and AIF assessment by Western blot. To assess the protein levels of PARP-1 (cleaved form) equal amounts of nuclear fraction (40 µg) were diluted in Laemmli sample buffer and boiled at 65 °C for 5 min. Proteins were separated on 10% SDS-PAGE and were transferred to PVDF Hybond membrane (Millipore Corporation, Billerica, MA, USA). The membrane was then incubated overnight at 4 °C with the highly specific (mouse) C₂₋₁₀ anti-PARP-1 monoclonal antibody (Alexis Biochemicals). Equal protein amounts of total homogenate (40 µg for caspase-3), of cytosolic fractions (45 µg for cytochrome c) and of nuclear fractions (40 µg

for AIF) were diluted in Laemmli sample buffer, boiled for 5 min and separated by SDS-PAGE on 15% gels. Proteins were transferred to PVDF Hybond membranes. The membranes were then incubated overnight at 4 °C with (rabbit) anti-caspase-3/CPP32 antibody (Biosource International, CA, USA), (mouse) anti-cytochrome c (Oncogene Research Products), and (rabbit) anti-AIF (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). After washing, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. The immunolabeled bands were then detected using a SuperSignal West Dura (Pierce, Rockford, IL, USA) and quantified using the above-mentioned software for image analysis. Results were expressed as ratios between the densitometry of the protein of interest and the densitometry of the loading control [β -tubulin (for homogenate and cytosol) and histone H1 (for nuclei)].

Statistical analysis. All data are expressed as mean \pm SEM. Comparisons between the different groups were performed by ANOVA followed by Bonferroni t-test. A p value of <0.05 was accepted as statistically significant.

Results

ROS production and oxidative-stress markers in HR cells. Compared to the normoxic cells our HR cardiomyoblasts exhibited a significant increase in ROS cellular levels. As indicated by the DCFH-DA fluorescence, this increase started at 5 min of reoxygenation and was prolonged until 90 min; at 120 min control levels were restored (Fig. 1). After 90 min of reoxygenation, the concentrations of MDA and 4-HNE (typical stable end products of the lipoperoxidative process), of protein carbonyls and of 8-OHdG also significantly increased, relative to the control value, in HR cells (Table 1). At this time, in correspondence with the presence of high levels of oxidative-stress markers, the total antioxidant capacity, assayed as an index of antioxidant defenses, was significantly reduced. Hypoxia alone did not induce significant changes in ROS production, nor in the levels of oxidative-stress markers and of total antioxidant capacity with respect to control cells.

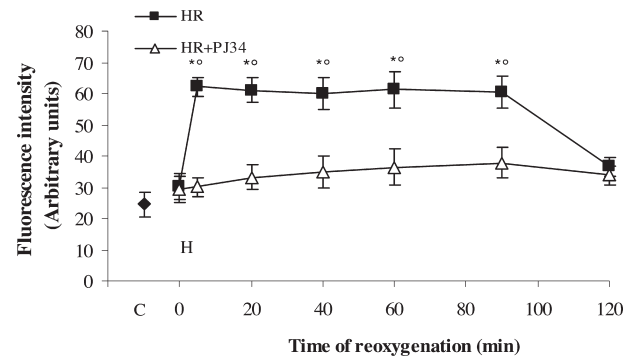


Figure 1. Time course of reactive oxygen species (ROS) generation in control (C), hypoxic (H), hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. ROS production was measured at λ ex 488 nm and λ em 520 nm using the ROS-sensitive fluorescent probe DCFH-DA (final concentration 10 μ M). Each value represents the mean \pm SEM of five different measures. *Significant difference ($p < 0.05$) from control, and ° significant difference ($p < 0.05$) from HR + PJ34.

Although insufficient to completely restore the conditions observed in the normoxic control cells, PJ34 treatment produced significant changes in all the above parameters (Table 1). Compared to the corresponding untreated cells, HR cardiomyoblasts treated with PJ34 exhibited a marked decrease in ROS production (Fig. 1) that was associated to reduced levels of all the considered oxidative-stress markers and to a higher total antioxidant capacity.

PARP-1 activity, intracellular levels of NAD⁺ and ATP, and MTT reduction assay. PARP-1 activity, which is triggered by DNA strand breaks, and may therefore represent an indirect marker of free radical-induced genomic damage, was enhanced by hypoxia-reoxygenation. A significant increase (about 80% above the control value) in the poly(ADP-ribosylation) of nuclear proteins was observed after 90 min of reoxygenation (Fig. 2), parallel with the other findings that indicate the development of oxidative stress. In the enzymatic assay for PARP activity (Table 2) HR cells showed a threefold increase with respect to controls. PARP-1 activation was associated to a marked and significant reduction of mitochondrial activity as judged by the MTT reduction test (Fig. 3A) with

Table 1. Oxidative-stress markers^a.

