NUCLEAR POLY(ADP-RIBOSE) POLYMERASE-1 RAPIDLY TRIGGERS MITOCHONDRIAL DYSFUNCTION

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Running title: PARP-1 and Mitochondrial Dysfunction

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To obtain further information on time course and mechanisms of cell death after poly(ADP-ribose) polymerase-1 (PARP-1) hyperactivation, we used HeLa cells exposed for 1 h to the DNA alkylating agent methyl-nitroso-guanidine (MNNG). This treatment activated PARP-1 and caused a rapid drop of cellular NAD(H) and ATP contents culminating 8-12 h later in cell death. PARP-1 antagonists fully prevented nucleotide depletion and death. Interestingly, in the early 60 min after MNNG challenge mitochondrial membrane potential and superoxide production significantly increased while cellular ADP contents decreased. Again, these events were prevented by PARP-1 inhibitors, suggesting that PARP-1 hyperactivity leads to mitochondrial state 4 respiration. Mitochondrial membrane potential collapsed at later time points (3h) when mitochondria released apoptosis inducing factor and cytochrome c. Using immunocytochemistry and targeted luciferase transfection we found that, in spite of an exclusive localization of PARP-1 and poly(ADP-ribose) in the nucleus, ATP levels first decreased in mitochondria and then in the cytoplasm of cells undergoing PARP-1 activation. PARP-1 inhibitors rescued ATP but not NAD(H) levels in cells undergoing hyper-poly(ADP-ribosylation). Glycolysis played a central role in the energy recovery, whereas mitochondria consumed ATP in the early recovery phase and produced ATP in the late phase after PARP-1 inhibition, further indicating that nuclear poly(ADP-ribosylation) rapidly modulates mitochondrial functioning. Together, our data provide evidence for rapid nucleus-mitochondria cross-talk during hyper-poly(ADP-ribosylation)-dependent cell death.

The nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) converts β-nicotinamide adenine dinucleotide (NAD) into polymers of poly(ADP-ribose) (PAR), which participate in regulating nuclear homeostasis (1). However, once hyperactivated by genotoxic stress, PARP-1 causes NAD and ATP depletion, eventually leading to irreversible cellular energy failure and necrotic death (2-6). The pathophysiological significance of PARP-1 hyperactivation is well exemplified by the remarkable therapeutic efficacy of PARP-1 inhibitors in experimental models of disorders characterized by DNA damage such as ischemia, diabetes, shock, inflammation and cancer (7). Recently, several studies have broadened the role of poly(ADP-ribosyl)ation in cell killing showing that PARP-1 activation also occurs during apoptosis, and inhibition of PAR formation impairs activation of the apoptotic machinery [for reviews see: (8;9)]. In particular, it has been reported that PARP-1 prompts a cascade of events leading to PAR-dependent mitochondrial dysfunction and rapid release of apoptosis inducing factor (AIF) (10-12).

Despite their pathogenetic relevance, however, molecular mechanisms underlying energetic derangement during PARP-1 hyperactivation still wait to be clarified. For instance, whether nuclear or mitochondrial PARP-1 hyperactivity triggers mitochondrial dysfunction and release of apoptogenic factors is unresolved (13-15). Similarly, the early events occurring in mitochondria during hyper-poly(ADP-ribosylation) are elusive. In light of the powerful cytoprotective efficacy of PARP-1 inhibitors, another major question waiting to be answered is whether, and with which spatio-temporal kinetics, energy rescue occurs in cells when PARP-1 is inhibited after hyperactivation. To address these issues, we used HeLa cells exposed to the PARP-1 activating agent N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) (1;4;10) or to PARP-1 inhibitors after MNNG exposure.

MATERIALS AND METHODS

Cells and culture conditions - HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2mM glutamine, 10% fetal bovine serum and antibiotics. Cultures were brought to 50-70% confluence and exposed to MNNG and other drugs. Cell viability was evaluated by measuring lactate dehydrogenase (LDH) release in the incubating media or reduction of

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methylthiazolyl tetrazolium (MTT). Data obtained by means of MTT reduction were always confirmed by microscopic evaluation of cell morphology. Annexin V staining was performed by using a kit from Molecular Probes (The Netherlands).

