Mitochondrial-to-nuclear translocation of apoptosis-inducing factor in cardiac myocytes during oxidant stress: potential role of poly(ADP-ribose) polymerase-1

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Abstract

Objective: Oxidant stress-induced activation of poly(ADP-ribose) polymerase (PARP) plays a role in the pathogenesis of various cardiovascular diseases. We have now investigated the role of PARP in the death of cardiac myocytes in response to oxidant stress induced by hydrogen peroxide, with focus on the mitochondrial function. Methods and results: Using wild-type and PARP-1-deficient murine myocytes challenged with hydrogen peroxide, we found that mitochondrial respiration and mitochondrial membrane potential were better preserved in PARP-deficient myocytes and cellular NAD+ levels were maintained. The release of the mitochondrial cell death factor cytochrome c, and the mitochondrial-to-nuclear translocation of apoptosis-inducing factor (AIF) were also attenuated in the PARP-deficient myocytes. Conclusion: PARP-1, directly or indirectly, regulates the translocation of AIF in myocytes subjected to oxidative stress. The current results are consistent with the view that PARP-1 activation, via induction of mitochondrial dysfunction and promotion of mitochondrial cell death pathways, plays a deleterious pathophysiological role under conditions of oxidative stress.

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1. Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1), a monomeric enzyme present in eukaryotes, is the major isoform of an expanding family of poly(ADP-ribosyl)ating enzymes [1–5]. The main isoform of the family, PARP-1, primarily functions as a DNA damage sensor in the nucleus. Upon binding to damaged DNA mainly through the second zinc finger domain, PARP-1 forms homodimers and catalyzes the cleavage of NAD+ into nicotinamide and ADP-ribose and uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins. The biological role of PARP-1 is complex and includes the regulation of DNA repair and maintenance of genomic integrity [1].

PARP-1 has been implicated in a variety of pathophysiological processes. PARP is an energy-consuming enzyme, which transfers ADP ribose units to nuclear proteins. As a result of this process, the intracellular nicotinamide dinucleotide (oxidized) (NAD+) and adenosine 5'-triphosphate (ATP) levels remarkably decrease, resulting in cell dysfunction and cell death via the necrotic route (overviewed in Ref. [1]).

PARP becomes activated in response to DNA single-strand breaks, which can develop as a response to free radical and oxidant cell injury. Oxidative and nitrosative stress triggers the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which contributes to the pathogenesis of various cardiovascular diseases including myocardial infarction and ischemia–reperfusion and heart failure [2–5]. Recent studies have implicated the importance of mitochondrial dysfunction and mitochondrial cell death factors in the process of oxidant-induced cell death,
and the potential role of PARP in regulating these factors [6–10].

The role of PARP in the oxidant-induced mitochondrial alterations has not yet been investigated in cardiac myocytes. Using wild-type and PARP-deficient myocytes, the aim of the present study was to investigate the role of PARP pathogenesis of oxidant-mediated mitochondrial dysfunction in the heart. The specific objectives of the study were to (1) determine whether PARP-deficient myocytes are resistant to oxidant-induced mitochondrial dysfunction, (2) whether oxidant challenge in cardiac myocytes induces mitochondrial-to-nuclear translocation of the cell death factor apoptosis-inducing factor (AIF) and (3) whether this latter process is regulated by PARP.

2. Methods

2.1. Cardiac myocyte preparation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The method of isolation of ventricular myocytes from mice was adapted from that of Zhou et al. [11] with some modifications. Briefly, 3–4 months old male wild-type or PARP-1-deficient mice of identical mixed genetic background of SV129xBl6, generated as described [12], were anesthetized with ketamine/xylazene. Hearts were excised and mounted in a Langendorff perfusion apparatus and perfused for 15–20 min with 95% O2–5% CO2, followed by a 15–20 min perfusion with same KRB buffer containing 25 mM CaCl2, 0.1% BSA, and 5 taurine (Sigma), gassed with O2/C02 and then the Ca2+ concentration was increased to 1.0 mM for 30 min. After washing twice with PBS, the ventricular tissue was chopped and dispersed by collagenase (Worthington Biochemical, Freehold, NJ) and incubated for 30 min in the presence of 5 U/mL collagenase ( Worthington Biochemical, Freehold, NJ) and 0.1% BSA. Thereafter, ventricular tissue was washed and incubated for 10 min in the same medium supplemented with 1% BSA. The ventricular tissue was then dispersed and filtered. The resultant myocytes were washed twice with fresh KRB buffer containing 1% BSA and 25 mM CaCl2, and then the Ca2+ concentration was increased to 1.0 mM gradually. Finally, the myocyte pellets were washed and suspended in MEM medium containing 1.2 mM Ca2+ (Sigma, M1080).