| | MDA+4-HNE pmol/mg protein | Protein carbonyls nmol/mg protein | Total antioxidant capacity pmol/mg protein | 8-OHdG ng/ml |
|---------|------------------------------|--------------------------------------|---|-------------------|
| Control | 0.42 \pm 0.04 | 3.62 \pm 0.13 | 112.4 \pm 4.21 | 1.14 \pm 0.14 |
| Hypoxia | 0.47 \pm 0.03 | 3.81 \pm 0.17 | 98.3 \pm 4.70 | 1.15 \pm 0.18 |
| HR | 0.98 \pm 0.07** | 7.9 \pm 0.25** | 65.12 \pm 5.41** | 3.32 \pm 0.17** |
| HR+PJ34 | 0.62 \pm 0.03*† | 5.15 \pm 0.14**† | 95.71 \pm 3.51† | 2.12 \pm 0.15*† |

^a Oxidative-stress markers levels in control, hypoxic, hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. Values represent mean \pm SEM of five independent experiments. ** $p < 0.001$ vs C; * $p < 0.05$ vs C; † $p < 0.001$ vs HR

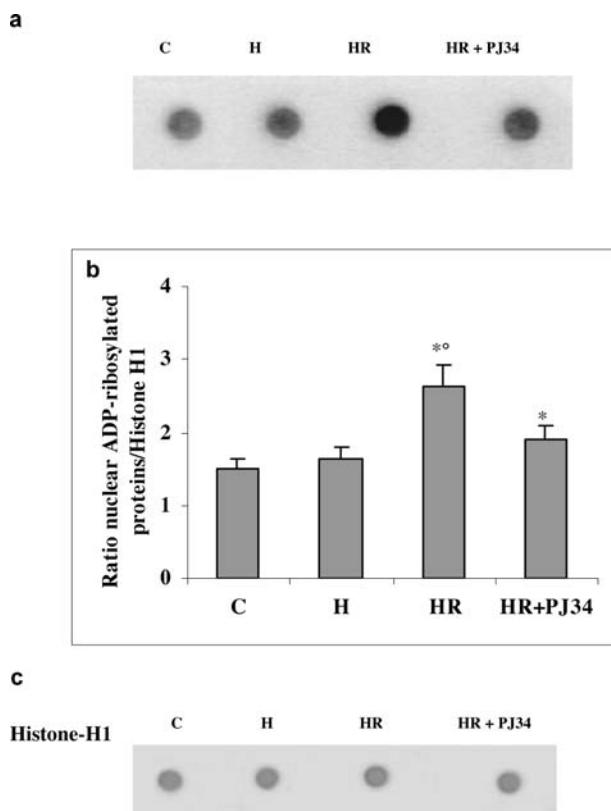


Figure 2. Immunodot blot of ADP-ribosylation levels of PARP nuclear substrates. (a) Representative dot blot showing poly(ADP-ribosylation) (pAR) level of nuclear proteins in control (C), hypoxic (H) and hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. (b) quantitative data. Each bar represents the mean \pm SEM of five different dot blots and relative densitometric quantifications. All signals were quantified by densitometric analysis, and are expressed as ratio of nuclear ADP-ribosylated proteins densitometry on histone H1 (loading control) densitometry. (c) Dot blot analysis of histone H1: a control for equal protein loading. *Significant difference ($p < 0.05$) from control, and °significant difference ($p < 0.05$) from HR + PJ34.

Table 2. PARP activity^a.

| | C | H | HR | HR+PJ34 |
|--------------------------|--------------|----------------|--------------------|----------------|
| Units of PARP/mg protein | 4 \pm 0.41 | 4.7 \pm 0.45 | 10.9 \pm 1.93**° | 4.9 \pm 0.50 |

^a PARP activity in control (C), hypoxic (H), hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. Unit definition: 1 U PARP incorporates 100 pmol poly(ADP) from NAD⁺ into acid-insoluble form in 1 min at 22 °C. Values represent mean \pm SEM of five independent experiments. **Significant difference ($p < 0.001$) from control and °significant difference ($p < 0.001$) from HR + PJ34.

respect to the control level and to a concomitant depletion of intracellular ATP and NAD⁺ pools (Fig. 3B, C).

