

Regulation of Mitochondrial Poly(ADP-Ribose) Polymerase Activation by the β -Adrenoceptor/cAMP/Protein Kinase A Axis during Oxidative Stress

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ABSTRACT

We investigated the regulation of mitochondrial poly(ADP-ribose) polymerase 1 (PARP1) by the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) system during oxidative stress in U937 monocytes. Oxidative stress induced an early (10 minutes) mitochondrial DNA damage, and concomitant activation of PARP1 in the mitochondria. These early events were followed by a progressive mitochondrial oxidant production and nuclear PARP1 activation (by 6 hours). These processes led to a functional impairment of mitochondria, culminating in cell death of mixed (necrotic/apoptotic) type. β -Adrenoceptor blockade with propranolol or inhibition of its downstream cAMP/PKA signaling attenuated, while β -adrenoceptor agonists and cAMP/PKA

activators enhanced, the oxidant-mediated PARP1 activation. In the presence of cAMP, recombinant PKA directly phosphorylated recombinant PARP1 on serines 465 (in the automodification domain) and 782 and 785 (both in the catalytic domain). Inhibition of the β -adrenergic receptor/cAMP/PKA axis protected against the oxidant-mediated cell injury. Propranolol also suppressed PARP1 activation in peripheral blood leukocytes during bacterial lipopolysaccharide (LPS)-induced systemic inflammation in mice. We conclude that the activation of mitochondrial PARP1 is an early, active participant in oxidant-induced cell death, which is under the control of β -adrenoceptor/cAMP/PKA axis through the regulation of PARP1 activity by PARP1 phosphorylation.

Introduction

Poly(ADP-ribose) polymerase 1 (PARP1), the major form of the PARP superfamily, is generally viewed as a constitutive nuclear enzyme with physiologic roles in the regulation of DNA repair, chromatin remodeling, and gene transcription (Tulin et al., 2003; Oei et al., 2005; Hottiger et al., 2011; DeVos et al., 2012). Its pathophysiologic overactivation, as a result of oxidative DNA injury, has been implicated in the pathogenesis of several diseases, including neuroinjury, inflammation, and ischemia/reperfusion, and various forms of critical illness (Tulin et al., 2003; Jagtap and Szabo, 2005; DeVos et al., 2012; Szanto et al., 2012; Curtin and Szabo, 2013; Burkle and Virag, 2013). In addition to the nuclear isoform of PARP1 several sets of studies have identified PARP1 in the mitochondrial compartment as well; it appears that, in addition to nuclear PARP1, mitochondrial PARP1 also plays roles in the regulation of various cellular functions—at least in some cell types (Masmoudi et al., 1988; Du et al., 2003; Rossi et al., 2009, reviewed in Burkle and Virag, 2013).

Protein kinase A (PKA), a cyclic adenosine monophosphate (cAMP)-dependent serine/threonine kinase, is one of the major effector proteins of cAMP and a regulator of cellular function in health and disease (Gancedo, 2013; Taylor et al., 2013; Valsecchi et al., 2013). The purpose of the current study was to determine whether PARP1 activity is regulated by the β -adrenoceptor/cAMP/PKA system during oxidative stress. The results demonstrate an early activation of the mitochondrial form of PARP1, unveil its regulation via phosphorylation by PKA through β -adrenoceptor/cAMP signaling and show the role of these processes in oxidant-induced cell death.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Cell Culture. U937 human monocyte histiocytic lymphoma cells, and C2C12 mouse myoblast cells were obtained from ATCC.

Western Blotting Analysis. Western blotting analysis was carried out as previously described (Gerö et al., 2013a) using anti-PARP1 antibody (Cell Signaling Technology, Beverly, MA), anti- β -receptor antibody (Abcam, Cambridge, MA), anti-protein kinase A (R&D Systems, Minneapolis, MN), anti- β -actin-horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology, Inc., Dallas, TX), anti-rabbit-IgG (Cell Signaling Technology), and anti-mouse-IgG (Cell Signaling Technology). PARP1 activity was quantified by detection of its product, poly

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ABBREVIATIONS: cAMP, cyclic adenosine monophosphate; DDA, 2,5-dideoxy-adenosine; FSK, forskolin; H₂O₂, hydrogen peroxide; HRP, horseradish peroxidase; ISP, isoproterenol; LC, liquid chromatography; LDH, lactate dehydrogenase; LPS, bacterial lipopolysaccharide; NAD⁺, nicotinamide adenine dinucleotide; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; PARP1, poly(ADP-ribose) polymerase 1; PCR, polymerase chain reaction; PJ34, *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylaminoacetamide hydrochloride; PKA, protein kinase A; PLA, proximity ligation assay; PP, propranolol; siRNA, small interfering RNA.

(ADP-ribose) (PAR), by Western analysis as described (Gerö et al., 2013a), using an anti-poly(ADP-ribose) antibody (anti-PAR) (Trevigen Inc., Gaithersburg, MD), anti-rabbit-IgG (Cell Signaling), and anti- β -actin-HRP conjugate (Santa Cruz Biotechnology). Equal loading was normalized with anti- β -actin-HRP conjugate antibodies (Santa Cruz Biotechnology). Ten micrograms of protein of each isolation was analyzed.

Measurement of Mitochondrial and Total Cellular Oxidant Production. U937 cells were incubated with MitoSox Red (5 μ M; Life Technologies, Grand Island, NY) 10 minutes at 37°C in dark or with 2',7'-dichlorofluorescein (5 μ M; 2',7'-dichlorofluorescein H₂DCF) 30 minutes at 37°C before hydrogen peroxide (H₂O₂) challenge. Fluorescence signal was measured as described (Gerö et al., 2013b) on Synergy 2 (Ex/Em of MitoSox Red: 510/580 nm; Ex/Em of reactive oxygen species detection reagent: 490/520 nm) (BioTek, Winooski, VT).

Measurement of Mitochondrial and Nuclear DNA Integrity. To measure DNA integrity (damage) we used gene-specific semi-quantitative polymerase chain reaction (PCR) assays using LongAmp Taq DNA Polymerase (New England BioLabs Inc., Ipswich, MA) as described (Szczesny et al., 2013). Briefly, total DNA was isolated using DNase Blood and Tissue Kit (QIAGEN, Hilden, Germany). Damage to nuclear DNA was estimated by quantification of the PCR amplification of the 9-kb nuclear-specific DNA fragment using Quant-iT PicoGreen ds DNA Reagent to detect double-stranded DNA (Life Technologies). Damage to the mitochondrial DNA was estimated by quantification of the PCR amplification of the 10-kb mitochondrial-specific DNA fragment using PicoGreen staining. Data were normalized by the PCR amplification of 117-bp mitochondrial genome-specific fragment for correction of the multiple copies of the mitochondrial genome.

AlamarBlue Conversion. AlamarBlue conversion was measured as described (Módis et al., 2013a).

Measurement of Mitochondrial Membrane Potential. U937 cells (5 \times 10⁴/ml) at the various time points after 400 μ M H₂O₂ treatment were used and mitochondrial membrane potential assay was performed using TMRE—Mitochondrial Membrane Potential Assay Kit (Abcam) according to manufacturer's recommendations, and fluorescence signal was measured on a Synergy2 reader (Ex/Em: 540/575 nm) (BioTek).

Comet Assay. Comet assay was performed using CometAssay (Trevigen) according to the manufacturer's recommendations and as described (Suzuki et al., 2011). At various time points after 400 μ M of H₂O₂, 1000 cells were used.

Quantification of Cell Death by Flow Cytometry. Detection of apoptosis/necrosis was performed using PE Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, CA). U937 cells (1 \times 10⁶/ml) at the 24-hour time point after 400 μ M H₂O₂ treatment were analyzed using a FACSArray Bioanalyzer (BD Biosciences, San Jose, CA).

Lactate Dehydrogenase (LDH) Release. Lactate dehydrogenase (LDH) release into the culture medium was performed as described (Gerö et al., 2013a).

Bioenergetic Analysis of Isolated Mitochondria. U937 cells were pretreated with propranolol (10 μ M) for 30 minutes, followed by 6 hours treatment with 400 μ M H₂O₂ at 37°C. Mitochondria were isolated and subjected to extracellular flux analysis as described (Módis et al., 2012, 2013b). Oxidative phosphorylation-coupled ATP production and uncoupler-stimulated maximal respiration were measured using 10 μ g of mitochondria per well.

Bioenergetic Analysis of Cultured Cells. U937 cells were plated in 24-well Seahorse culture plates coated with 3.5 μ g/cm² Cell-Tak (BD Biosciences Discovery Labware, Bedford, MA). After H₂O₂ treatment, bioenergetic function was measured by XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) as described (Módis et al., 2012, 2013b).

Subcellular Localization of PARylated Proteins and PARP1. Cells were treated with the various pharmacological agents for 10 minutes, followed by exposure to H₂O₂ (400 μ M) for various time points. For analysis, cells were fixed in slide chambers (Lab-Tek)

and triple labeling was used to detect the subcellular localization of PARylated proteins and PARP1 by immunocytochemistry. For subcellular compartments detection, nuclei were stained with DAPI (Life Technologies), while mitochondria were labeled by anti-Ndudf3 antibody (mitochondria complex I subunit; Abcam) labeled by Alexa Fluor 488 anti-mouse antibody (Life Technologies). For the detection of PARylation, anti-PAR antibody (Trevigen) labeled by Alexa Fluor 647 anti-rabbit antibody was used, whereas PARP1 subcellular localization was detected by an anti-PARP1 antibody (Cell Signaling Technologies) labeled by Alexa Fluor 647 anti-rabbit antibody. Images were visualized using Nikon Eclipse 80i fluorescent microscope with CoolSNAP HQ camera and analyzed with NIS-Elements BR3.10 software.