2.2. Cell culture and H2O2 treatment

Myocytes were plated in laminin-precoated plates or glass coverslips at a density of 10^5 cells/cm². After 1 h plating in MEM medium with 5% fetal calf serum, the medium was changed to FBS-free MEM. Following overnight culture, myocytes were subjected to H2O2 treatment at 50, 100 or 150 μM.

2.3. Cardiomyocyte injury and viability assays and measurement of cellular NAD+

2.3.1. MTT reduction

The metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma), to a colored formazan product [6]. At the end of H2O2 treatment, MTT (0.5 mg/ml) was added to MEM and incubated for 30 min. The medium was then carefully aspirated, and 1 ml of dimethylsulfoxide was added to solubilize the colored formazan product. A sample (200 μl) from each duplicate well was then transferred to a 96-well microplate, and the absorbance at 570 nm was determined by spectrophotometer (Molecular Devices).

2.3.2. Determination of cellular NAD+

In an additional series of experiments, cellular NAD+ levels from wild-type and PARP-deficient myocytes were measured prior and after hydrogen peroxide (150 μM) exposure using the alcohol dehydrogenase colorimetric method, as described previously [13]. Briefly, cells were extracted in 0.5 N HClO4, scraped, neutralized with 3 M KOH and centrifuged for 2 min at 10,000 × g. The supernatant was assayed for NAD+, using a colorimetric method, in which NADH, produced by enzymatic cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediate of phenazine methosulfate. The rate of increase in the absorbance was read immediately after addition of the NAD samples and after 10 and 20 min incubation at 37 °C against a blank at 560 nm in the Spectramax spectrophotometer.

2.3.3. Propidium iodide staining of cell nuclei

Myocytes were stained with propidium iodide (PI) (Molecular Probes) to identify nonviable cells. Myocytes were incubated for 30 min in the presence of 5 μg/ml PI, washed with PBS and analyzed using flow cytometry (Becton Dickinson).

2.4. Mitochondrial membrane potential (ΔΨm) determination

Mitochondrial membrane potential (ΔΨm) was assessed by a mitochondrial voltage-sensitive dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbicyanocarbocyanine iodide (JC-1, Molecular Probes). Mitochondria with intact membrane potential concentrate JC-1 into aggregates that show red fluorescence. De-energized mitochondria cannot concentrate JC-1 and show green fluorescence. After H2O2 treatment, myocytes were incubated with 10 μM JC-1 in media for 30 min in the dark, and then collected by scraping. After washed in PBS, cells were subjected to FACS analysis [14]. The ratio of JC-1 aggregate (FL2, green) to monomer (FL1, red) intensity was calculated.
An increase in this ratio was interpreted as decrease of \( \Delta \psi_m \). As a positive control for loss of \( \Delta \psi_m \), cells were treated with 200 nmol/l carbonylcyanide-trifluoro-methoxyphenylene hydrazone (FCCP, Molecular Probes) at 37 °C for 30 min.

### 2.5. Detection of cytochrome c release

Cytosol and mitochondria from myocytes were prepared using a cell fractionation kit (BD Bioscience). Myocytes were washed with ice-cold washing buffer, resuspended in fractionation buffer supplemented with 10 mM DTT and protease inhibitors and homogenized by 60 strokes using a glass Dounce homogenizer and a pestle. The cell suspension was centrifuged at 60,000 \( \times \) g for 10 min to remove nuclear fraction. The resulted supernatant was further centrifuged at 100,000 \( \times \) g for 10 min to separate the mitochondria from the cytosolic fraction. The protein content of each fraction was determined by the BioRad Bradford assay. About 2 \( \mu \)g protein of each fraction was subjected to electrophoresis on 4–20% gradient polyacrylamide gels (Invitrogen) and then transferred to nitrocellulose membrane (Millipore), incubated with primary and secondary antibodies, and developed by chemiluminescence. Rabbit anti-mouse cytochrome c (Pharmingen) was used as a primary antibody.