**Western Blotting and immunocytochemistry** - Cell fractionation, Western blotting and immunocytochemistry were performed as previously described (16). The anti-PARP and anti-PAR antibodies (C2-10 and 10H, respectively) were from Alexis (Vinci, Italy), the anti-caspase-3 antibody was from Cell Signaling (Beverly, MA, USA), the anti AIF was from Santa Cruz (Santa Cruz, CA, USA), and the anti cytochrome c (Cyt-c) was from BD Biosciences (Lexington, KY, USA). Imaging was performed using a Nikon fluorescence microscope and a CCD camera.

**Nucleotide and superoxide measurement** - NAD contents were quantified by means of an enzymatic cycling procedure according to Shah et al. (17). NADH was quantified by HPLC and UV detection (Perkin Elmer model LC 90). Briefly, cells seeded in a 24 well plate were killed with 50 µl of KOH 50 mM and the lysate transferred to eppendorf tubes containing 75 µl of Buffer A (see below) and centrifuged at 13,000 g/5 min. 100 µl of the supernatant were injected in a C18 Pinnacle-II column. The mobile phases were 100 mM KH2PO4 pH 6 (Buffer A) and 100 mM KH2PO4 + 10 % methanol pH 6 (Buffer B). The gradient used was: 14 min 100% Buffer A, 25% Buffer B in 6 min, 60% Buffer B in 5 min, 100% Buffer B in 5 min and a final step of 7,5 min at 100% Buffer B. Absorbance was measured at 340 nm. ATP was measured by means of a kit from Perkin Elmer (Belgium). ADP was quantified by HPLC and UV detection according to Kawamoto et al (18). Superoxide production was measured by means of the nitroblue tetrazolium colorimetric assay as described (19).

**Evaluation of Mitochondrial membrane potential and cell autofluorescence** - Mitochondrial membrane potential (ΔΨm) was evaluated by means of digital imaging microscopy or flow cytometry. Cells seeded onto 24-mm coverslips were incubated with Krebs-Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM NaH2PO4, 5.5 mM glucose, 5 mM NaHCO3, 2 mM L-glutamine, 1mM CaCl2 and 20 mM HEPES, pH 7.4 at 37°C containing TMRE 25 nM for 10 min at 37°C. Coverslips were then mounted into a Leyden chamber containing Krebs-Ringer modified buffer plus 2.5 nM TMRE at 37°C and cells allowed to equilibrate for 5 min before imaging with a Nikon microscope equipped with piezoelectric motorization and a CCD camera. Drugs were added directly to the incubating solution. Stacks of images were acquired through the depth of the cell and deconvoluted using the EPR software as described (20). For each experiment, an intensity threshold was chosen, and the thresholded stack was projected to form a two-dimensional mask. This mask was used to identify mitochondrial pixels in the subsequent time series, thereby avoiding computation of noise by cytosolic TMRE fluorescence. Analysis of the resulting TMRE images and quantification of intensity optical density (IOD) were performed using custom designed software. For each time point, values correspond to the mean of at least 6 different microscopic fields containing the same number of cells. For analysis of ΔΨm by flow cytometry, cells seeded in 48 well plates were incubated with TMRE 2.5 nM in complete DMEM, exposed or not to MNNG 100 µM and analyzed. Briefly, cells were detached with trypsin and then diluted in complete DMEM. After gentle pipetting, 200 µl of the cell suspension were further diluted with PBS and analyzed by the flow cytometer Coulter EPICS XL (Beckman Coulter, Inc) equipped with the EXP032 Flow Cytometry ADC software (Beckman Coulter, Inc). TMRE 2.5 nM was present in all the solutions used for cell preparation and measurement. Cell autofluorescence was measured with the microscopic apparatus described above equipped with an UV filter. A stack of 6-8 images per microscopic field was acquired and processed for deblurring and tri-dimensional reconstruction.