In HR cardiomyoblasts treated with PJ34 PARP-1 activity (assessed by dot blot analysis and by enzymatic as-

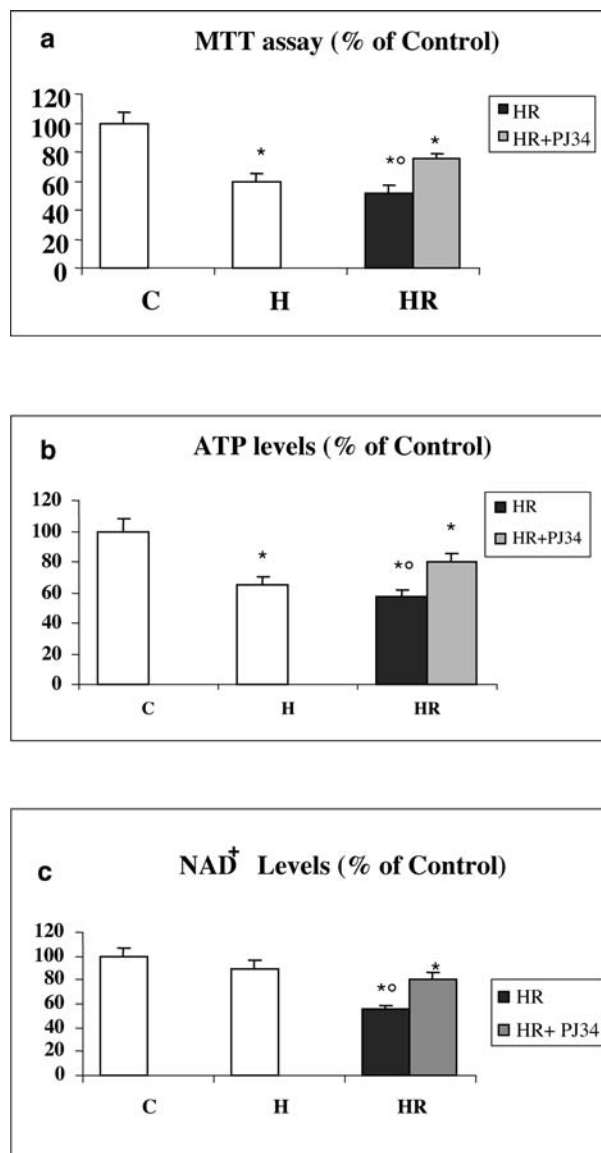


Figure 3. (a) Mitochondrial activity, assessed by MTT test, in control (C), hypoxic (H), hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. Results were expressed as the percentage of MTT reduction, assuming the absorbance of control cells as 100%. (b) ATP levels determined by a bioluminescence assay in control (C), hypoxic (H), hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. Results were expressed as a percentage of control values (assumed as 100%). (c) NAD⁺ levels assayed by a method based on its conversion to NADH by alcohol dehydrogenase in control (C), hypoxic (H), hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. Results were expressed as a percentage of control values (assumed as 100%). All values represent mean \pm SEM of five independent experiments. *Significant difference ($p < 0.05$) from control and °significant difference ($p < 0.05$) from HR + PJ34.

say) was significantly lower than in the corresponding untreated cells, resulting only about 25% over the control level. Correspondingly, the impairment of mitochondrial activity (Fig. 3A) was significantly attenuated, as was the cellular depletion of both ATP and NAD⁺ (Fig. 3B, C).

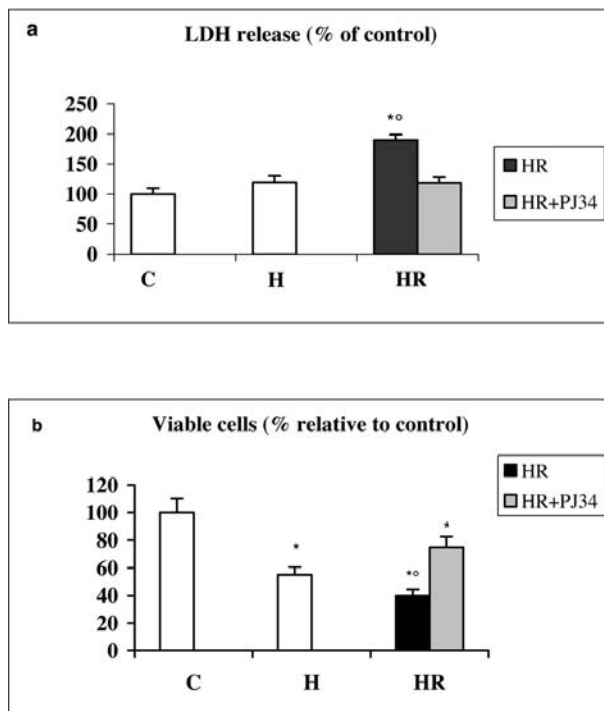


Figure 4 (a) LDH activity, as an index of necrotic death, released into the medium in control (C), hypoxic (H), untreated and PJ34-treated hypoxic-reoxygenated (HR) cardiomyoblasts. LDH activity was assessed in the culture medium and in adherent cells (to obtain total LDH content) after hypoxia-reoxygenation and in the control cells. LDH release was calculated as a percentage of total LDH content. (b) Trypan blue exclusion assay. The cellular suspension treated with trypan blue solution was counted with a hemocytometer where non-viable cells are stained and viable cells exclude the stain. The percentage of viable cells in the different groups was determined from cell counts in H or HR cells divided by cell counts of controls. Values represent mean \pm SEM of five independent experiments. * Significant difference ($p < 0.05$) from control, and $^{\circ}$ significant difference ($p < 0.05$) from HR + PJ34.

