Pivotal Role of Akt Activation in Mitochondrial Protection and Cell Survival by Poly(ADP-ribose)polymerase-1 Inhibition in Oxidative Stress

Antal Tapodi, Balazs Debreceni, Katalin Hanto, Zita Bognar, Istvan Wittmann, Ferenc Gallyas, Jr., Gabor Varbiro, and Balazs Sumegi

From the Department of Biochemistry and Medical Chemistry, First Department of Medicine, Division of Cardiology, Second Department of Medicine, Faculty of Medicine, and the Hungarian Academy of Sciences, Research Group for Mitochondrial Function and Mitochondrial Diseases, Faculty of Medicine, University of Pecs, 12 Szigeti Street, Pecs 7624, Hungary

According to the classical view, the cytoprotective effect of inhibitors of poly(ADP-ribose)polymerase (PARP) in oxidative stress was based on the prevention of NAD⁺ and ATP depletion, thus the attenuation of necrosis. Our previous data on PARP inhibitors in an inflammatory model suggested that PARP-catalyzed ADP-ribosylations may affect signaling pathways, which can play a significant role in cell survival. To clarify the molecular mechanism of cytoprotection, PARP activity was inhibited pharmacologically by suppressing PARP-1 expression by a small interfering RNA (siRNA) technique or by transdominantly expressing the N-terminal DNA-binding domain of PARP-1 (PARP-DBD) in cultured cells. Cell survival, activation of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt system, and the preservation of mitochondrial membrane potential were studied in hydrogen peroxide-treated WRL-68 cells. Our data showed that suppression of the single-stranded DNA break-induced PARP-1 activation by pharmacological inhibitor, siRNA, or by the transdominant expression of PARP-DBD protected cells from oxidative stress and induced the phosphorylation and activation of Akt. Furthermore, prevention of Akt activation by inhibiting PI3-kinase counteracted the cytoprotective effect of PARP inhibition. Microscopy data showed that PARP inhibition-induced Akt activation was responsible for protection of mitochondria in oxidative stress because PI3-kinase inhibitors diminished the protective effect of PARP inhibition. Similarly, Src kinase inhibitors, which decrease Akt phosphorylation, also counteracted the protection of mitochondrial membrane potential supporting the pivotal role of Akt in cytoprotection. These data together with the finding that PARP inhibition in the absence of oxidative stress induced the phosphorylation and activation of Akt indicate that PARP inhibition-induced Akt activation is dominantly responsible for the cytoprotection in oxidative stress.

Under several pathological conditions, reactive oxygen species-induced damages play important roles in pathogenesis (1–3). High levels of reactive oxygen species are generated from a variety of sources such as the xanthine oxidase system (1), the leakage of electrons from the mitochondrial respiratory chain (2, 4), the cyclooxygenase pathway of arachidonic acid metabolism (3, 5), and the respiratory burst of phagocyte cells (6, 7), and they can cause DNA damage-generating single-stranded DNA breaks (8). Poly(ADP-ribose)polymerase (PARP-1, EC 2.4.2.30) is a multifunctional nuclear enzyme (9) that is activated by DNA strand breaks and catalyzes the covalent coupling of branched chains of ADP-ribose units to various nuclear proteins such as histone proteins and PARP-1 itself. PARP-1 is involved in chromatin remodeling, DNA repair, replication, transcription, and the maintenance of genomic stability by, in part, poly(ADP-ribose)lation (9). With moderate amounts of DNA damage, PARP-1 is thought to participate in the DNA repair process (10, 11). However, oxidative stress, which induces a large amount of DNA damage, can cause excessive activation of PARP-1, leading to depletion of its substrate NAD⁺; and in an effort to resynthesize NAD⁺, ATP is also depleted, resulting in cell death as a consequence of energy loss (12–15). PARP inhibitors show pronounced protection against myocardial ischemia (16), neuronal ischemia (17, 18), acute lung inflammation (19), acute septic shock (20), zymogen-induced multiple organ failure (21), and diabetic pancreatic damage (22–24), providing evidence for the role of excessive PARP-1 activation in cell death. It is believed that by preventing excessive NAD⁺ and ATP utilization, PARP inhibitors protect cells against oxidative damage, but some recent data suggest a more complex mechanism for the cytoprotection (25, 26).