**Mitochondrial and cytosolic ATP measurement** - ATP was indirectly quantified by means of cytosol- or mitochondria- targeted luciferase (21;22). Transfection with cytLuc or mtLuc cDNA (4 µg/ml) or mitochondria- targeted luciferase (21;22). was performed by the standard calcium-phosphate procedure. Cell luminescence was evaluated 36h after transfection by means of a luminometer as previously described (22). Cells were perfused with Krebs-Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM KH2PO4, 1mM CaCl2, 20 µM luciferin, 4.5 g/l glucose, and 20 mM HEPES, pH 7.4 at 37°C. Under these conditions, the light output was in the range of 1.000-10.000 cps/coverslip of cytLuc-transfected cells and 1000-3000 cps/coverslip of mtLuc-transfected cells vs a background lower than 30 cps. As previously reported, recordings did not exceed 15 min because longer measurements may be biased by factors other than intracellular ATP such as Coenzyme A or luciferin availability, pH as well as oxyluciferin and oxygen radicals formation (21;22).
concentrations are consistent with the drugs’ IC50 on %, respectively. Considering that these exposure increased survival to 89±8, 98±4 and 82±9 incubating medium during and after MNNG amidino-1,2-benzopyrone (INH2BP, 100 µM) in the phenanthridinone (PHE, 30µM) and 5-Iodo-6-amino-1,2-benzopyrone (INH2BP, 100 µM) in the incubating medium during and after MNNG exposure increased survival to 89±8, 98±4 and 82±9 %, respectively. Considering that these concentrations are consistent with the drugs’ IC30 on PARP-1 activity (23-25), our findings suggest that the hyperactivity of PARP-1 was mainly responsible for MNNG-induced cell death. This assumption is corroborated by evidence that PARP-1−/− fibroblasts, astrocytes and neurons do not die after MNNG exposure (10;11). Although PARP-2 is DNA damage-dependent (26), and BZD and PHE might inhibit its enzymatic activity, to date there is no evidence that PARP-2 hyperactivation causes cell death. This is consistent with the fact that almost 80% of cellular poly(ADP-ribose) is synthesized by PARP-1 (27). Cell death was evident 8 h after MNNG exposure (not shown) and became almost complete at 12 h, when cells became round-shaped and nuclei showed typical apoptotic hallmarks such as chromatin condensation, nuclear pyknosis and fragmentation, as well as phosphatidylserine exposure outside the plasmamembrane (Fig. 1A). As further evidence of apoptosis, cells released only ~4% of total LDH 8 h after MNNG (not shown). MNNG-treated cells, however, did not undergo caspase-3 activation and PARP-1 cleavage (Fig. 1B), and were not protected by the pancaspase inhibitor zVAD-fmk (not shown). In control cells and 1 h after MNNG exposure, intracellular distribution of AIF was punctate, indicating mitochondrial localization (28). AIF immunoreactivity appeared cytoplasmic 3 h after MNNG exposure in ~30% of cells, while massive cytoplasmic and nuclear AIF redistribution occurred after 4 h. Protein relocation was prevented by PHE (Fig. 1C) or BZD (not shown) added to the incubating media during and after MNNG challenge. Similar time courses of AIF and Cyt-c release in cytosol were obtained by Western blotting (Fig. 1D).

RESULTS

Effects of PARP-1 hyperactivation on cell death - Cells were exposed to the PARP-1 activator MNNG 100 µM/1 h, washed and incubated in culture medium for different times. Only 23±2% of cells survived 12 h after MNNG exposure. The presence of PARP-1 inhibitors of different chemical classes such as benzamide (BZD, 1mM), 6-(5H)-phenanthridinone (PHE, 30µM) and 5-lodo-6-amino-1,2-benzopyrone (INH2BP, 100 µM) in the incubating medium during and after MNNG exposure increased survival to 89±8, 98±4 and 82±9%, respectively. Considering that these concentrations are consistent with the drugs’ IC30 on PARP-1 activity (23-25), our findings suggest that the hyperactivity of PARP-1 was mainly responsible for MNNG-induced cell death. This assumption is corroborated by evidence that PARP-1−/− fibroblasts, astrocytes and neurons do not die after MNNG exposure (10;11). Although PARP-2 is DNA damage-dependent (26), and BZD and PHE might inhibit its enzymatic activity, to date there is no evidence that PARP-2 hyperactivation causes cell death. This is consistent with the fact that almost 80% of cellular poly(ADP-ribose) is synthesized by PARP-1 (27). Cell death was evident 8 h after MNNG exposure (not shown) and became almost complete at 12 h, when cells became round-shaped and nuclei showed typical apoptotic hallmarks such as chromatin condensation, nuclear pyknosis and fragmentation, as well as phosphatidylserine exposure outside the plasmamembrane (Fig. 1A). As further evidence of apoptosis, cells released only ~4% of total LDH 8 h after MNNG (not shown). MNNG-treated cells, however, did not undergo caspase-3 activation and PARP-1 cleavage (Fig. 1B), and were not protected by the pancaspase inhibitor zVAD-fmk (not shown). In control cells and 1 h after MNNG exposure, intracellular distribution of AIF was punctate, indicating mitochondrial localization (28). AIF immunoreactivity appeared cytoplasmic 3 h after MNNG exposure in ~30% of cells, while massive cytoplasmic and nuclear AIF redistribution occurred after 4 h. Protein relocation was prevented by PHE (Fig. 1C) or BZD (not shown) added to the incubating media during and after MNNG challenge. Similar time courses of AIF and Cyt-c release in cytosol were obtained by Western blotting (Fig. 1D).