Effects on cellular viability, necrosis and apoptosis. Reoxygenation for 90 min of hypoxic H9c2 cardiomyoblasts markedly and significantly increased (about twofold over the control level) the LDH release in the culture medium (Fig. 4A), which is considered to account for cell mortality and, especially, for necrotic cell death. These results are consistent with those obtained by the commonly used cell viability assay based on trypan blue exclusion (Fig. 4B). Under these conditions, however, we also found a significant enhancement of oligonucleosomal DNA fragmentation, a finding that is generally accepted as an hallmark of apoptosis (Fig. 5). To assess the caspase dependence of this DNA fragmentation pattern, HR cells were treated with the caspase inhibitor Z-VAD-fmk: this treatment did not produce significant changes, so that DNA fragmentation may be considered, at least in these conditions, a caspase-independent process. Consistent with these findings, our determinations revealed that, in HR cells, cytochrome c (an activator of the caspase pathway) was significantly

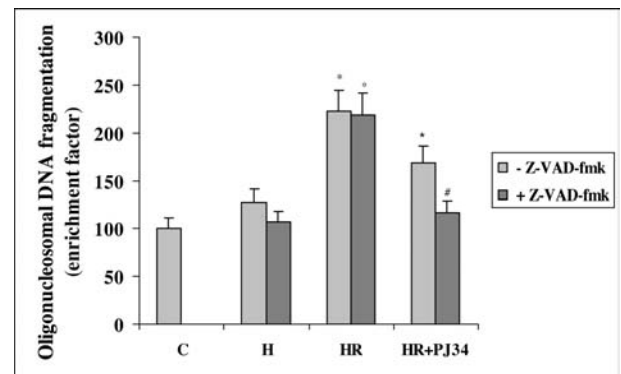


Figure 5. DNA fragmentation, as an index of apoptotic death, in control (C), hypoxic (H), hypoxic-reoxygenated (HR) cardiomyoblasts with and without PJ34. DNA fragmentation was expressed as the enrichment of histone-associated mono- and oligonucleosomes released into the cytoplasm by measuring the absorption at 405 nm. The enrichment factor is considered to be proportional to the number of apoptotic cells present in the population. Each value is the mean \pm SEM of five independent experiments. * Significant difference ($p < 0.05$) from control, $^{\circ}$ significant difference ($p < 0.05$) from HR + PJ34 and $^{\#}$ significant difference ($p < 0.05$) from HR + PJ34 without Z-VAD-fmk.

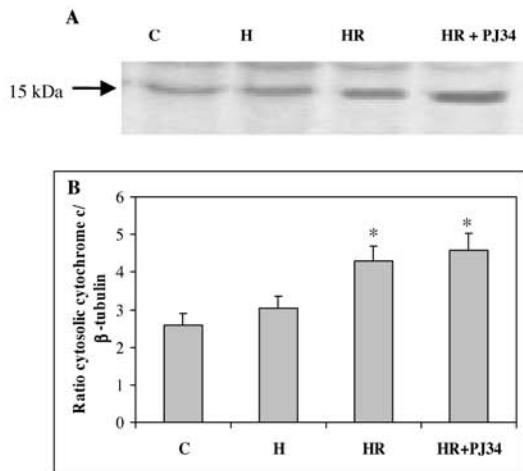
released into the cytosol (Fig. 6a), but the levels of both the catalytically active form of caspase-3 (Fig. 6b), a downstream effector of caspase-dependent apoptosis, and those of the typical fragment of PARP-1 (Fig. 7a), produced through the proteolytic cleavage of this enzyme by caspase-3, did not significantly differ from the control values. Thus, it appears unlikely that the cytochrome c/caspase pathway was involved in the apoptosis of our HR cardiomyoblasts. These cells, on the other hand, exhibited a significant translocation of AIF (Fig. 7b), as indicated by the strong nuclear signal for this factor. PJ34 treatment of HR cardiomyoblasts resulted in several considerable changes with respect to the corresponding untreated cells: LDH release was drastically decreased and apoptosis also appeared to be significantly reduced, as indicated by the differences observed in oligonucleosomal DNA fragmentation. Moreover, as for the mechanisms potentially involved in the apoptotic processes occurring in these conditions, we found that AIF nuclear translocation was nearly absent, while cytochrome c release into the cytosol, caspase-3 activation, and PARP-1 cleavage were significantly higher in the PJ34-treated, rather than in the untreated, HR cells. DNA fragmentation observed in the PJ34-treated HR cardiomyoblasts was significantly reduced by Z-VAD-fmk treatment, which suggests a caspase dependence for this process.