Intracellular cAMP. Intracellular cAMP was measured by the cAMP complete ELISA Kit (Enzo Life Sciences, Inc., Farmingdale, NY).

PKA Silencing by Small-Interfering RNA. Cells were transfected with control and PKA-specific siRNA (50 nM, OriGene, Rockville, MD) using Lipofectamine 2000 (Life Technologies) for 6 hours at 37°C in Opti-MEM. Medium was then replaced to RPMI 1640 (Life Technologies). Cells were challenged with H₂O₂ at 48 hours post-transfection.

Immunoprecipitation of PARP1. Cells were lysed in lysis buffer [50 mM Tris-HCl, pH7.5; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; Protease Inhibitor Cocktail (Roche Diagnostics Corporation, Indianapolis, IN); PhosSTOP (Roche Diagnostics Corporation), 100 mM NaF; 20 mM β -glycero-phosphate; 2 mM Na₃VO₄], and incubated on ice for 2 hours followed by clear-up centrifugation (16,000g, 5 minutes, at 4°C). Total lysate (1.5 mg) was incubated with anti-PARP1 (1/100; Cell Signaling Technology) antibody for 3 hours at 4°C followed by addition of Dynabeads Protein G (Life Technologies) and further incubated for 3 hours at 4°C. Membranes were subsequently probed with anti-PARP1, anti-PAR, anti-pSerine, anti-pThreonine, anti-pTyrosine, and Protein-A HRP-conjugate as a secondary antibody (Life Technologies).

Proximity Ligation Assay. In situ protein/protein proximity/interaction studies were performed with Duolink in situ (Olink Bioscience, Uppsala, Sweden). Cells were fixed in slide chambers (Lab-Tek) and incubated with the same antibodies as for Western analysis. Images were visualized using Nikon Eclipse 80i fluorescent microscope with CoolSNAP HQ camera and analyzed with NIS-Elements BR3.10 software.

In Vitro PARP1 Phosphorylation Assay. Experiments were performed using PKA Assay Kit (EMD Millipore, Billerica, MA) with 6 μ M recombinant PARP1 and PKA. Auto-PARylation of PARP1 was stimulated by 500 ng of sonicated sperm DNA and 500 nM nicotinamide adenine dinucleotide (NAD⁺). PJ34 (10 μ M) was used as a positive control PARP inhibitor. The reaction was terminated after 30 minutes at 37°C by addition of SDS loading buffer. Proteins were separated by SDS-PAGE and membrane probed subsequently with specific antibodies against phospho-serine, phospho-tyrosine, phospho-threonine, PARP1 (Santa Cruz Biotechnology), PAR (BD Pharmingen), and PKA C alpha/beta (R&D Systems).

Analysis of the Post-Translational Modification of PARP1. Human recombinant PARP1 treated in cell-free phosphorylation reactions in vitro with NAD⁺/DNA (or vehicle), followed by treatment with poly(ADP-ribose) glycohydrolase (PARG) (6 μ M) (or its vehicle) for 30 minutes at 37°C. The reaction was stopped by SDS-loading buffer and samples were separated on SDS-PAGE (4–12%). After staining with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA), bands were excised and subjected to phosphorylation detection after trypsin digestion. Mass spectra of digested peptides were obtained using electrospray ionization liquid chromatography-tandem mass spectrometry (ESI-LC-MS/MS) (Applied Biosystems 4000 QTRAP with LC Packings capillary LC system; Foster City, CA). Proteins and phosphorylation from peptide mass fingerprint were identified by Mascot (Matrix Science, Boston, MA) database search and PEAKS mass spectrometry software (BSi, Waterloo, ON, Canada).

LPS-induced inflammation model. The experiments were carried out according to UTMB's AICUC protocol. C57BL6 male mice were injected with propranolol (10 mg/kg) or vehicle (saline) i.p. every 12 h for 3 days. Next, mice were injected with *E. coli* LPS (50 mg/kg) or vehicle (saline) i.p. and sacrificed 6 h later. Peripheral blood mononuclear

cells (PBMCs) were isolated from peripheral blood and analyzed by Western analysis.

Statistical Analysis. All data are presented as means \pm S.E.M. and were analyzed using GraphPad Prism software. Statistical analyses included ANOVA followed by Bonferroni's multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

Oxidative Stress Induces Early-Onset Mitochondrial DNA Injury and PARP1 Activation, Leading to Subsequent Intracellular Oxidant Production, Mitochondrial Dysfunction, and Cell Death. Oxidative stress (H_2O_2 , 400 μM) induced a rapid-onset (within 10 minutes) activation of PARP1 in U937 cells (Fig. 1A), resulting in the PARylation of multiple proteins (Fig. 2). In situ proximity ligation assay (PLA) showed that 10 minutes after H_2O_2 challenge, the activation of PARP1 exclusively occurs in the extranuclear/mitochondrial compartment, but not in the nucleus (Fig. 1A), and is associated with the auto-PARylation of PARP1 (Fig. 1B). Mitochondrial PARP1 activation coincides with an early, transient damage to the mitochondrial DNA (Fig. 2C). At 3–24 hours, consistently with prior studies (see *Introduction*), there was a significant degree of nuclear PARP1 activation (Fig. 1, A and B), which probably results from a progressive increase in the endogenous cellular oxidant production (Fig. 2, A and B). By 3–6 hours, the progressive intracellular oxidant production and PARP activation leads to mitochondrial functional impairment (Fig. 3), which, by 12 hours, induces a breakdown of cell membrane integrity, culminating in cell death of mixed (necrotic/apoptotic) type by 24 hours (Fig. 3F). This response is not associated with an overt degree of nuclear DNA damage (Fig. 2D), which may be attributable to the constant, relatively low-level production of endogenous oxidants, which does not overwhelm the capacity of the nuclear DNA repair. The above findings are consistent with the role of PARP1 in cell death (see Jagtap and Szabo, 2005; Burkle and Virag, 2013) but now also incorporate an early, selective intra-mitochondrial PARP1 activation component into the pathophysiological sequence of events.

β -Adrenoceptor Signaling Regulates Mitochondrial PARP1 Activation during Oxidative Stress. Pretreatment with the β -receptor antagonist propranolol (PP, 10 μM) (Fig. 4A), the adenylyl cyclase inhibitor 2,5-dideoxy-adenosine (DDA, 3 μM) (Fig. 4B), or a protein kinase A (PKA) inhibitor (PKAi, 1 μM) (Fig. 4C) markedly reduced the oxidant-induced increase in the PARylation of multiple proteins, including the auto-PARylation of PARP1 at 116 kDa, as measured at 10 minutes after H_2O_2 challenge. Similarly, PKA depletion via siRNA yielded a significant reduction of the oxidant-induced early-onset PARylation response (Fig. 5). Conversely, the β -receptor agonist isoproterenol (ISP, 10 μM) (Fig. 4D), the adenylyl cyclase activator forskolin (FSK, 3 μM) (Fig. 4E), or the PKA activator 8-bromo-adenosine-3',5'-cyclic monophosphate (8Br-cAMP, 30 μM) (Fig. 4F) each enhanced the degree (Fig. 5) and the onset (Fig. 6) of oxidant-induced PARylation. The β -receptor modulators failed to affect the total cellular expression levels of PARP1 or of the β receptor (Fig. 7A). The functionality of the β -adrenergic receptor on the cells was confirmed by measuring cAMP levels, the primary second messenger of β -adrenoceptor signaling (Gancedo, 2013; Taylor et al., 2013; Valsecchi et al., 2013). In line with prior observations (Chen et al., 1993; Lopez-Hellin et al., 1998),

oxidative stress decreased cAMP levels in U937; cAMP levels were further reduced by propranolol, while they were increased by isoproterenol (Fig. 7B). As expected, the adenylyl