There is evidence that PARP activation can contribute to exaggeration of mitochondrial damage (27) and mitochondrial reactive oxygen species production (28), indicating that PARP activation can modulate processes outside of the nucleus. Recent works reported the existence of mitochondrial PARP that can be blocked with PARP-1 inhibitors (29); therefore, it would be important to clarify whether the mitochondrial protection by PARP inhibitors is a direct consequence of the inhibition of mitochondrial ADP-ribosylation or the inhibition of nuclear PARP modulation by yet unidentified processes that are responsible for the mitochondrial protection. Our previous works demonstrated that PARP inhibitors induced the phosphorylation and activation of Akt in the liver, lung, and spleen of lipopolysaccharide-treated mice, raising the possibility that the protective effect of PARP inhibition can be mediated through the PI3-kinase/Akt pathway (30). These observations indicate that the protective effect of PARP inhibitors should be far more

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1 To whom correspondence should be addressed. Tel.: 36-72-536-276; Fax: 36-72-536-277; E-mail: balazs.sumegi@aok.pte.hu.

2 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; C1 and N3, mammalian expression vector construct detailed in Fig. 1a; FCS, fetal calf serum; GFP, green fluorescent protein; GSK, glycogen synthase kinase; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MEM, minimum Eagle’s medium; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ECL, enhanced chemiluminescence; PAR, poly(ADP-ribose); PARP-DBD, N-terminal DNA binding domain of PARP; PI3-kinase, phosphatidylinositol 3-kinase; siRNA, small interfering RNA.
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complex than a mere NAD$^+$ and ATP depletion because Akt kinase can phosphorylate several regulatory proteins, including GSK3β, caspase-9, BAD, or FKHR (31), which can be involved in the stabilization of mitochondrial membrane system (32, 33).

In the present study, we analyzed the effect of PARP inhibition by pharmacologic agents, by the transdominant expression of the PARP N-terminal DNA-binding domain (PARP-DBD), and by the suppression of PARP expression by small interfering RNA (siRNA) in cultured cells during oxidative stress. We also analyzed the effect of PARP inhibition on the PI3-kinase/Akt pathway as well as on the preservation of cells during oxidative stress. We also analyzed the effect of PARP inhibition on the PI3-kinase/Akt pathway as well as on the preservation of cells during oxidative stress.

EXPERIMENTAL PROCEDURES

Materials—PI3-kinase inhibitors LY 294002 and wortmannin, PARP-1 inhibitor PJ-34, protease inhibitor mixture, and all of the chemicals for cell cultures were purchased from Sigma. Fluorescent dyes JC-1, fluorescein-conjugated annexin V, and propidium iodide were from Molecular Probes. The following antibodies were used: anti-phospho-Akt (Ser473) and anti-phospho-GSK3β (Cell Signaling Technology, Beverly, MA); anti-PAR and anti-PARP (Alexis Biotechnology, London, U.K.); anti-β-actin, anti-mouse IgG, and anti-rabbit IgG (Sigma).

Cell Culture—WRL-68 human liver cells were from the American Type Culture Collection (Wesel, Germany). The cells were maintained as monolayer adherent culture in minimum Eagle’s medium containing 10% fetal bovine serum (FCS) in a humid 5% CO2 atmosphere at 37 °C.

Transdominant Expression of the DNA-binding Domain of PARP—The coding region of the N-terminal DNA-binding domain of PARP (PARP-N214, amino acid residues 1–214 (34)) was amplified by PCR and cloned in-frame into pEGFP-C1/N3 vectors (Clontech) after cutting with HindIII and EcoRI restriction enzymes (Fermentase, Vilnius, Lithuania). For enabling active nuclear transport of the green fluorescent protein (GFP)-tagged PARP-N214, the nuclear localization signal was added to the N terminus of the PARP-N214 sequence using PCR primers coding for the nuclear localization signal sequence. The recombinant pPARPGFP-C1/N3 vectors were purified by a plasmid purification kit (Qiagen, Valencia, CA) and utilized for transient transfection of WRL-68 cells using LipoFectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For an effective transdominant expression of PARP-DBD, the transfection step was repeated 48 h after the first transfection, and the experiments on the cells were performed 40 h after the second transfection.