PAR and nucleotide contents during PARP-1 hyperactivation - PAR accumulated in the nucleus of HeLa cells 5 min after MNNG exposure and returned to control levels after 15 min and for the following 8h (Fig. 2A and not shown). Findings are in keeping with prompt PAR hydrolysis by poly(ADP-ribose) glycohydrolase (PARG), and prevention of PARP-1 inhibition by auto-(ADP-ribosyl)ation (1). Under resting conditions, contents of NAD, NADH and ATP in HeLa cells were 8.2±0.9, 3.9±0.8 and 16±2.3 nmol/mg prot, respectively. Upon MNNG exposure, NAD contents decreased to 11.7±2% of control after 15 min, whereas those of ATP reached 9.9±1.5% of control after 30 minutes. Despite MNNG wash out, nucleotide contents remained at these levels for the following 6 h and became hardly detectable at 8 h (Fig 2B). Notably, PHE and BZD efficiently prevented MNNG-induced NAD and ATP depletion in a concentration-dependent manner (not shown), indicating that PARP-1 and not MNNG caused energy derangement. In keeping with prior findings in cardiac myocytes (29) and fibroblasts (30), we found that NAD contents were higher in mitochondria than in cytosol of Hela cells (54±7 and 2.8±0.4 nmol/mg prot in mitochondria and cytosol, respectively). These results, along with evidence that mitochondrial permeability transition pore opening triggers NAD depletion in mitochondria isolated from cardiac myocytes (29) or in intact astrocytes exposed to MNNG (11), prompted us to evaluate the effect of the permeability transition pore inhibitor cyclosporin-A on reduction of NAD contents in HeLa cells undergoing PARP-1 hyperactivation. Of note, cyclosporin-A (0.3-30 µM) had no effects on MNNG-triggered, PARP-1-mediated NAD shortage (not shown), suggesting that the alkylating agent and/or PARP-1/PAR did not cause transition pore opening in HeLa cells under our experimental conditions. Contents of NADH also decreased upon exposure to MNNG in a PHE-sensitive manner (Fig 2Ca), indicating that PARP-1 hyperactivation triggers cellular NADH depletion. In principle, two hypotheses can be advanced to explain this result. One is that PARP-1 indirectly decreases NADH content by simply consuming NAD and therefore shifting the NAD ↔ NADH equilibrium to the left. The other is that PARP-1 directly triggers NADH decrease through unknown mechanisms. The finding that rotenone (an inhibitor of NADH conversion into NAD by respiratory Complex I), increased NADH content in cells undergoing PARP-1 activation (Fig.
analysis revealed that the majority of MNNG-treated medium 1 h before (not shown). Cytofluorimetric
∆Ψ Complex I and II, respectively. Rotenone decreased nitroproprionic acid, inhibitors of respiratory
MNNG in the presence of rotenone or 3-
fluorescence was evaluated in HeLa cells exposed to To investigate how electrons entered the respiratory
activation (Fig. 3B).

Importantly, at all the time points investigated, the presence of PHE in the medium prevented increase of TMRE fluorescence (Fig. 3Ad) indicated that PARP-1 was responsible for early increase of ∆Ψ m. Mean mitochondrial membrane potential returned to control values 1 h after MNNG wash out (Fig. 3Aa) and the same occurred in cells not washed from MNNG (not shown). Significant drop of mean ∆Ψ m took place only 4 h after MNNG exposure (Fig. 3Aa), and such a collapse was inhibited (50.9±6.7% inhibition) by PHE (30 µM) added to the incubating medium 1 h before (not shown). Cytofluorimetric analysis revealed that the majority of MNNG-treated cells had ∆Ψ m higher than controls up to 3 h after PARP-1 activation. However, a fraction of cells with decreased ∆Ψ m appeared after 2 and 3 h (6.5±3.5% and 27.5±7.4%, respectively), whereas most of the cells (70.2±12%) lost ∆Ψ m only 4 h after PARP-1 activation (Fig. 3B).