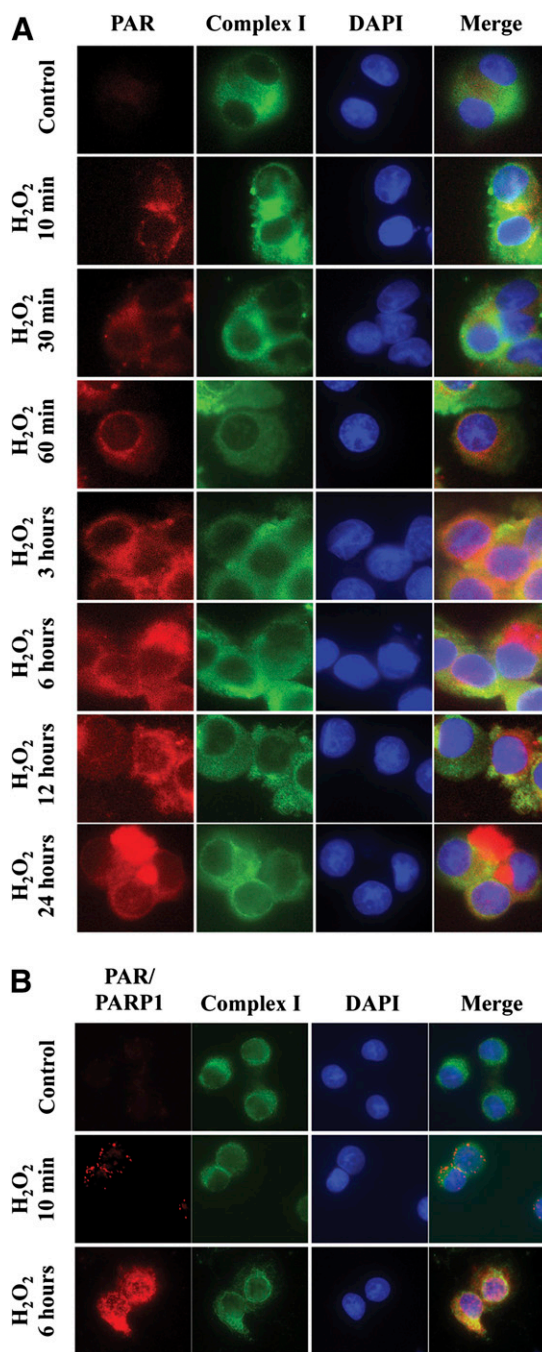


Fig. 1. Time course of PARP1 activation in the extranuclear and nuclear compartments of U937 cells subjected to oxidative stress. (A) H_2O_2 -induced PARP activation appears initially extranuclearly (10–60 minutes), while at subsequent time points nuclear PARP activation develops. Immunostaining images for poly(ADP-ribose) (PAR), Complex I (a mitochondrial localization marker), and DAPI (a nuclear localization marker) are shown, as well as merged images. (B) Auto-PARylation of PARP1, as assessed by PAR/PARP1 in situ PLA analysis, was found extranuclearly at the early time point (10 minutes) after H_2O_2 exposure, while it was localized both to the nuclear and extranuclear compartments by 6 hours. Figures show representative images of at least $n = 3$ independent determinations conducted on different experimental days.

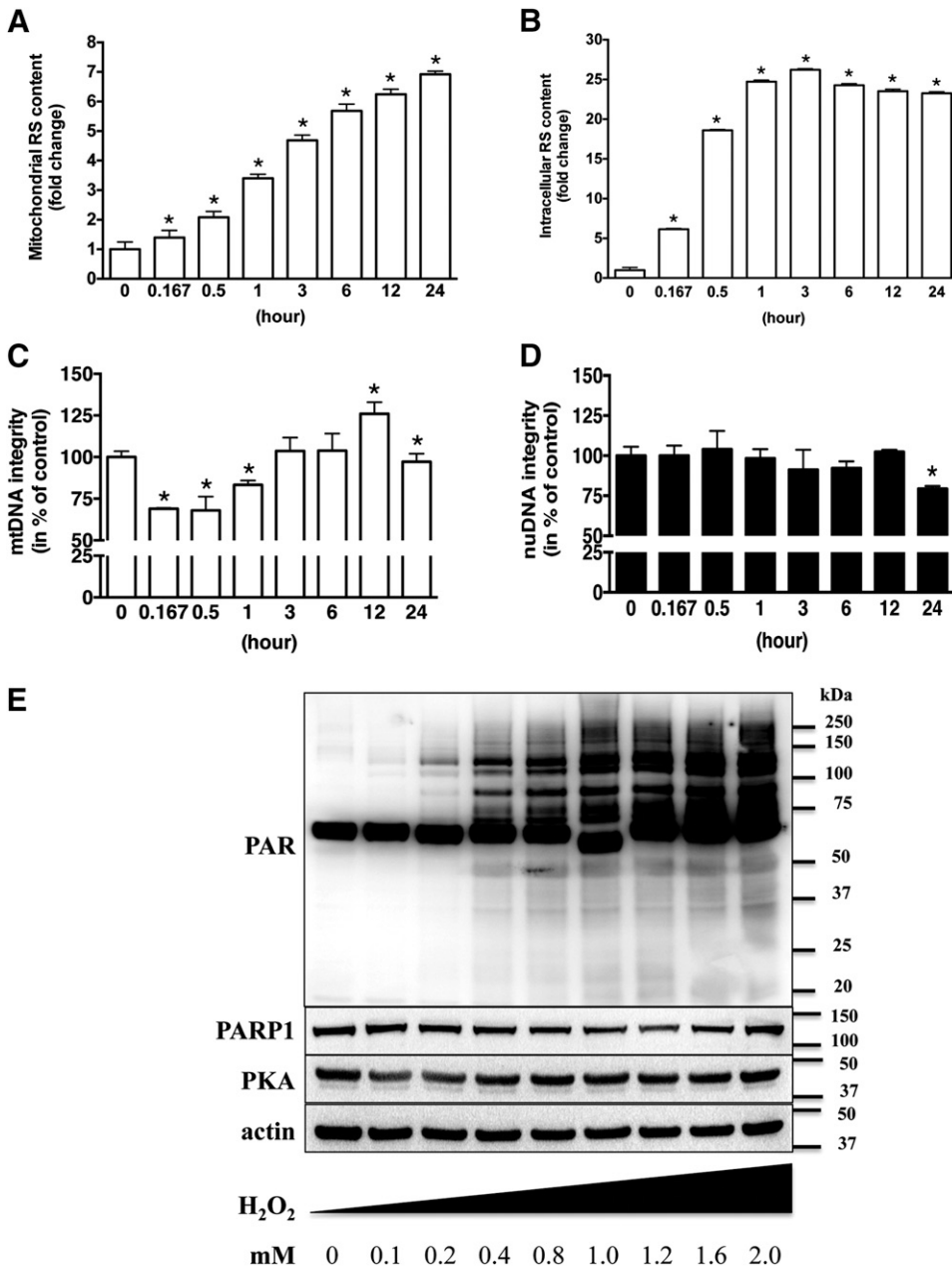


Fig. 2. H_2O_2 challenge induces early mitochondrial and late nuclear DNA damage and a progressive increase in cellular reactive oxygen species (ROS) production in U937 cells. H_2O_2 ($400 \mu M$) induces a time-dependent increase in mitochondrial reactive oxygen species (RS) content (A) and total intracellular RS content (B). Mitochondrial DNA integrity (C) decreased shortly after H_2O_2 challenge and recovered at late time points. In contrast, nuclear DNA integrity (D) was not significantly affected until the last (24-hour) time point, when a significant decrease was noted. Data represent mean \pm S.E.M. of three independent experiments; * $P < 0.05$ shows a significant change compared with baseline control (time 0). (E) Dose-dependence of H_2O_2 induced PARylation (PARP1 activation) in U937 cells, as detected at 10 minutes. Please note that the threshold of PARP1 activation occurs at $200 \mu M$. Control lanes show unchanged protein levels of PARP1 and PKA under the same experimental conditions. A representative Western blot of $n = 3$ determinations is shown.

cyclase inhibitor DDA reduced, while the adenylyl cyclase activator FSK increased, intracellular cAMP (Fig. 7C). These results confirm the functionality of the β -receptor-mediated signaling in the current experimental system of U937 cells. The regulation of PARP1 activation by propranolol and the PKAi appears to be a general phenomenon, as it was also demonstrated in C2C12 cell lines (Fig. 8). Based on these data we conclude that β -adrenoceptor signaling, via cAMP and PKA, regulates the early-onset mitochondrial PARP1 activation during oxidative stress.

β -Adrenoceptors, Adenylyl Cyclase, and PKA Regulate Mitochondrial PARP1 Phosphorylation during Oxidative Stress. To explore the potential phosphorylation of PARP1 during oxidative stress, we have conducted PLA assays. The results demonstrate that phosphorylation of PARP1 (similarly to the PARylation of PARP1) occurs early

(10 minutes) in response to oxidative stress, and is localized to the extranuclear (mitochondrial) compartment (Fig. 9A). Immunoprecipitation of PARP1 from control and H_2O_2 -treated U937 cells showed that oxidant exposure resulted in an increase in PARP1 phosphorylation on serine residues, whereas phosphorylation on threonine and tyrosine residues remains unchanged (Fig. 9B). Additional PLA analysis showed that oxidative stress induces both the auto-PARylation and the phosphorylation of PARP1, with phosphorylation being exclusive to the extranuclear (mitochondrial) compartment (Fig. 9C). Propranolol, PKAi and siRNA-mediated depletion of PKA all reduced the phosphorylation of PARP1 (Fig. 9, C and D), together with an attenuation of the auto-PARylation of PARP1 (Fig. 9C). No stable proximity/interaction was detected between PKA and PARP1 in baseline conditions, while an interaction may occur in the extranuclear component during