Suppression of PARP-1 Expression by siRNA Technique—WRL-68 cells were transiently transfected with siRNA designed for PARP suppression by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA) in Opti-MEM I Reduced Serum Medium (Invitrogen) using LipoFectamine 2000. For an effective suppression of PARP, the transfection step was repeated twice with a 48-h interval between the transfections, and the experiments on the cells were performed 40 h after the third transfection.

Cell Viability Assay—The cells were seeded into 96-well plates at a starting density of 10^4 cell/well and cultured overnight before H2O2 or different inhibitors modulating the effect of the H2O2 were added to the medium at a concentration and composition indicated in the figure legends. After 3 h of treatment, the medium was removed, and fresh MEM/FCS containing 0.5% of the water-soluble yellow mitochondrial dye MTT was added. Incubation was continued for an additional 3 h, and the MTT reaction was terminated by adding HCl to the medium at a final concentration of 10 mm. The amount of water-insoluble blue formasan dye formed from MTT was proportional to the number of live cells and was determined with an Anthos Labtech 2010 enzyme-linked immunosorbent assay reader at 550 nm wavelength after dissolving the blue formasan precipitate in 10% SDS. All experiments were run in at least four parallels and repeated three times.

Western Blot Analysis—The cells were seeded and treated as for the cell viability assay. After 1 h of treatment, the cells were harvested in a chilled lysis buffer of 0.5 mM sodium metavanadate, 1 mM EDTA, and protease inhibitor mixture in phosphate-buffered saline. The proteins were precipitated by trichloroacetic acid, washed three times with −20 °C acetone, and subjected to SDS-PAGE. Proteins (30 μg/lane) were separated on 12% gels and then transferred to nitrocellulose membranes. The membranes were blocked in 5% low fat milk for 1 h at room temperature, then exposed to the primary antibodies at 4 °C overnight at a dilution of 1:1,000 in blocking solution. Appropriate horseradish peroxidase-conjugated secondary antibodies were used for 2 h at room temperature and a 1:5,000 dilution. Peroxidase labeling was visualized with enhanced chemiluminescence (ECL) using an ECL Western blotting detection system (Amersham Biosciences). The developed films were scanned, and the pixel values of the bands were determined using NIH Image J software. All experiments were repeated four times.

Determination of NAD$^+$—The cells were seeded and treated as for the cell viability assay. All experiments were run in two (for transfection experiments) or three parallels and repeated twice. Harvesting and sample processing were performed according to Du et al. (29). Cellular NAD$^+$ levels were measured by the microplate version of the enzymatic cycling method using alcohol dehydrogenase exactly as described by Shah et al. (36) except for using iodonitrotetrazolium chloride instead of MTT in the assay buffer, the former having the advantage of being water-soluble. The reaction was monitered at 550 nm and was allowed to run for 10 min. A standard curve was generated using known concentrations of NAD$^+$ for the calculation of the cellular NAD$^+$ levels. Cellular protein contents of the cell homogenates were determined with the BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as standard.

Fluorescent Microscopy—Wild type or transfected WRL-68 cells were seeded to poly-l-lysine-coated (2.5–5 μg/cm²) glass coverslips and cultured at least overnight before the experiment. After subjecting the cells to the appropriate treatment (indicated in the figure legends), the coverslips were rinsed twice in phosphate-buffered saline then placed upside down on the top of a small chamber formed by a microscope slide and a press-to-seal silicone isolator filled with phosphate-buffered saline containing 4.5 g/liter glucose and 20 mM HEPES pH 7.4. Cells were imaged with an Olympus BX61 fluorescent microscope equipped with a ColorView CCD camera and analySISR software using a 60 × objective and epifluorescent illumination. For GFP fluorescence, 450–490 nm excitation and >520 nm emission (green) filters were used. For JC-1 fluorescence, the cells were loaded with the dye for 10 min, then the same microscopic field was imaged first with 546 nm bandpass excitation and >590 nm emission (red), then with green filters. Under these conditions we did not observe considerable bleed-through between the red and green images.