### 2.6. Immunofluorescence cell staining for AIF

For immunofluorescence staining, cells were plated and cultured on glass coverslips, treated with \( \mathrm{H}_2\mathrm{O}_2 \) for an indicated time and fixed with 3% paraformaldehyde in PBS. After blocking with 1% bovine serum, cells were probed with an anti-AIF rabbit polyclonal antibody (Chemicon International) followed by goat anti-rabbit antibody conjugated to FITC. Coverslips were mounted to slides with propidium iodide containing mounting media. The cells were visualized using fluorescence microscopy.

### 2.7. Statistical analysis

Results are expressed as mean ± S.E.M. Differences between means were evaluated by unpaired two-tailed Student’s t-test.
3. Results

3.1.  \( \text{H}_2\text{O}_2 \)-induced mitochondrial dysfunction and cell death in myocytes from PARP\(^{+/+}\) and PARP\(^{-/-}\) mice

In the first sets of experiments, we compared \( \text{H}_2\text{O}_2 \)-induced mitochondrial dysfunction followed by cell death between two populations of myocytes isolated from wild-type (PARP\(^{+/+}\)) and PARP-deficient (PARP\(^{-/-}\)) mice. Mitochondrial dysfunction was assessed by the MTT method, which is reduced to formazan when the function of the electron transport chain is intact. Fig. 1a shows that 1 h treatment with different concentrations of \( \text{H}_2\text{O}_2 \) induced a dose-dependent reduction of formazan in myocytes from both PARP\(^{+/+}\) and PARP\(^{-/-}\) mice. However, myocytes from PARP\(^{-/-}\) mice preserved significant higher MTT-reducing activity than myocytes from PARP\(^{+/+}\) mice. Cellular viability at different time points after exposure to 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) was quantitated by FACS analysis of PI-positive cells. As shown in Fig. 1b, the number of PI-positive cells, which increased progressively during \( \text{H}_2\text{O}_2 \) treatment, these changes were markedly less pronounced in PARP\(^{-/-}\) cells, when compared to the PARP\(^{+/+}\) cells, at each time point. Data from four independent experiments are summarized in Fig. 1c. There was also a marked maintenance of cellular NAD levels in response to hydrogen peroxide (150 \( \mu \text{M} \)) challenge. While in the wild-type myocytes, cellular NAD levels decreased to 44 \( \pm \) 15\% of control \((p < 0.01, n = 3)\), in the PARP-deficient myocytes only a modest tendency for a decrease (to 72 \( \pm \) 26\% of control) was noted \((n = 3)\).

3.2. Changes in mitochondrial membrane potential (\( \Delta \psi_m \)) in response to \( \text{H}_2\text{O}_2 \) treatment in myocytes from PARP\(^{+/+}\) and PARP\(^{-/-}\) mice

To determine \( \Delta \psi_m \) loss, myocytes were stained with JC-1. JC-1 concentrates in the mitochondria as red fluorescent aggregates at high membrane potentials and then it converts to green monomers as \( \Delta \psi_m \) is lost. This is associated with a decrease in fluorescence at 585 nm (FL2) and an increase in fluorescence intensity at 530 nm (FL1). As displayed in dot plots (Fig. 2a–e), 1 h exposure to \( \text{H}_2\text{O}_2 \) (200 \( \mu \text{M} \)) caused a marked decrease of fluorescence of FL2 and a slight increase in the fluorescence of FL1 in PARP\(^{+/+}\) myocytes, a change that was similar to that induced by FCCP (positive control). In contrast, in PARP\(^{-/-}\) cells FL2 was maintained at a high level under the same oxidant stress conditions. The