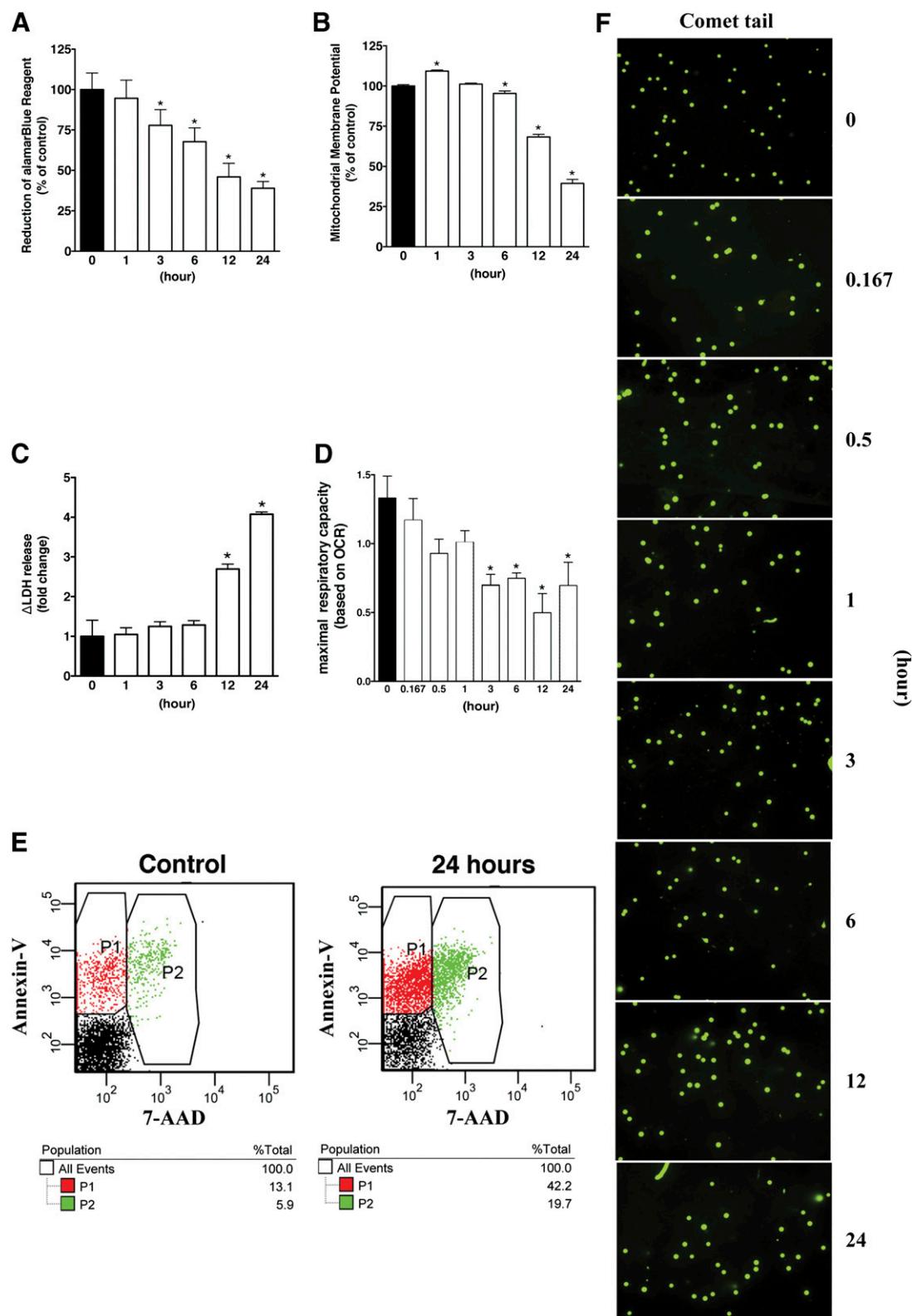


Fig. 3. H₂O₂ challenge induces a progressive decline of mitochondrial function, culminating in cell death of mixed type in U937 cells. (A) Time-dependent alterations in mitochondrial metabolic activity loss; (B) decrease in mitochondrial membrane potential; (C) decreased cell membrane integrity, evidenced by the release of LDH to the tissue culture medium; (D) decreased mitochondrial respiratory capacity (FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone)- mitochondrial oxidative phosphorylation inhibitor-induced maximal respiratory rate, indicating the bioenergetic parameter known as “spare respiratory reserve capacity”). Data represent mean ± S.E.M. of three independent experiments; **P* < 0.05 shows a significant change compared with baseline control (time 0). (E) Flow cytometry analysis shows that by 24 hours after H₂O₂ challenge, cells undergo death via a mixed (necrotic/apoptotic) route. Despite these mitochondrial alterations, and evidence of cell death, there was no significant full-scale nuclear DNA fragmentation, as evidenced by Comet assay throughout the 24-hour experimental period (F).

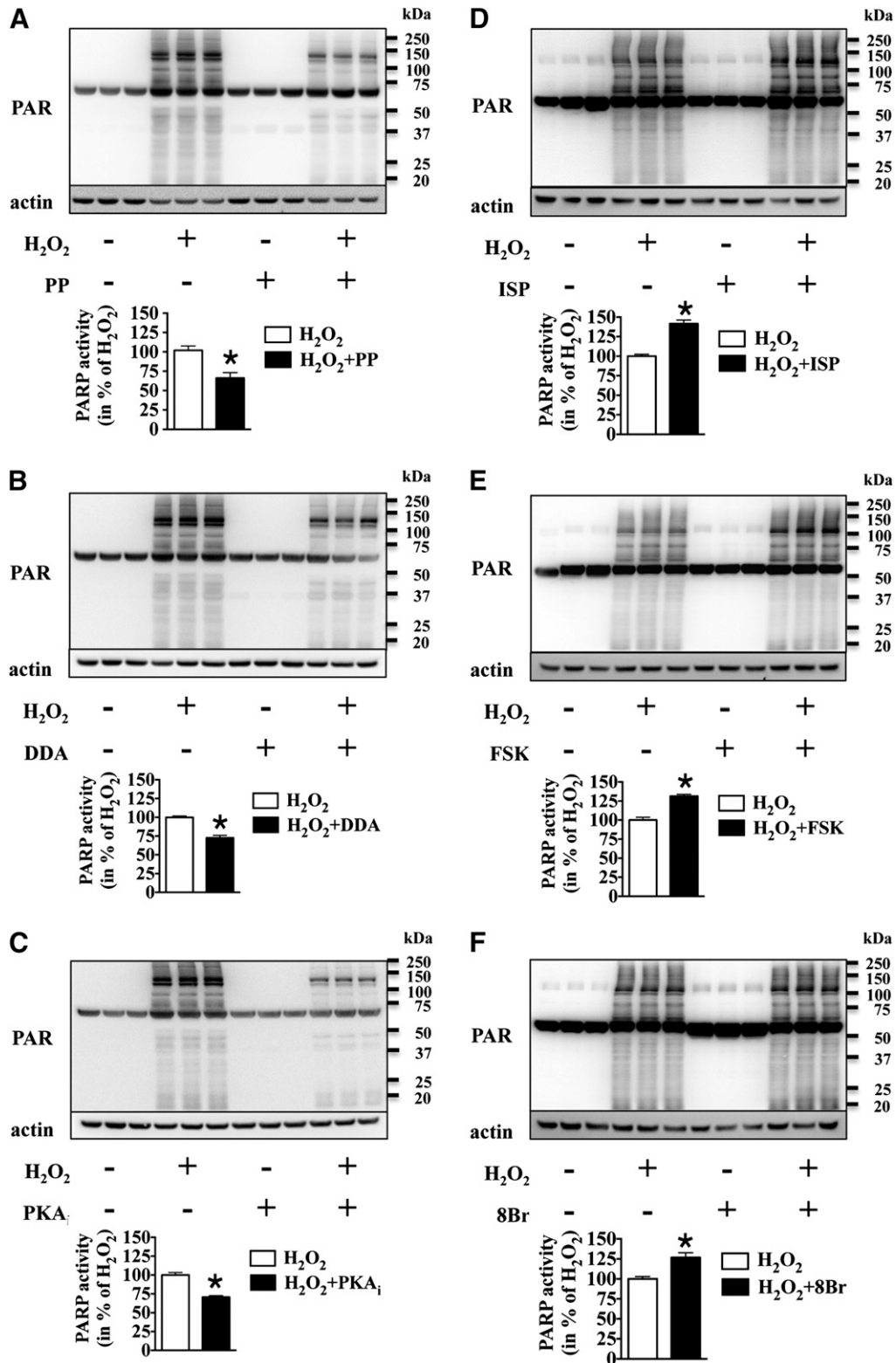


Fig. 4. β -adrenoceptor/cAMP/PKA signaling regulates PARP activation in U937 cells during oxidative stress. The β -adrenoceptor antagonists propranolol (PP) (A), the adenylyl cyclase inhibitor DDA (B), or the PKA inhibitor PKAi (C) decrease cellular PARylation in U937 cells treated with H₂O₂. The β -adrenoceptor agonist isoproterenol (ISP) (D), the adenylyl-cyclase activator FSK (E), and the PKA activator 8Br-cAMP (F) enhance cellular PARylation during oxidative stress. The Western blot shown includes three separate technical replicates (lanes) for each experimental condition. Densitometric analysis of PARylation in H₂O₂-treated cells was set as 100% and effect of pharmacological modulators is presented as mean \pm S.E.M. of three independent experiments; * P < 0.05.