RESULTS

Transdominant Expression of PARP-DBD in WRL-68 Liver Cell Line and Its Effect on the PARP Auto-ADP-ribosylation in Oxidative Stress—To achieve nonpharmacological competitive inhibition of the PARP-1 enzyme, we transiently transfected WRL-68 liver cells with constructs expressing the hybrid proteins consisting of a nuclear localization signal and the PARP-DBD attached either to the N (N3) or the C terminus...
(C1) of GFP (Fig. 1A). As expected, the C1 and N3 proteins were localized to the nucleus (Fig. 1, B–D), their electrophoretic mobility corresponded to the theoretical value calculated from the molecular weight of GFP and PARP-DBD (Fig. 1E), and they inhibited both the unstimulated and the oxidative stress-induced PARP activity (Fig. 2).

Effect of Transdominant Expression of PARP-DBD on the Viability of WRL-68 Cells in Oxidative Stress—To evaluate the effect of the transdominant expression of PARP-DBD on the viability of human hepatocyte WRL-68 cells, we examined the direct cytotoxic effect of 0.3 mM H2O2 for 3 h (Fig. 3). The cell viability was detected by MTT assay and expressed as percent of the viability of untreated wild type cells. The transdominant expression of PARP-DBD protected WRL-68 cells from the H2O2-induced oxidative stress that proved to be significant (p < 0.01). These results suggest that the enzymatic activity of nuclear PARP-1 is involved in the cytotoxicity of H2O2, and the inhibition of PARP-1 either by a pharmacological agent or by a nonpharmacological way protects against oxidative stress-related injuries.

Comparison of the Effect of Transdominant Expression of PARP-DBD and of Pharmacological Inhibition of PARP on Akt Activation in Oxidative Stress—To establish the role of nuclear PARP-1 in regulating proteomic signal transduction pathways, we analyzed the activation of the Akt/protein kinase B pathway during oxidative stress in the presence of H2O2 (data not shown). The transfection of wild type WRL-68 liver cells or cells transfected with pEGFP, C1pEGFP (C1), or N3pEGFP (N3) plasmids were treated with 0, 0.3, 1, or 3 mM H2O2 for 3 h. Cell viabilities were detected by MTT assay and were expressed as a percent of the untreated wild type cells (Fig. 4). Wild type and transfected cells (C1, N3) was investigated under normal conditions without the induction of oxidative stress, its level was found to be elevated compared with the wild type WRL-68 cells. Transfection by pEGFP plasmid had no effect on the viability of WRL-68 cells in oxidative stress. WRL-68 liver cells transfected with pEGFP, C1pEGFP (C1), or N3pEGFP (N3) plasmids were treated with 0.3 mM H2O2 for 3 h as indicated below. Auto-ADP-ribosylation of PARP in the cell homogenates was detected by Western blotting utilizing an anti-ADP-ribose antibody. Even protein loadings were confirmed by an anti-actin antibody and Western blotting. ADP-ribosylation in pEGFP-transfected cells was identical to that in untransfected cells either in the presence or the absence of H2O2 (data not shown). Lane 1, untransfected control cells; lane 2, C1 cells; lane 3, N3 cells; lane 4, control cells + H2O2; lane 5, C1 cells + H2O2; lane 6, N3 cells + H2O2.