To characterize more deeply the rate and the mode of cell death during hypoxia-reoxygenation, and the effects of PJ34 in this connection, apoptotic, necrotic and living cells were differentiated and quantified by flow cytometry (annexin/PI staining). The results of these determinations (Fig. 8) indicate that, after hypoxia-reoxygenation,

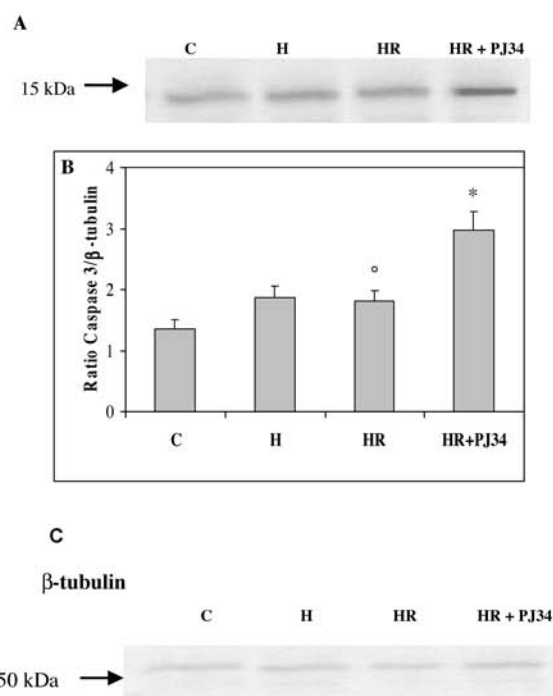
the living cells were significantly reduced with respect to the control level and that both necrosis and apoptosis contributed, almost in the same measure, to this effect. Compared with the corresponding untreated cells, HR

cardiomyoblasts subjected to PJ34 treatment exhibited a reduction in apoptotic and, even more, in the necrotic processes, an effect that may account for the concomitant increase that we found in the number of living cells.

a



b



Discussion

A first consideration that is suggested by the results of the present study regards the effectiveness of our hypoxia/re-oxygenation treatment to enhance ROS production and to induce oxidative stress, the occurrence of which was indicated by typical biochemical markers. As for PARP-1 activity, the low levels detectable in control cells suggest, in agreement with previous reports [22], that a baseline oxidative DNA damage is occurring within cell nuclei. Poly(ADP-ribosyl)ation of nuclear proteins and PARP-1 activity were unchanged during the hypoxic phase but underwent a marked increase after 90 min reoxygenation, consistent with the view that PARP-1 is primarily involved in the reperfusion injury. The discrepancy between the increase in PARP-1 activity (about threefold with respect to controls value) and that observed for the poly-(ADP ribosyl)ation level of nuclear protein (about 80% above the control value) may be due to the fact that the latter represents the result of two enzymatic activities: PARP-1 and poly(ADP-ribose) glycohydrolase (PARG), an enzyme that can act with high efficiency poly(ADP-ribose) immediately upon the initiation of its synthesis; furthermore, PARG has a higher specific activity than PARP-1, and its catalytic activity rises with the increasing length of poly(ADP-ribose) chain [23]. To see whether the degree of PARP-1 activation affected the rate and/or the mode of death in HR cardiomyoblasts, our determinations were performed in the absence and in the presence of PJ34. This belongs to a class of recently synthesized bicyclic and tricyclic PARP-1 inhibitors that have proved to be much more selective and potent in comparison with

Figure 6. (a) Western blot analysis of cytosolic cytochrome c in control (C), hypoxic (H) and hypoxic-reoxygenated (HR) cells. (A) Representative Western blot; (B) quantitative data. All signals were quantified by densitometric analysis and are expressed as ratio of cytochrome c densitometry on β -tubulin (loading control) densitometry. Each bar represents the mean \pm SEM of five different blots and relative densitometric quantifications. (C) Western blot analysis of β -tubulin: a control for equal protein loading. (b) Western blot analysis of caspase-3 active fragment in control (C), hypoxic (H) and hypoxic-reoxygenated (HR) cells. (A) representative immunoblot. (B) quantitative data. All signals were quantified by densitometric analysis and are expressed as ratio of caspase-3 active fragment densitometry on β -tubulin (loading control) densitometry. Each bar represents the mean \pm SEM of five different blots and relative densitometric quantifications. (C) Western blot analysis of β -tubulin: a control for equal protein loading. * Significant difference ($p < 0.05$) from control and ° significant difference ($p < 0.05$) from HR + PJ34.