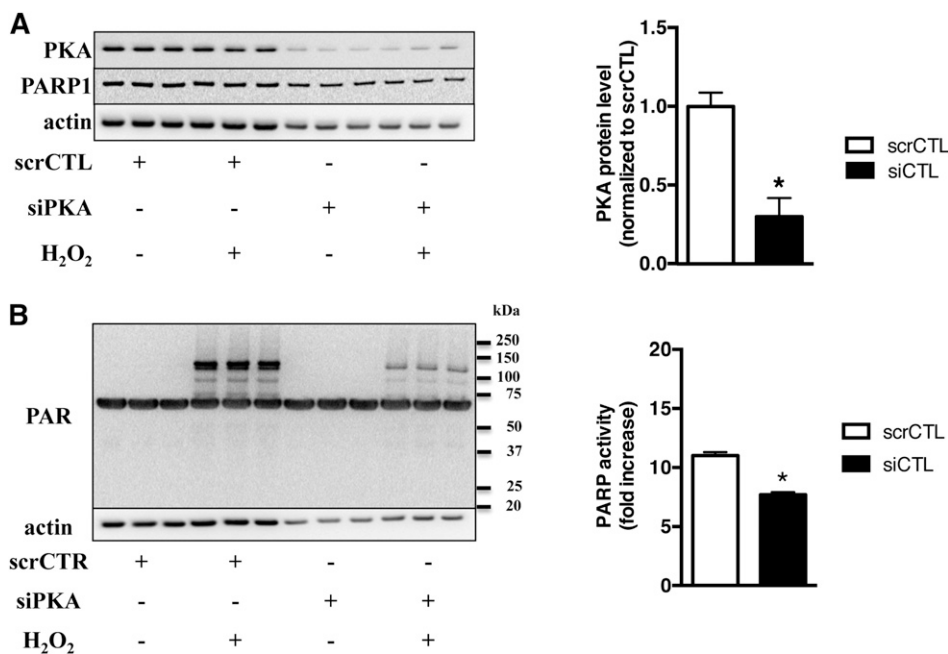


Fig. 5. PKA-depletion decreases cellular PARylation in H₂O₂ treated U937 cells. (A) The level of PKA-depletion in untreated and H₂O₂ treated cells was analyzed by Western blotting. PARP-1 protein level remains unchanged in scrCTL and siCTL transfected U937 cells after 48 hours. (B) PKA-depletion reduces cellular PARylation in H₂O₂ treated U937 cells. Quantification is based on three independent experiments, all normalized to the corresponding beta-actin control, and expressed as changes (%) compared with siCTL transfected U937 cells followed H₂O₂ treatment. **P* < 0.05.

oxidative stress (Fig. 9A). Based on these findings, we conclude that during oxidative stress PKA specifically phosphorylates mitochondrial PARP1 on serine residues, thereby increasing its catalytic activity.

PKA Directly Phosphorylates PARP1 in Vitro. To directly investigate the phosphorylation of PARP1 by PKA, we next conducted in vitro phosphorylation assays using recombinant PARP1 and PKA. As expected, activation of PARP1 by the combined addition of NAD⁺ and sonicated DNA resulted in its auto-PARylation and its slower migration (<250 kDa, lanes 1, 3, 5), which was prevented with the PARP inhibitor (Jagtap et al., 2002) PJ34 (lane 4) (Fig. 10A). In the presence of NAD⁺/DNA, but in the absence of added cAMP, only very low levels of PKA-mediated phosphorylation of monomeric PARP1 were detected (116 kDa, lane 3), but phosphorylation was seen at >250 kDa, most probably indicating the presence of phosphorylated, and PARylated, perhaps polymeric PARP1 units (Fig. 10A). cAMP triggered an increase in PARP1 phosphorylation, particularly for the hyper-PARylated high-molecular-weight band (>250 kDa, lane 5). The phosphorylation of PARP1 was reduced by the addition of the PKA inhibitor (lane 1). In the absence of NAD⁺/DNA, PARylation was very low (lane 2), and reduced PARP1 phosphorylation was

seen at 116 kDa, but not at >250 kDa (Fig. 10, A and B). In a subsequent set of studies we first allowed the PARylation and phosphorylation reactions to occur, and then added recombinant PARG to remove the PAR adducts from PARP1. As expected, PARG reduced the PARylation signal of PARP1 at the >250 kDa lanes. Moreover, the addition of PARG resulted in an increased phosphorylation band of PARP1 at 116 kDa PARP1, which we interpret as the result of a relative enrichment of the 116 kDa PARP1 under these conditions (Fig. 10B).

Mass spectrometry analysis confirmed the direct phosphorylation of PARP1 and localized it to serines 465 (in the auto-modification domain) and 782 and 785 (both in the catalytic domain) (Fig. 10C). Taken together, these data directly confirm 1) the phosphorylation of PARP1 by PKA and 2) the functional consequence of this interaction in terms of PARylation capacity of the enzyme. In addition, these findings suggest that optimal PARP1 phosphorylation by PKA requires its concomitant auto-PARylation.

Phosphorylation of PARP1 Regulates Oxidant-Induced Cell Death. One of the important pathophysiologic roles of PARP1 activation is its importance in mediating cellular bioenergetic dysfunction and an active form of nonapoptotic cell death during oxidative stress (see Jagtap and Szabo, 2005;

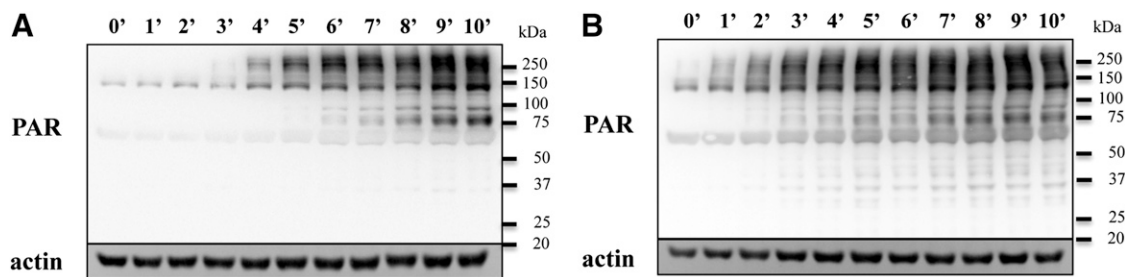


Fig. 6. Time-dependent activation of PARP upon H₂O₂-treatment in U937 cells is accelerated in the presence of isoproterenol. (A) The earliest detectable level of PARylation occurring at 4 minutes postchallenge with H₂O₂ (400 μM). (B) Pretreatment of U937 cells with β-adrenoceptor activator isoproterenol (ISP) accelerates the onset of PARylation. Representative Western blots of *n* = 3 determinations are shown.

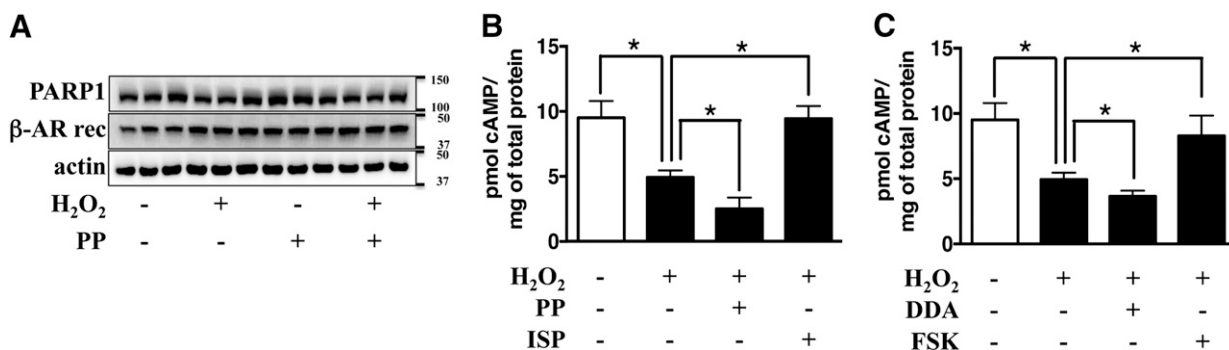


Fig. 7. Intracellular cAMP levels are regulated by β -adrenoceptor signaling in U937 cells during oxidative stress. (A) The expression of the β -adrenoceptor is unaffected by propranolol (PP) or H₂O₂ exposure. The Western blot shows three separate technical replicates (lanes) for each experimental condition. (B) cAMP levels decrease in response to H₂O₂; this response is further enhanced by inhibition of β -adrenoceptor by propranolol (PP). In contrast, cAMP levels are elevated by the β -adrenoceptor agonist isoproterenol (ISP). (C) The adenylyl cyclase inhibitor DDA reduces, while the adenylyl cyclase activator FSK increases intracellular cAMP levels. Data represent mean \pm S.E.M. of three independent experiments; * $P < 0.05$.