Western blotting utilizing an anti-PARP antibody. The transfection of PARP-siRNA inhibited the PARP expression both under normal conditions as well as in oxidative stress (Fig. 5). We investigated the effect of the suppression of PARP by the siRNA technique on the viability of WRL-68 cells in oxidative stress. WRL-68 liver cells transfected with PARP-siRNA as well as wild type cells were treated with 0, 0.3, 1, or 3 mM H2O2 for 3 h (Fig. 5). The cell viability was detected by MTT assay and expressed as percent of the untreated wild type cells. The suppression of PARP by the siRNA technique on the viability of WRL-68 cells in oxidative stress proved to be significant (p < 0.001, n = 24). ***: significantly different from H2O2-treated wild type (WT H2O2), p < 0.001 (n = 24); n.s., not different from WT H2O2.
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Effect of PARP Inhibition and Kinase Inhibitors on the Viability and NAD⁺ Content of WRL-68 Cells in Oxidative Stress—To establish further the role of the Akt pathway among the molecular mechanisms in the protective effect of PARP inhibition in H₂O₂-induced oxidative stress, we analyzed the effect of a specific PI3-kinase inhibitor, LY 294002, and an Src kinase inhibitor, Pp2 on the viability and NAD⁺ content of WRL-68 cells. The cells were treated for 3 h with 0.3 mM H₂O₂, 10 μM PJ-34, 10 μM LY 294002, or 10 μM Pp2, or with different combinations of these compounds as indicated. Cell viabilities were detected by MTT assay and were expressed as a percent of the untreated cells. PJ-34 significantly (p < 0.001) decreased the toxicity of H₂O₂ in WRL-68 cells. However, when LY 294002 at a concentration of 10 μM was also present, the protective effect of PJ-34 was significantly (p < 0.001) diminished (Fig. 8A). Similar results were obtained in the presence of 1 μM wortmannin (data not shown). The specific inhibitor of Src kinase; Pp2, at a concentration of 10 μM, significantly decreased the protective effect of PJ-34 on the H₂O₂-induced oxidative stress (Fig. 8A), the same way as the PI3-kinase inhibitors did. The specific PI3-kinase inhibitor LY 294002 and the Src kinase inhibitor diminished the protective effect of both the transdominant expression of PARP-DDB and suppression of PARP by siRNA compared with the pEGFP plasmid-transfected cells that were used as a negative control (Fig. 8B). When added alone to the cells, PJ-34, LY 294002, and Pp2 did not affect the viability of the cells.

To compare the significance of Akt activation with that of prevention of NAD⁺ depletion among the mechanisms of the cytoprotective effect of PARP inhibition, we determined the NAD⁺ content of the cells treated identically to those used for viability measurements. We found that the 3-h treatment with 0.3 mM H₂O₂ induced about an 80% drop in the cellular NAD⁺ content of both unprotected WRL-68 cells and the cells transfected with the pEGFP plasmid (Fig. 9). PJ-34 treatment, transdominant expression of the PARP-DDB, or suppression of PARP by siRNA partially, although significantly (p < 0.05 and p < 0.001) diminished this NAD⁺ depletion, resulting in levels of about 50% that of untreated cells (Fig. 9). In contrast to the cell viability measurements,
the PI3-kinase and Src kinase inhibitors did not interfere with the protective effect of PARP inhibition (Fig. 9). PARP inhibition or the PI3-kinase and Src kinase inhibitors did not affect the cellular NAD$^+$ levels in the absence of oxidative stress (Fig. 9). These data suggest that the diminishing of NAD$^+$ depletion alone could not account for the cytoprotective effect of PARP-1 inhibition, and the activation of the Akt/protein kinase B pathway should indeed be involved in the molecular mechanisms of it.

Effect of the PI3-Kinase and Src Kinase Inhibitors on the Oxidative Stress- and PARP Inhibition-induced Akt Pathway Activation—To confirm the previous conclusion, we analyzed the effect of PI3-kinase and Src kinase inhibitors in combination with that of PARP inhibition on oxidative stress-induced activation of Akt/protein kinase B and the phosphorylation of its downstream target GSK3β. The experimental setup was similar to that used for the viability and NAD$^+$ measurements. Wild type WRL-68 cells were treated for 1 h with 1 mM H$_2$O$_2$, 10 μM PJ-34, 10 μM LY 294002, or 10 μM Pp2, or with different combinations of these compounds as indicated in Fig. 10. Both LY 294002 and Pp2 decreased the PJ-34-induced Akt or GSK3β phosphorylation during oxidative stress (Fig. 10A) as did 1 μM wortmannin (data not shown).

When added alone to the cells, LY 294002, wortmannin, and Pp2 gave results identical to those of untreated cells (data not shown). When the expression of PARP enzyme was blocked by PARP-siRNA in WRL-68 cells, or the cells were transfected with C1 or N3 plasmids, we observed the same effect as in the case of the pharmacological inhibitor PJ-34 during oxidative stress. Akt and its downstream target GSK3β were phosphorylated, and this effect was diminished because of the specific PI3-kinase inhibitor LY 294002 or Src kinase inhibitor Pp2. Transfection by pEGFP plasmid had no effect on the phosphorylation of Akt (Ser$^{473}$) or GSK3β (Fig. 10B).