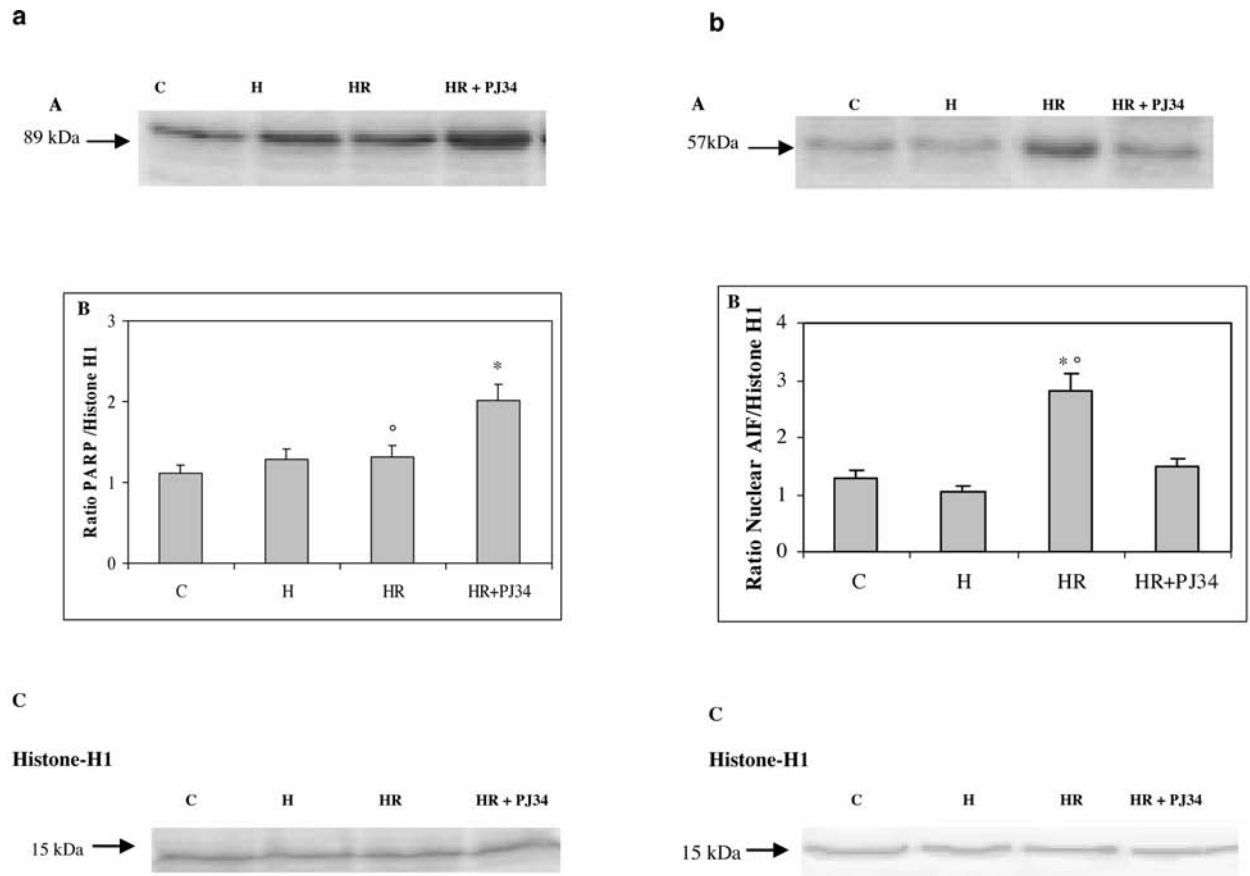


Figure 7. (a) Western blot analysis of 89 kDa PARP fragment in control (C), hypoxic (H) and hypoxic-reoxygenated (HR) cells. (A) representative immunoblot. (B) quantitative data. All signals were quantified by densitometric analysis and are expressed as ratio of 89 kDa PARP fragment densitometry on Histone H1 (loading control) densitometry. Each bar represents the mean value \pm SEM of five different blots and relative densitometric quantifications. (C) Western blot analysis of histone-H1: a control for equal protein loading. (b) Western blot analysis of nuclear AIF in control (C), hypoxic (H) and hypoxic-reoxygenated (HR) cells. (A) representative Western blot; (B) quantitative data. All signals were quantified by densitometric analysis and are expressed as ratio of AIF densitometry on histone-H1 (loading control) densitometry. Each bar represents the mean value \pm SEM of five different blots and relative densitometric quantifications. (c) Western blot analysis of histone-H1: a control for equal protein loading. * Significant difference ($p < 0.05$) from control and ^o significant difference ($p < 0.05$) from HR + PJ34.

the classical substrate analogues such as nicotinamide or 3-aminobenzamide [24]. These new compounds, devoid of inherent antioxidant activity, are characterized by low-micromolar to mid-nanomolar inhibitory potencies in whole-cell-based assays, besides producing relevant effects when administered to animals in dosing range of few milligrams per kilogram of body weight [25]. Owing to its high inhibitory power, hydrosolubility and oral bio-availability, PJ34 appears as one of the most promising among the recent PARP-1 inhibitors, especially in view of a possible clinical utilization [26, 27].