Burkle and Virag, 2013). At 6 hours after H₂O₂ challenge there was a significant suppression of mitochondrial function (ATP turnover and maximal respiratory capacity), as detected by Extracellular Flux Analysis of the mitochondria isolated from the cells (Figs. 3D and 11A); this was prevented by pretreating the cells with propranolol (Fig. 11B). To investigate the possible physiologic relevance of β -adrenoceptor signaling pathway via PKA-mediated PARP1 phosphorylation, we measured LDH release, an index of cell necrosis. Oxidative stress induced a delayed-onset cell death, with LDH release starting to increase at 12 hours after H₂O₂ challenge and becoming highly significant by 24 hours (Fig. 3B). Pharmacological inhibitors of β -adrenoceptor signaling pathway reduced, while activators of the same pathway increased, the oxidant-induced cellular LDH-release response, indicative, respectively, of a suppression or enhancement of oxidant-induced cell necrosis (Fig. 11C). The magnitude of the protective effect produced by the inhibitors of the β -adrenoceptor/cAMP/PKA axis was comparable with the effect of the pharmacological PARP1 inhibitor PJ34.

The inhibition of PARP1 activation by propranolol was also confirmed *in vivo*, in a model of systemic inflammation induced by the bacterial cell wall component lipopolysaccharide (LPS) (Fig. 11D). Taken together, these data point to a functional role of PARP1 phosphorylation by PKA in modulating an early-stage mitochondrial PARP1 activation (Fig. 12), which, in turn, modulates the subsequent, oxidant-mediated mitochondrial dysfunction and cell death.

Discussion

The main findings of the current study are the following: 1) An early and specific activation of PARP1 occurs in the mitochondrial compartment in oxidatively stressed U937 cells; 2) PARP1 is simultaneously phosphorylated and catalytically activated by PKA during oxidative stress; 3) the phosphorylation of PARP1 by PKA primarily occurs in the extranuclear (mitochondrial) component and occurs on serines 465 (in the automodification domain) and 782 and 785 (both in the catalytic domain); 4) the functional consequences of this mechanism are reflected by the modulation of oxidant-mediated mitochondrial bioenergetic dysfunction and cell death by pharmacological modulators of the β -adrenoceptor/cAMP/PKA axis.

PARP1 activity has previously been shown to be regulated by various endogenous factors (including xanthines, purines, estrogen) (Virag and Szabo, 2001; Mabley et al., 2005; Geraets et al., 2006; Szabo et al., 2006; Mabley et al., 2007) and has been shown or suggested to be regulated/phosphorylated by protein kinase C, mitogen-activated protein kinase, and extracellular signal-regulated kinases 1 and 2 (Tanaka et al., 1987; Kauppinen et al., 2006; Hegedus et al., 2008). Based on the current findings, we conclude that the cAMP/PKA system represents an additional mode of the regulation of its activity via phosphorylation. The (patho)physiologic significance of this system is highlighted by the fact that the phosphorylation and PARylation responses, as well as the functional responses (e.g., cell death), are modulated not only by pharmacological modulators of PKA (such as forskolin, or PKAi), but also by agonists and antagonists of β -adrenoceptors, which are functionally coupled to the cAMP system. Interestingly, the β -adrenoceptor blocker propranolol reduced intracellular cAMP levels and exerted functional effects on PKA and PARP1 in the absence of any exogenously added adrenergic agonists (Fig. 3 B and C). Thus, the responses noted here are interpreted as the consequence of a basal cellular activity of the β -receptor/cAMP system, perhaps as maintained/stimulated by the physiologic levels of catecholamines in the 10% fetal calf serum present in the culture medium.

PARP1 is a constitutive nuclear, as well as—at least in some cell types—a mitochondrial enzyme that is catalytically activated by DNA strand breaks (see Jagtap and Szabo, 2005; Burkle and Virag, 2013). Recognition of DNA strand breaks

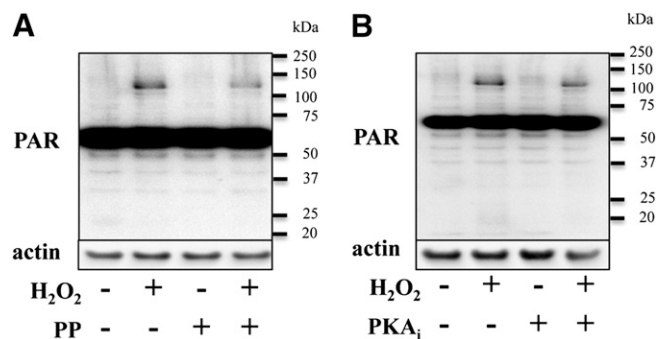


Fig. 8. The β -adrenoceptor/cAMP/PKA system regulates PARP activity in C2C12 cell line. The β -adrenergic receptor antagonist propranolol (PP) (A) or the protein kinase inhibitor PKAi (B) decrease cellular PARylation induced by H₂O₂ in C2C12 cells. Representative Western blots of $n = 3$ determinations are shown.

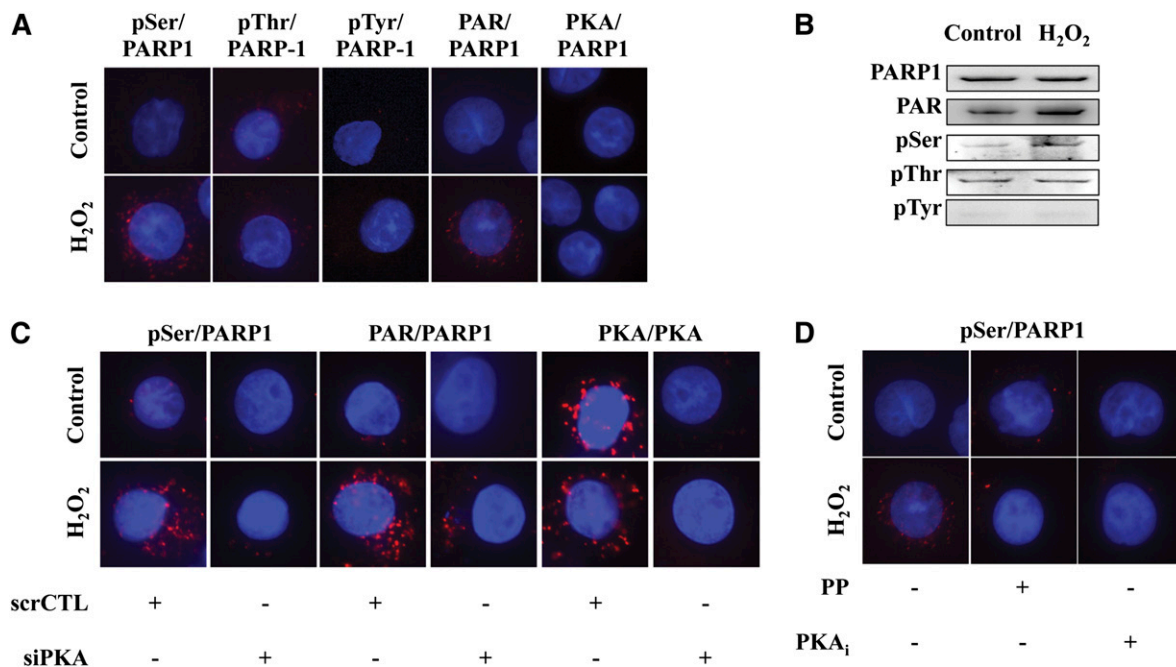


Fig. 9. PARP1 is specifically phosphorylated by PKA on serine residues in U937 cells during oxidative stress. (A) Serine-specific phosphorylation of PARP1, as evidenced by in situ PLA analysis. Control experiments show the auto-PARylation of PARP1. (B) H₂O₂ induces serine-specific (but not threonine- and tyrosine-specific) phosphorylation of PARP1. (C) Serine-specific phosphorylation of PARP1 is reduced by propranolol (PP) and PKA_i in H₂O₂-treated cells. (D) PKA silencing reduces the phosphorylation of PARP1 on serine residues and attenuates the level of PARP1 auto-PARylation. Figures show representative images of at least *n* = 3 independent determinations conducted on different experimental days.

by their zinc fingers induces a conformational change, which increases the catalytic activity of the enzyme; the enzyme converts its substrate, NAD⁺ to (ADP-ribose) groups, which

are attached to acceptor proteins, as well as to itself, and releases its byproduct, nicotinamide, which acts as a feedback inhibitor of the enzyme. The results of the current study