Effect of PARP Inhibition, Kinase Inhibitors, PARP Suppression, and Transdominant Expression of PARP-DBD on the Mitochondrial Membrane Potential of WRL-68 Cells in Oxidative Stress—We investigated the effect of the suppression of PARP by PARP-siRNA and PARP inhibition by transdominant expression of PARP-DBD as well as by PJ-34 on the mitochondrial membrane potential of WRL-68 cells utilizing a specific, mitochondrial membrane potential-dependent fluorescent dye; JC-1. WRL-68 liver cells as well as pEGFP, C1, N3 plasmid, or PARP-siRNA-transfected cells were treated for 3 h with 0.3 mM H$_2$O$_2$, 10 μM
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FIGURE 10. Modulation of the activation of Akt pathway by oxidative stress, PARP inhibition, and different kinase inhibitors. Activation of Akt and phosphorylation of its downstream target GSK3β were studied in WRL-68 liver cells by Western blotting utilizing phosphorylation-specific primary antibodies (P-Ser473Akt and P-Ser9GSK3β, respectively). A, the wild type WRL-68 cells were treated for 1 h with 1 mM H2O2, 10 μM PJ-34, 10 μM LY 294002, or 10 μM Pp2, or with different combinations of these compounds as indicated. B, WRL-68 liver cells were transfected with PARP-siRNA or pEGFP, C1, or N3 plasmids and were treated for 1 h with 1 mM H2O2, 10 μM LY 294002, or 10 μM Pp2, or with different combinations of these compounds as indicated. When added alone to the cells, LY 294002 and Pp2 gave results identical to those of untreated cells for both antibodies (data not shown). Even protein loadings were confirmed by an anti-actin antibody and Western blotting.

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FIGURE 11. Effect of PARP inhibition, different kinase inhibitors, PARP suppression, and transdominant expression of PARP-DBD on the mitochondrial membrane potential of WRL-68 cells in oxidative stress. Green and red fluorescence images were merged and used to demonstrate the effect of the following treatments on the mitochondrial membrane potential of WRL-68 liver cells for 3 h with 0.3 mM H2O2, 10 μM PJ-34, 10 μM LY 294002, or 10 μM Pp2, or with different combinations of these compounds as indicated. A, WRL-68 liver cells were transfected with PARP-siRNA or pEGFP, C1, or N3 plasmids and were treated for 3 h with 0.3 mM H2O2, 10 μM LY 294002 or 10 μM Pp2, or with different combinations of these compounds as indicated. B, WRL-68 liver cells were transfected with PARP-siRNA and treated for 3 h with 0.3 mM H2O2, 10 μM LY 294002, or 10 μM Pp2, or with different combinations of these compounds as indicated. After the treatment, the medium was replaced with fresh medium without any agents and containing 1 μM JC-1 membrane potential-sensitive fluorescent dye. After 10-min loading, digital images were acquired using a fluorescent microscope equipped with a digital camera.
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PJ-34, 10 μM LY 294002, or 10 μM Pp2. After the treatment, cells were loaded with JC-1, and fluorescence images were acquired in the green and red channels of the microscope. Images of healthy cells with intact undamaged mitochondria were sharp and rich in both red and green components appearing on the merged image as yellow (Fig. 11A, first picture). However, cells with damaged mitochondria showed characteristically different images; they appeared blurred and completely lacking red components because of the loss of mitochondrial membrane potential (Fig. 11A, second picture). The pharmacological inhibitor PJ-34 protected the mitochondrial membrane potential from the H$_2$O$_2$-induced injury, and the specific kinase inhibitors LY 294002 or Pp2 counteracted the protection of PJ-34 (Fig. 11A) as did wortmannin (data not shown). The C1 or N3 plasmid-transfected cells exerted a greater resistance against H$_2$O$_2$-induced injury compared with the wild type WRL-68 cells, and this protective effect was decreased by the specific kinase inhibitor, LY 294002, or wortmannin. Transfection by pEGFP plasmid could not influence the mitochondrial membrane potential of the WRL-68 cells (Fig. 11B). The suppression of PARP by the siRNA technique had an effect on the mitochondrial membrane potential of WRL-68 cells identical to that of the transdominant expression of PARP-DBD, and the resistance of the PARP-siRNA-transfected cells against H$_2$O$_2$-induced oxidative stress could also be decreased by the specific kinase inhibitors, LY 294002 or Pp2 (Fig. 11C).