To investigate how electrons entered the respiratory chain during PARP-1 hyperactivation, TMRE fluorescence was evaluated in HeLa cells exposed to MNNG in the presence of rotenone or 3-nitropropionic acid, inhibitors of respiratory Complex I and II, respectively. Rotenone decreased ∆Ψ m in cells exposed 1 and 2 h to MNNG, whereas ∆Ψ m became sensitive to 3-nitropropionic acid only 2 h after exposure to the alkylation agent (Fig. 3C). Importantly, at all the time points investigated, addition of the protonophore FCCP (1µM) to the incubating solution never led to increase of TMRE fluorescence but always to rapid (seconds) and complete loss of mitochondrial TMRE accumulation (Fig. 3Ac and not shown), indicating that the dye was indeed working in a non-quenching mode.

Increase of ∆Ψ m in spite of lack of ATP production occurs during ADP shortage-dependent mitochondrial state 4 respiration (34). Accordingly, ADP contents decreased in HeLa cells exposed to MNNG, and PHE prevented ADP depletion (Fig. 4A), indicating that PARP-1 was causative in the adenine nucleotide fall. Furthermore, in line with increased production of O2 during transition from state 3 to state 4 respiration (34), we found a PHE-sensitive burst of O2 production in cells exposed to MNNG (Fig. 4B).

ATP depletion in cytosol and mitochondria during PARP-1 hyperactivation - Because ATP levels in cellular sub-compartments during hyper-poly(ADP-ribosyl)ation have not been investigated so far, we monitored kinetics of ATP depletion in MNNG-treated HeLa cells transfected with the ATP-sensitive photoprotein luciferase targeted to cytosol or mitochondria. Comparison of signal from the two cell compartments was feasible because recombinant luciferases display similar Km values (21). MNNG caused a time-dependent reduction of photon emission form cells transfected with cytosolic or mitochondrial luciferase (Fig. 5A). Addition of PHE or BZD to the incubation medium prevented reduction of photon emission (not shown), indicating that decrease of luciferase activity was dependent on PARP-1 activity. Surprisingly, photon emission decreased earlier and for a higher extent in mitochondria than in cytosol (54±16.8% reduction in mitochondria, 28.1±6.7% in cytosol, 15 min after MNNG exposure), indicating more rapid impact of poly(ADP-ribosyl)ation on mitochondrial than cytoplasmic ATP homeostasis. These findings, along with possible presence of mitochondrial PARP-1 in several cells [for a recent review see (15)], prompted us to investigate whether PARP-1 also localized within mitochondria of HeLa cells. As shown in Fig. 5B, Western blotting revealed that PARP-1 was exclusively present in the nuclear fraction. Consistently, both a mouse monoclonal and a rabbit polyclonal anti-PARP-1 antibodies recognized PARP-1 strictly confined into the nucleus and not colocalizing with Cyt-c- or AIF-positive mitochondria (Fig. 5C). These findings are in line with prior work showing exclusive nuclear localization of PARP-1 (1;10;35-44).

Energy dynamics following inhibition of PARP-1 hyperactivation - ATP contents promptly increased in cells treated with MNNG, washed and exposed to PHE (W+PHE), reaching control levels within 1 h. Surprisingly, NAD contents did not rise within the first hours of W+PHE (Fig. 6A), reaching only 30.2±3% and 38.5±2 % of control after 7 and 12 h, respectively (not shown). Likewise, NADH contents did not rise upon W+PHE (not shown). PARP-1 inhibitors different from PHE such as BZD, PJ-34 (45) and TIQ-A (24) also prompted selective ATP

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rescue at concentrations consistent with their relative potencies on PARP-1 activity [EC$_{50}$ (µM) on ATP rescue at 1 h: PHE, 3±0.8; BZD, 342±41; PJ-34, 0.6±0.09; TIQ-A, 2±0.4]. To identify the contribution of glycolysis or mitochondria to the energetic rescue, we measured ATP recovery in cells treated with MNNG and then subjected to W+PHE in the presence or absence of a cocktail of mitochondrial inhibitors (oligomycin, rotenone and antimycin, ORA). Notably, ATP rescue still occurred in the presence of ORA. In particular, 5 and 10 min after MNNG wash out ATP contents were higher in cells exposed to PHE+ORA than in those exposed to PHE only, whereas the opposite occurred after 60 min (Fig 6B). Also, W+PHE prompted immediate and linear increase of photon emission by cytosolic luciferase (56±3.6% of recover at 15 min), whereas photon emission by mitochondrial luciferase increased upon W+PHE but reached a plateau after 5 min (32±9.2% of recover at 15 min) (Fig. 6C). These findings suggest that upon inhibition of PARP-1 activation mitochondria consumed ATP due to inversion of $F_0F_1$, but then became ATP producers because of glycolysis-dependent ADP production and mitochondrial recoupling.