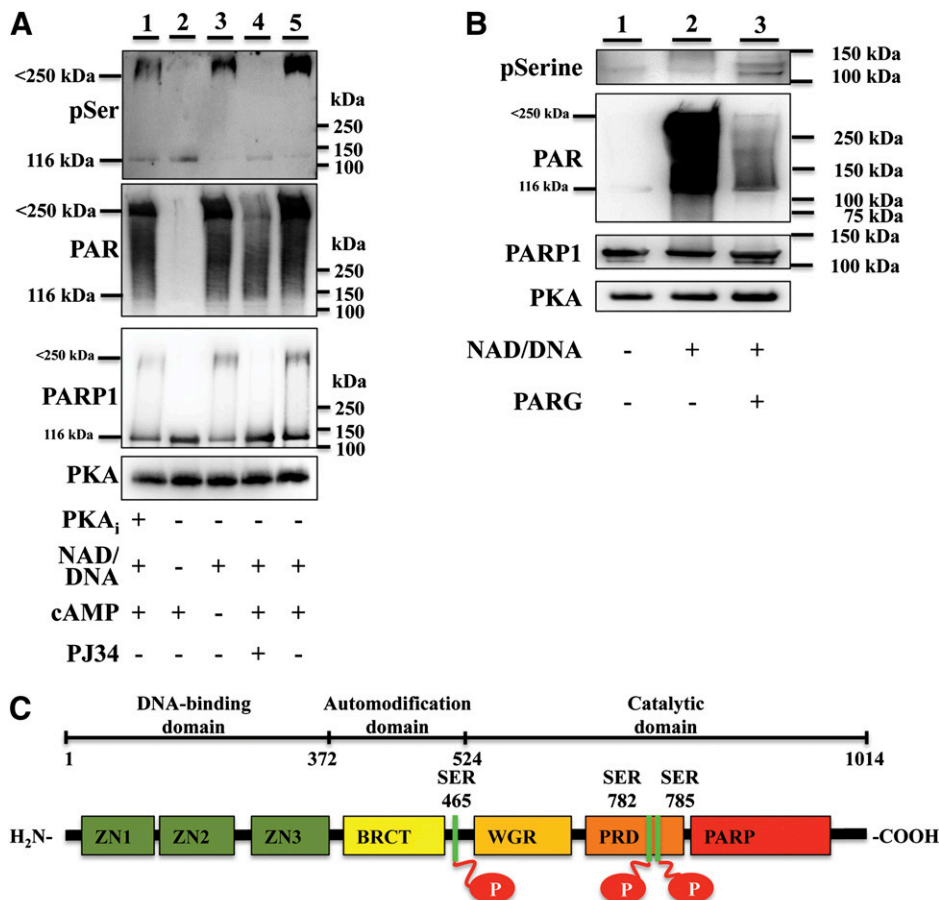


Fig. 10. Phosphorylation of PARP1 requires its auto-PARylation. (A) In vitro phosphorylation assay with PKA and PARP1 recombinant proteins was analyzed by Western blotting. The catalytic activity of PKA increased by the addition of cAMP or NAD⁺/DNA and decreased by PKA_i or the PARP1 inhibitor PJ34. (B) The phosphorylation of the 116-kDa auto-PARylated PARP1 was enhanced by the removal of PAR-residues with PARG. Figures show representative blots of at least *n* = 3 independent determinations conducted on different experimental days. (C) The in vitro phosphorylation data were extended by LC-tandem mass spectrometry analysis showing three different phosphorylations of PARP on serine residues Ser465, 782, and 785.

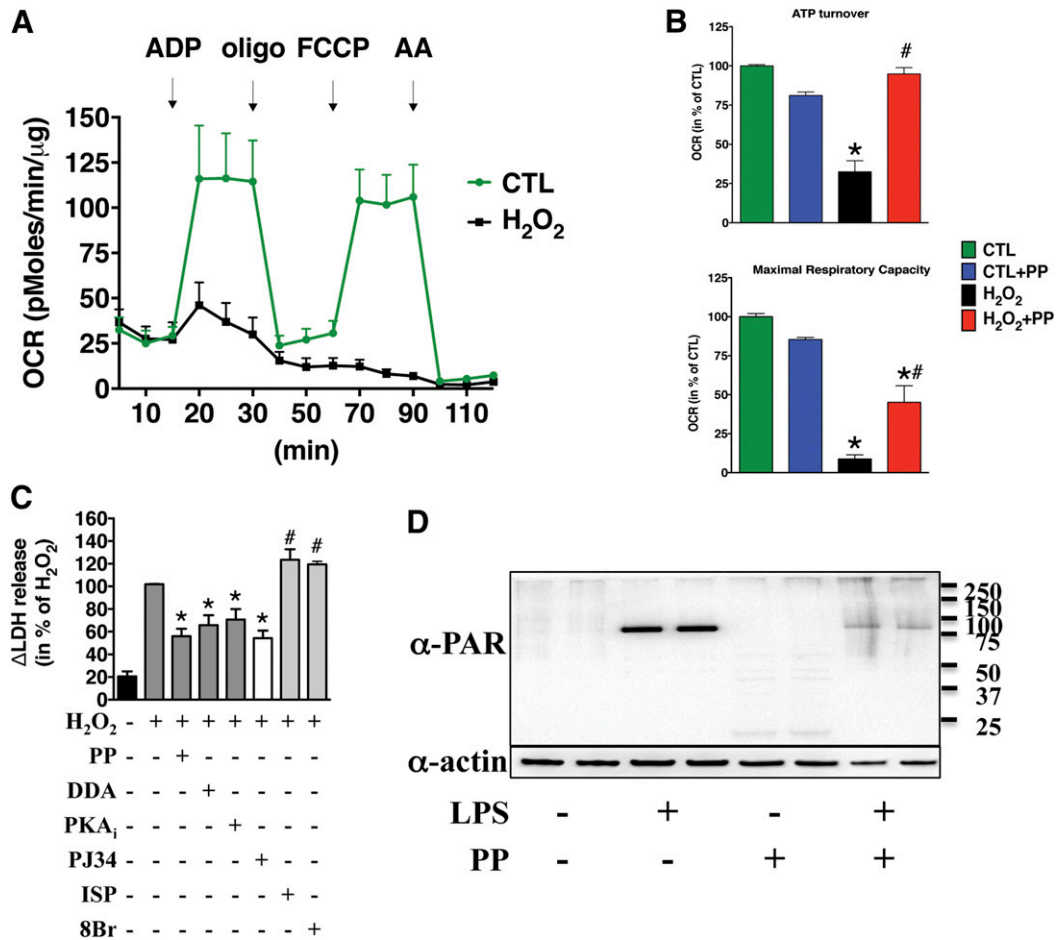


Fig. 11. The β -adrenoceptor/cAMP/PKA axis regulates oxidant-induced cell damage in U937 cells. (A) Extracellular flux analysis conducted on isolated mitochondria indicates a strong dysfunction in ADP-induced oxygen consumption and maximal respiratory capacity in cells exposed to oxidative stress for 6 hours. (B) Pretreatment of cells with propranolol prevents the decline in oxygen consumption and the suppression of maximum respiratory capacity of mitochondria. $*P < 0.05$ shows a decrease in the bioenergetic parameter in response to oxidative stress; $^{\#}P < 0.05$ shows a protective effect of propranolol pretreatment; $n = 4$. (C) H₂O₂-induced LDH release was reduced by the β_2 -adrenoceptor inhibitor propranolol (PP), the adenylyl cyclase inhibitor DDA, or by the PKA inhibitor (PKAi), as well as by the positive control PARP1 inhibitor (PJ34), while it was increased by the β_2 -adrenoceptor activator isoproterenol (ISP) and by the PKA activator 8Br-cAMP. Percent changes are shown as mean \pm S.E.M. of three independent experiments; $*P < 0.05$ shows a significant reduction, while $^{\#}P < 0.05$ shows a significant enhancement of H₂O₂-mediated LDH release by the various pharmacological interventions. (D) Auto-PARylation of PARP1 is reduced by propranolol treatment in an LPS model of systemic inflammation in mice. Propranolol (PP) attenuated the LPS-induced PARylation of PARP1. Representative experiments (two bands showing isolations from two separate sets of mice) of $n = 3$ sets of studies are shown. Each lane represents peripheral blood mononuclear cells (PBMCs) isolated from five animals and pooled for analysis.

demonstrate that mitochondrial PARP, during the early stage of oxidant-induced cell death, undergoes a rapid catalytic activation, which is enhanced by a PKA-mediated rapid-onset phosphorylation of PARP1 in specific serine residues Ser465, 782 and 785. We hypothesize that the phosphorylation of PARP1 may put the enzyme in a more active conformational form, and/or may increase the binding of the enzyme to DNA strand breaks, thereby providing a more sustained stimulus for its activation. In line with our current results, prior studies by Poirier and coworkers have previously proposed several putative phosphorylation sites in PARP1, including S⁴⁶⁵, S⁷⁸² and S⁷⁸⁵ (Gagné et al., 2009).

Although PARP1 activation and the associated cell death response were initially attributed to events in the nuclear compartment, subsequent studies demonstrated the mitochondrial presence of PARP1 and an independent role for mitochondrial PARP1 in mediating cell death. (Masmoudi et al., 1988; Du et al., 2003; Rossi et al., 2009). Based on the results of the PLA assays, we conclude that the mitochondrial PARP1 activation

is an early event in oxidant-induced cell injury and, in line with the extranuclear/mitochondrial localization of PKA (Gancedo, 2013; Taylor et al., 2013; Valsecchi et al., 2013), the PKA/PARP1 interactions primarily occur in the extranuclear/mitochondrial compartment. The current findings are consistent with prior studies demonstrating the stimulation of mitochondrial PKA activity by treatment of the cells with β -receptor ligands such as isoproterenol (Lefkimmatis et al., 2013) and are also consistent with prior data showing the direct regulation of mitochondrial PKA activity by cytosolic cAMP (Lefkimmatis et al., 2013). Based on the results of our cell-free assays, we conclude that the phosphorylation of PARP1 by PKA does not require additional “scaffolding” or “connector” proteins, as it occurs in response to cAMP stimulation in a simple reconstituted system that consists of PKA, PARP1, and NAD⁺ (the substrate of PARP1) and sonicated (broken) DNA (used to activate PARP1).