**DISCUSSION**

It is well documented that PARP inhibitors or knock-out of the PARP-1 gene protects cells from different types of oxidative stress (37). Although PJ-34 is a well characterized PARP-1 inhibitor, the specificity of a small molecular weight synthetic inhibitor is always questionable because of the presence of several enzymes with mono- and poly(ADP-ribo)sylation activity in the cells (37). Suppression of the expression of PARP-1 in WRL-68 cells clearly protected the cells from oxidative stress (Fig. 6) and induced Akt phosphorylation and activation indicated by GSK3 phosphorylation (Figs. 7 and 10A). Because this method specifically suppressed PARP-1 synthesis, it was clear that PARP-1 was responsible for Akt phosphorylation and activation, although the question remained whether the suppression of PARP-1 catalytic activity or the absence of PARP-1 protein was responsible for the observed phenomenon. The transdominant expression of PARP-DBD inhibited the self-ADP-riboisolation of PARP because binding to single-stranded DNA breaks was essential for the activation of PARP-1, and the PARP-DBD competed with PARP-1 in binding to single-stranded DNA breaks, only the former did not have catalytic activity. Monitoring intracellular localization of the PARP-DBD construct was enabled because of the presence of GFP in the construct, and the PARP-DBD was found to be present almost exclusively in the nucleus (Fig. 1) so clearly in position to compete with PARP-1. Besides the inhibition of self-ADP-riboisolation of PARP-1, the expression of PARP-DBD significantly protected the cells from H$_2$O$_2$-induced cell death (Fig. 3) and induced Akt phosphorylation and activation (Fig. 4). Taking together all these data, it is unequivocal that Akt activation was the consequence of the inhibition of the single-stranded DNA break-induced PARP-1 activation and not of protein-protein interaction between PARP-1 and other regulatory proteins or of an other mechanism that was regulated by the pharmacological inhibitor.

The significance of the PARP inhibition-induced Akt activation in the survival of cell in oxidative stress can be assessed by the inhibition of PI3-kinase-mediated Akt activation by LY 294002 or by wortmannin. These data show that PI3-kinase inhibitors almost completely blocked the PARP inhibitor-induced cytoprotection and inhibited the PARP inhibitor-activated Akt and GSK phosphorylation; that is, Akt activation played a pivotal role in the cytoprotective effect of PARP inhibitor in oxidative stress under our experimental conditions. Inhibition of Src kinase, which can have a role in Akt activation (51, 52), also decreased Akt phosphorylation and reduced the cytoprotective effect of PARP inhibitor, indicating that Src kinase has been involved in the PARP inhibition-induced Akt activation process. Specificity and possible side effects of a pharmacological agent are always an issue; however, LY 294002 was reported to inhibit all isoforms of PI3-kinase but not to affect other kinases such as protein kinases C and A, mitogen-activated protein kinase, S6 kinase, epidermal growth factor tyrosine kinase, c-Src kinase, PI4-kinase, and diacylglycerol kinase (53). Wortmannin, although affecting myosin light chain kinase, is widely recognized as a selective and specific PI3-kinase inhibitor too with reportedly no effect on PI4-kinase, protein kinase C, and protein-tyrosine kinases (54). Pp2 was characterized as a selective inhibitor of the Src family of tyrosine kinases (55), so the effect of the above mentioned kinase inhibitors on the PARP inhibition-induced phosphorylation processes and cytoprotection was most likely the result of their main pharmacological effect on their respective kinases rather than a side effect.