In this study PJ34, used at a final concentration of 0.5 μ M (about fivefold the concentration needed to produce a 50% inhibition in PARP-1 activity tests based on NAD⁺ consumption [28]), strongly reduced not only PARP-1 activation but also ROS production and oxidative stress in HR cardiomyoblasts, at the same time preserving the antioxidant defenses of these cells. Since PJ34 does not *per*

se possess antioxidant activity, a plausible explanation for this finding may be that this inhibitor suppresses the ability of activated PARP-1 to promote mitochondrial damage and dysfunction, and hence a secondary increase in ROS generation. In this connection, it has been reported that the exposure of cultured cells to oxidative stress produces a time- and dose-dependent decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$), which is associated with an increased ROS production, probably due to an enhanced univalent leakage of the respiratory chain [29]; under these conditions PARP-1 inhibition has been found to attenuate both the oxidative stress-induced $\Delta\Psi_m$ reduction and the secondary ROS generation [30]. Mitochondrial dysfunction associated with PARP-1 activation is mainly ascribed to the depletion of NAD⁺ [31], the pool of which is largely compartmentalized within these organelles. Some authors [32], however, have suggested that molecular signals released from the nucleus of cells

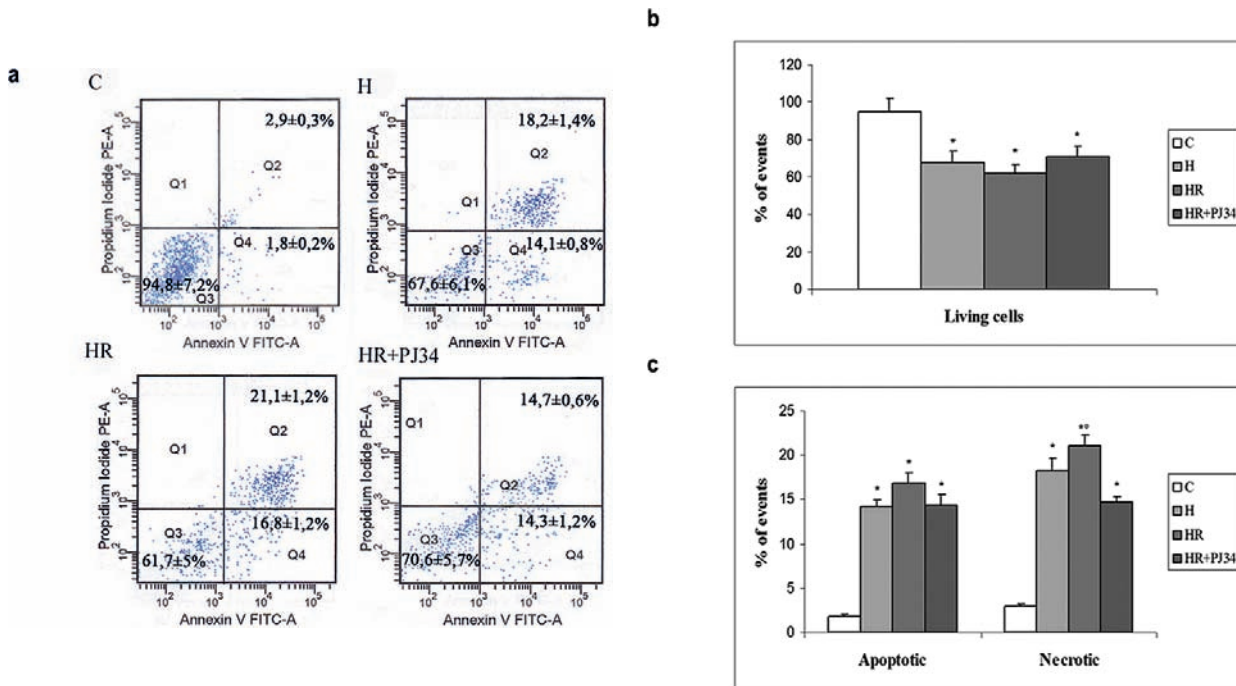


Figure 8. Flow cytometric analysis of apoptosis and necrosis in H9c2 cardiomyoblasts labeled with FITC-annexin V-PI. Dot plot shows three populations: Q2 = necrotic cells, Q3 = living cells and Q4 = apoptotic cells in control, in hypoxic cells (H) and in hypoxic-reoxygenated (HR) cells with and without PJ34 (A). These data are represented in histograms showing percentages \pm SEM (five different experiments performed) of living (B), apoptotic and necrotic cells (C): * Significant difference ($p < 0.05$) from control and ° Significant difference ($p < 0.05$) from HR+PJ34 (B).