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Fig. 2. Loss of \( \Delta \psi_m \) induced by \( \text{H}_2\text{O}_2 \) (JC-1 FACS analysis) in cardiomyocytes from PARP\(^{+/+}\) and PARP\(^{-/-}\) mice. Myocytes were exposed to 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 1 h, stained with JC-1, and analyzed as described in Materials and methods. Dot plots of red fluorescence (FL2) versus green fluorescence (FL1) showed that in \( \text{H}_2\text{O}_2 \)-untreated myocytes of PARP\(^{+/+}\) and PARP\(^{-/-}\) mice (a, b) FL2 levels were high indicating intact \( \Delta \psi_m \), and that after \( \text{H}_2\text{O}_2 \) treatment FL2 decreased remarkably in PARP\(^{+/+}\) myocytes (c) but only partially in PARP\(^{-/-}\) myocytes (d), (e) FCCP-induced loss of \( \Delta \psi_m \) in PARP\(^{+/+}\) cells as a positive control. (f) The changes of FL2/FL1 ratio after \( \text{H}_2\text{O}_2 \) treatment in myocytes of PARP\(^{+/+}\) and PARP\(^{-/-}\) mice, data are expressed as mean \( \pm \) S.E.M. of \( n = 3 \) independent experiments, \(*p < 0.05\) vs. corresponding values of PARP\(^{+/+}\) myocytes.)
changes of FL2/FL1 ratio after H2O2 (200 μM) treatment for 0.5 and 1 h of both PARP+/+ and PARP−/− myocytes are shown in Fig. 2f. PARP−/− myocytes maintained a significant higher FL2/FL1 ratio than that of PARP+/+ myocytes after 1 h exposure to H2O2, indicating a better preservation of mitochondrial membrane potential.

Fig. 3. Cytochrome c release and AIF translocation after H2O2 treatment in cardiomyocytes from PARP+/+ and PARP−/− mice. Myocytes were exposed to 200 μM H2O2 for 1 h. (a) Cytochrome c release, revealed by Western blot analysis of cytochrome c in cytosolic and mitochondrial fractions, was detected in PARP+/+ myocytes but not in PARP−/− myocytes. (b) Nuclear AIF staining after H2O2 treatment was demonstrated by the co-localization of AIF (green) and nuclei (red) only in PARP+/+ cells but not in PARP−/− cells.
3.3. Release of cytochrome c and translocation of apoptosis-inducing factor (AIF) after H_2O_2 treatment in myocytes from PARP^+/+ and PARP^-/- mice

Cytochrome c release and AIF translocation in response to the H_2O_2 treatment are shown in Fig. 3a and b. Small amounts of cytochrome c were detected in the cytosol of H_2O_2-ununtreated myocytes from both PARP^+/+ and PARP^-/- mice. The preparation of primary murine myocytes is an involved procedure, which utilizes perfused heart preparations, and the small levels of cytochrome c detected in the cytosol presumably reflect the result of the myocyte isolation process. After 1 h H_2O_2 (200 μM) treatment, there was a substantial increase in cytosolic cytochrome c concentrations in PARP^+/+ myocytes but not in the cytosol of PARP^-/- myocytes (Fig. 3a).

AIF immunostaining revealed a cytoplasmic distribution of AIF in control myocytes. Within 30 min of H_2O_2 exposure, however, a strong nuclear AIF staining was detected in myocytes from PARP^+/+. Nuclear AIF translocation was absent in PARP^-/- myocytes (Fig. 3b).