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Nuclear translocation of mitochondrial apoptosis-inducing factor in response to excess PARP-1 activation triggering chromatin condensation, DNA fragmentation, nuclear shrinkage, and caspase-independent apoptotic cell death was found to represent a major cell death pathway in various neuronal and cardiovascular excitotoxicity and oxidative stress models (35, 56–59). NAD$^+$ depletion and induction of mitochondrial permeability transition were implicated as intermediate steps linking PARP-1 activation to apoptosis-inducing factor translocation (56). We observed a significant NAD$^+$ depletion in wild type and pEGFP-transfected WRL-68 cells as a consequence of H$_2$O$_2$ treatment which was significantly attenuated by pharmacological or nonpharmacological PARP-1 inhibition (Fig. 9). However, and in contrast to the viability and Akt pathway experiments, the PI3-kinase and Src kinase inhibitors did not counteract, in fact did not affect at all (Fig. 9), the protection of NAD$^+$ pool by PARP inhibition, which suggests that Src and PI3-kinase activities are not involved in the regulation of the intracellular NAD$^+$ level and that prevention of NAD$^+$ depletion and Akt pathway activation are two independent mechanisms of the cytoprotective effect PARP inhibition as is described schematically in Fig. 12. Whether or not nuclear translocation of apoptosis-inducing factor plays a role in our model system is still not clear; however, cell death was predominantly necrotic as revealed by positive propidium iodide and negative fluorescence of annexin V fluorescence (data not shown) and the absence of apoptotic morphology on the fluorescence images (Fig. 11).

Previously, it was reported that PARP inhibitors protect mitochondrial membrane integrity in oxidative stress (32, 33). Because there is evidence for the existence of mitochondrial PARP polymerase (29), when using pharmacological PARP inhibitors, it is hard to determine whether indeed PARP-1 or other PARPs are responsible for the collapse of mitochondrial membrane potential. Using our model system, it was evident that PARP inhibitor PI3-4, suppression of PARP-1 protein expression by siRNA, and the inhibition of single-stranded DNA break-induced PARP-1 activation by the transdominant expression of PARP-DBD preserved mitochondrial integrity and membrane potential the same way in H$_2$O$_2$-induced oxidative stress (Fig. 11); that is, the enhanced catalytic activity of PARP-1 in oxidative stress is responsible through a mechanism, which is as yet uncharacterized in detail, for the collapse of mitochondrial membrane potential.

It is known that Akt phosphorylates and so inactivates the proapoptotic BAD protein, which process can contribute to the stabilization of the mitochondrial membrane system. Therefore, it was important to determine whether Akt activation was involved in the protection of mitochondrial membrane potential by PARP inhibition. Fig. 11 shows that the PARP inhibition-mediated protection of mitochondrial membrane potential was compromised by PI3-kinase inhibitor LY 294002 (or wortmannin), suggesting that PARP inhibition-induced protection of mitochondrial membrane potential was mediated by the PI3-kinase/Akt system. A similar effect was observed in the case of Pp2, an Src kinase inhibitor, which was in accord with our previous data showing that Pp2 inhibited the PARP inhibition-induced Akt phosphorylation too. These data show that in oxidative stress, the activation of PARP-1-initiated poly(ADP-ribosyl)ation processes, which somehow negatively influenced Akt activation, were responsible in a significant extent for the collapse of mitochondrial membrane potential. Inhibition of PARP-1 activation by three different methods blocked the excessive poly(ADP-ribosyl)ation, so abolished the suppression of Akt activation, and the activation of Akt participated in the protection of the mitochondrial membrane system (Fig. 12).

In conclusion, we provided evidence for undermining the classical view that cytoprotection by PARP inhibitors relies exclusively on the preservation of NAD$^+$ and consequently the ATP stores in oxidative stress. Our data established that suppression of the single-stranded DNA break-induced PARP-1 activation by small molecular weight inhibitor, by siRNA method, or by the transdominant expression of PARP-DBD protected cells from oxidative stress and induced the phosphorylation and activation of Akt protein kinase. Inhibition of Akt activation by specific inhibitors in a significant extent counteracted the cytoprotective effect of PARP inhibitor, indicating that the PARP inhibition-induced Akt activation was very significantly responsible for the cytoprotective property of PARP inhibitors. Furthermore, the PARP inhibition-induced Akt activation was mainly responsible for the mitochondrial protection in oxidative stress, which could also be abolished by PI3-kinase inhibitors under our experimental conditions. These data indicate that PARP-1 inhibition-induced Akt phosphorylation and activation are responsible to a large extent for the cytoprotective properties of PARP inhibitors.

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Antal Tapodi, Balazs Debreceni, Katalin Hanto, Zita Bognar, Istvan Wittmann, Ferenc Gallyas, Jr., Gabor Varbiro and Balazs Sumegi

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