undergoing massive poly(ADP-ribosyl)ation can rapidly modify mitochondrial functioning; a possible candidate messenger for these effects could be free ADP-ribose, the main degradation product of poly(ADP-ribose). It should also be noted that, as recently reported by Halmosi et al. [33], the compounds of the PJ34 family, besides their primary effect of inhibiting PARP-1, seem to have additional mitochondrial targets and to protect the respiratory complexes from ROS-induced inactivation. This might be a further mechanism accounting for a reduced secondary ROS generation by mitochondria, thus the attenuation of oxidative stress induced by PJ34 treatment. Whatever the case, all the above-proposed mechanisms implicate an action at the mitochondrial level that is consistent with our data showing a significant improvement of mitochondrial respiration owing to the treatment with this PARP-1 inhibitor.

As for the effects of PARP-1 activity on the rate and the mode of cell death, the present results suggest that both necrosis and apoptosis account for the considerable mortality of HR cardiomyoblasts not subjected to PARP-1 inhibition, as clearly highlighted by cytofluorometric quantification of live, necrotic and apoptotic cells (Fig. 8). Furthermore, the marked translocation of AIF to the nuclear fraction that we found in these cells, in agreement with previous reports [34], suggests that this factor is probably responsible for their apoptotic death.

The release of both cytochrome c and AIF (effects that may be ascribed to the mitochondrial dysfunction produced by PARP-1 activation through the mechanisms discussed above) was evident when HR cardiomyoblasts did not undergo treatment with PJ34; under these conditions, however, cytochrome c appeared unable to activate the corresponding apoptotic pathway. As a possible explanation for this finding we propose that energy loss due to a strong PARP-1 activation prevents the energy consuming cytochrome c/caspase-dependent apoptosis, but allows the development of a (relatively) energy inexpensive process such as the AIF-dependent apoptotic pathway.

This interpretation implies that the oligonucleosomal DNA fragmentation that we found in HR cells represents a caspase-independent and AIF-dependent process. We are aware that this does not agree with most published literature that generally depicts AIF as an apoptotic effector protein inducing chromatin condensation and large-scale DNA fragmentation [35]. In a recent report, however, Yuste et al. [36] have challenged this view, providing evidence that AIF, which lacks intrinsic nuclease activity, can affect chromatin condensation but does not control the pattern of DNA degradation. The same authors propose that high or low molecular weight DNA fragmentation depends on the activity of endogenous nucleases, notably (at least in their model) on the combined effect of caspase-activated DNase (CAD) and of its inhibitor

(ICAD). In our experimental conditions, owing to the established caspase independence of the observed DNA fragmentation, we should invoke the involvement of other DNases. A stimulating, although at present only speculative, hypothesis may be that mitochondrial dysfunction, besides AIF, can also induce the release of endonuclease G, a mitochondrion-specific enzyme that during apoptosis translocates to the nucleus, inducing nucleosomal DNA fragmentation independently of caspases [37, 38]. In HR cardiomyoblasts subjected to PJ34 treatment, the observed changes in the rates of LDH leakage, in the trypan blue exclusion assay, in oligonucleosomal DNA fragmentation and in the cytofluorometric analysis indicate that PARP-1 inhibition can induce a significant decrease in cell mortality, mainly due to a reduction of necrotic death but also to an attenuation of the apoptotic processes; these, however, did not appear to proceed through AIF, whose nuclear translocation was undetectable, but through the caspase-dependent pathway, as suggested by the fact that, in this case, the mitochondrial release of cytochrome c was associated with caspase-3 activation and with the typical PARP-1 cleavage. Probably, energy saving due to PARP-1 inhibition allowed the development of the ATP-requiring caspase-mediated apoptosis, while, as regards to the relationships between PARP-1 activity and the release of cytochrome c and AIF, these may be tentatively attributed to the different molecular size of these proteins and to their different organization in the mitochondrial structure [39]. As proposed in a recent report by Goldstein et al. [40], it is possible that, because of its lower molecular weight and its weaker binding to mitochondrial inner membrane, cytochrome c is more easily released, thus mild mitochondrial changes induced by moderate levels of PARP-1 activation might be sufficient to trigger the release of cytochrome c but not that of AIF.

In conclusion, the data reported here support the view that PARP-1 is an important regulator of cell survival and death in the myocardial injury due to post-hypoxic reoxygenation. Our results suggest that changes in the level of energy depletion and in the mitochondrial release of cell death factors, owing to the degree of PARP-1 activation, may result in varying combination of necrosis and apoptosis. From this point of view PARP-1 inhibition by PJ34 appears to produce beneficial effects, principally by reducing the overall mortality of the tested cells. This encourages us to undertake further studies aimed at probing the potentialities of this class of PARP-1 inhibitors more deeply, so as to optimize, if necessary even through additional treatments, their protective effect against myocardial reperfusion injury.

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