PARP1 activation during oxidative stress promotes an active form of cell necrosis (Szabo et al., 1996; Virag et al.,

Oxidative stress

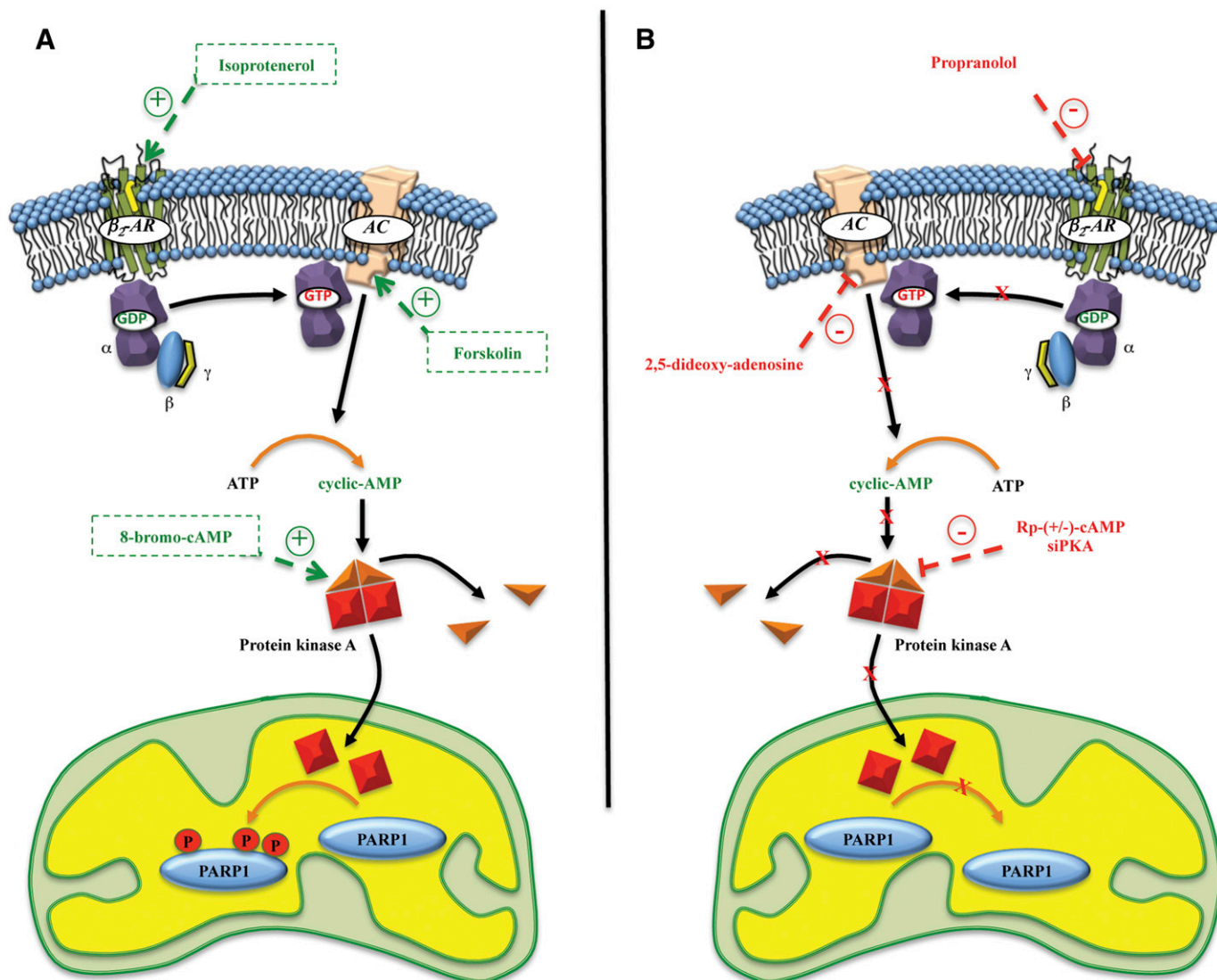


Fig. 12. Proposed model of PKA-PARP1 interactions in the early stage of oxidative stress in U937 cells. Upon oxidative stress, mitochondrial DNA strand breakage occurs, which activates PARP1 in the mitochondria. (A) The cAMP/PKA axis is stimulated by β -adrenoceptor agonists (such as isoproterenol), cAMP analogs (such as 8-bromo-cAMP), and by adenylyl cyclase activators (such as forskolin), leading to mitochondrial PARP1 phosphorylation and increased PARP activity. (B) The cAMP/PKA axis is inhibited by β -adrenoceptor antagonists (such as propranolol), by adenylyl cyclase inhibitors (such as 2,5-dideoxy-adenosine), and by inhibition/downregulation of PKA (by the pharmacological inhibitor 8-Bromo-adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer or by genetic silencing of PKA) leading to the inhibition of mitochondrial PARP1 phosphorylation and decreased PARP activity.

1998; Ha and Snyder, 1999). It is also an essential part of a form of cell death called as parthanatos, which also involves mitochondrial dysfunction and exhibits features of both necrosis and apoptosis (Zhang et al., 1994; Yu et al., 2006; Wang et al., 2009). In the current assays, by measuring LDH release (which occurs when the integrity of the cell membrane is broken down, and cells release their intracellular content), our experiments quantified an end-point of the process of cell necrosis, which, according to our flow cytometry data (Fig. 3E), occurs in a significant portion of the cell population by 24 hours. In line with prior findings (Jagtap et al., 2002), the positive control pharmacological PARP1 inhibitor (PJ34) afforded cytoprotective effects that were comparable, in magnitude, to the effects seen with the various pharmacological

inhibitors of the cAMP/PKA axis. Previous studies have demonstrated the cytoprotective effect of PKA inhibition in various experimental systems (Leadsham and Gourlay, 2010; Alamdary et al., 2013). Based on our data, we hypothesize that the prevention of mitochondrial PARP1 phosphorylation/overactivation may be a component of these protective actions. It should also be noted that recent studies have demonstrated that oxidative stress, on its own, activates mitochondrial PKA (Srinivasan et al., 2013). Thus, the interactions described in the current report probably represent the functional consequence of an interaction between oxidative-stress-activated PKA and oxidative-stress-activated PARP1.

Several lines of studies emphasize the independent causative roles of early mitochondrial events in the pathogenesis of

subsequent cell death response (Tann et al., 2011; Guicciardi et al., 2013; Virag et al., 2013; Dorn, 2013). In the current experimental model, early oxidant-mediated mitochondrial PARP1 activation, as well as other mitochondrial events, such as a progressive mitochondrial oxidant production (Fig. 2A) and mitochondrial electron transport defects (Fig. 11), probably contribute to the cell death. The results of the current research demonstrate that these processes are attenuated by blocking the early-onset PKA activation and the subsequent PARP1 activation. The working model outlined in Fig. 12 shows one possible interpretation of the findings of the current report: the cAMP/PKA axis is stimulated by β -adrenoceptor agonists (such as isoproterenol), cAMP analogs (such as 8-bromo-cAMP), and by adenylyl cyclase activators (such as forskolin), leading to mitochondrial PARP1 phosphorylation and increased PARP activity. Moreover, the cAMP/PKA axis is inhibited by β -adrenoceptor antagonists (such as propranolol), by adenylyl cyclase inhibitor (such as DDA) and by inhibition/downregulation of PKA (by the pharmacological inhibitor Rp-cAMP or by genetic silencing of PKA) leading to the inhibition of mitochondrial PARP1 phosphorylation and decreased PARP activity. In the Fig. 12, mitochondrial PKA is shown to be stimulated by cytosolic cAMP. It must be pointed out that multiple lines of data indicate that the cytosolic and mitochondrial cAMP pools do not communicate in most cases. At the same time, several sets of data also indicate that cell membrane and cytosolic signals can elevate intramitochondrial PKA activity, followed by phosphorylation of intramitochondrial proteins (reviewed in Lefkimiatis and Zaccolo, 2014). Yet another possibility may be the extramitochondrial phosphorylation of PARP1, followed by its transport into the mitochondria, even though the early time course of extranuclear PARP1 phosphorylation (see Fig. 9) tends to speak against this possibility.

Although many additional details of the underlying mechanisms remain to be explored, the current findings may have implications for a number of cellular processes that are known to be regulated by PARP1, including cellular metabolism and DNA repair, given the fact that both of these processes can be regulated by the β -adrenoceptor/cAMP system (Carlucci et al., 2008; Cho and Juhn, 2012; Valsecchi et al., 2013; Lefkimiatis and Zaccolo, 2014). However, these aspects remain to be directly investigated in further studies. The current findings may also provide a mechanistic explanation for our previously observed clinical/translational findings (Olah et al., 2011) showing that treatment of patients with severe burn injury with propranolol suppresses PARP1 activation in endothelial cells and tissue-resident mononuclear cells. Further studies are needed to determine whether modulation of PARP1 phosphorylation and its consequent catalytic activity by various drugs targeting the β -adrenoceptor/cAMP/PKA system may be used for therapeutic modulation of PARP1 in various pathophysiologic conditions associated with oxidative stress.

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Authorship Contributions

Participated in research design: Szabo, Brunyanszki and Szczesny.
Conducted experiments: Brunyanszki, Coletta, Oláh, Szczesny.
Performed data analysis: Brunyanszki, Szczesny.
Wrote or contributed to the writing of the manuscript: Szabo, Brunyanszki, Szczesny.

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