4. Discussion

Multiple reports indicate the importance of PARP activation in the development of mitochondrial dysfunction under conditions of oxidative stress [6–10]. Even though the major isoform of the PARP family, PARP-1, is widely considered as a nuclear enzyme, there is apparently a nuclear-to-mitochondrial signaling process, which initiates early mitochondrial alterations, as demonstrated in thymocytes [6] and in neurons [10]. Although there were early reports suggesting that pharmacological PARP inhibitors maintain mitochondrial respiration in cardiac myocytes subjected to oxidative and nitrosative stress [15,16], the interpretation of these findings is problematic, as many of the commercially available PARP inhibitors exert nonspecific antioxidant effects, and are not specific to the various isoforms of PARP [17,18]. In order to specifically and selectively investigate the role of PARP-1 in the changes in mitochondrial function in oxidant-challenged cardiac myocytes, we have now prepared myocytes for PARP-1-deficient mice, and from their corresponding wild-type counterparts. The results demonstrated that the absence of PARP-1 exerts a marked protection against oxidant-induced mitochondrial dysfunction (decrease in mitochondrial respiration and loss of mitochondrial membrane potential). These findings are consistent with prior studies demonstrating that PARP-1-deficient mice are protected against myocardial infarction [3], and hearts from PARP-1-deficient mice are protected against hypoxia/reoxygenation in vitro [19,20].

Importantly, in the PARP-1-deficient myocytes, the release of the mitochondrial cell death factors cytochrome c and AIF were attenuated. As cytochrome c is known to induce the activation of caspases, the current results may provide an explanation for recent findings demonstrating reduced caspase activation and apoptosis in the hearts of PARP-1-deficient mice subjected to myocardial ischemia and reperfusion [21].

Recently, much attention has been paid to the role of AIF in cell necrosis and apoptosis. AIF appears to be an important factor involved in the regulation of this caspase-independent neuronal cell death. Recent immunofluorescence studies in neuronal cell lines demonstrate that AIF is released from the mitochondria by a mechanism distinct from that of cytochrome c in neurons undergoing p53-mediated cell death [22]. Enforced expression of AIF can induce neuronal cell death in a Bax- and caspase-independent manner. A study from Yu et al. [10], using in vitro models of neuronal cell death, demonstrated that the mitochondrial-to-nuclear translocation of AIF, as it occurs during NMDA-receptor activation mediated neuronal death, can be reduced in the absence of functional PARP.

The present report is consistent with recent findings demonstrating that oxidants induce the mitochondrial to nuclear translocation of AIF in myocytes [23,24]. In addition, the current data demonstrate that the translocation of AIF is regulated by PARP-1. How, then, is PARP-1, a primarily nuclear enzyme, able to regulate the rapid mitochondrial release of AIF? One possibility may be related to a mitochondrially localized PARP-1 [9] may play in the process. Another possibility may be that the process is mediated by changes in intracellular NAD^+ concentrations. In support of this hypothesis, we are presenting data in the current manuscript that demonstrate that PARP^-/- myocytes maintain their NAD^+ levels in response to oxidant stress, as opposed to wild-type myocytes where NAD^+ levels are markedly depleted. Also, Alano et al. [25] have recently demonstrated that repletion of cytoplasmic NAD^+ can suppress the translocation of mitochondrial AIF to the nucleus in oxidatively challenged astrocytes. A third possibility is that a product of poly(ADP-ribosyl)ation—a poly(ADP-ribosyl)ated nuclear-to cytoplasmic second messenger, possibly poly(ADP-ribosel) itself—may signal to the mitochondria. It is noteworthy in this context that recent studies demonstrated the poly(ADP-ribosyl)ation of the cytoplasmic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) under conditions of oxidative stress in endothelial cells placed in high extracellular glucose milieu [26]. Clearly, further work remains to be conducted to delineate the early signaling processes between the nucleus and the mitochondria under conditions of oxidative stress. With respect to PARP, AIF and oxidant stress, further work remains to be conducted in order to determine whether mitochondrial-to-nuclear translocation of AIF also occurs in the heart in vivo, under various pathophysiological conditions that are associated with acute or chronic oxidative stress. Indeed, Komjáti et al. [27] have recently reported that there is a mitochondrial-to-nuclear translocation of AIF in stroke, which is reduced after pharmacological inhibition of PARP.
Recent work has demonstrated the importance of PARP activation and the protective effect of PARP inhibitors in various forms of ischemia/reperfusion, hypoxia/reoxygenation, heart transplantation, cardiotoxicity, and chronic heart failure (overviewed in Ref. [1]). Based on the current results we put forward the hypothesis that protection against mitochondrial dysfunction and release of mitochondrial cell death factors are important modes of cardioprotection elicited by PARP inhibition.

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References