To establish whether the integrity of the bioenergetic apparatus after hyper-poly(ADP-ribosyl)ation was permanent or temporary, ATP rescue was quantified in cells exposed to PHE at different times after MNNG wash out. One-three h delay of PHE exposure led to full ATP recover, whereas only partial rescue occurred with a 4 h delay (Fig. 7A). Exposure to PHE 5 h after MNNG wash out did not lead to significant ATP recover (not shown). Together, these findings indicate that PARP-1 activity, when unrestrained for times longer than 3 h, irreversibly impaired the energetic apparatus, and confirm that PARP-1 remained active despite transitory burst of PAR accumulation. Of note, exposure to PHE 2-3 h after MNNG wash out, although able to give full ATP rescue, provided only partial protection from cell death (Fig. 7B). Post-treatments longer than 4 h did not afford any protection (not shown). Data suggest that the point of no return in PARP-1-dependent cell death preceded irreversible impairment of the energetic apparatus.

**DISCUSSION**

The main goal of the present study was to obtain insight into the time course of the processes leading to PARP-1-dependent cell death. We show that PARP-1 hyperactivity in the nucleus rapidly impairs ATP production in mitochondria, whereas release of the pro-apoptotic factors AIF/Cyt-c from mitochondria only occurs several hours after PARP-1 hyperactivation. Machinery responsible for mitochondrial release of proapoptotic factors and its sensitivity to energy dynamics are not well understood. By showing PARP-1-dependent rapid energy collapse and delayed AIF/Cyt-c relocation, our findings indicate that this machinery is not directly prompted by NAD/ATP depletion and can stand prolonged nucleotide shortage. However, given that NAD repletion rescues mitochondria and cells from PARP-1-dependent failure (11;46), caution must be exercised when generalizing data obtained in a specific cell type/line. It is also worth noting that ability of PARP-1 inhibitors to prevent MNNG-induced nucleotide depletion, $\Delta \Psi_m$ loss, AIF release and cell death suggests that nuclear hyperpoly(ADP-ribosyl)ation and not the alkylating agent per se is responsible for mitochondrial dysfunction. This assumption is corroborated by evidence that MNNG does not cause NAD depletion or AIF release in PARP-1$^{-/-}$ cells (10).

We report here that upon PARP-1 activation ATP depletion in mitochondria precedes that in the cytosol. Given that in our model PARP-1 and PAR exclusively localize in the nucleus, our findings indicate that molecular signals released from the nucleus of cells undergoing massive poly(ADP-ribosyl)ation can rapidly modify mitochondrial functioning. A possible candidate messenger could be ADP-ribose, the main PAR degradation product. Accordingly, ADP-ribose exits the nucleus and causes impairment of ATP-dependent membrane transporters in conditions of PARP-1 hyperactivation (47). It is also worth noting that PARP-1 hyperactivation could alter some parameters of mitochondrial matrix including pH which in turn might impair intramitochondrial luciferase activity.

The present data help answering the open question of whether/how mitochondria respire during PARP-1 hyperactivation. By carefully monitoring $\Delta \Psi_m$, we found that PARP-1 hyperactivation leads to mitochondrial hyperpolarization. In cells, three main mechanisms can lead to increase of $\Delta \Psi_m$: a) state 4 respiration (typically characterized by ADP deficiency); b) Cyt-C oxidase dephosphorylation; c) $F_0F_1$ inhibition (typically characterized by ADP accumulation) (48). Evidence that ADP contents were reduced by PARP-1 concomitant to $\Delta \Psi_m$ increase (Fig. 4A) suggests that state 4 respiration underpins mitochondrial hyperpolarization upon
PARP-1 activation. Such a bioenergetic state is likely due to PARP-1 activity-dependent neosynthesis of NAD from ATP which prevents the latter to be re-transformed into ADP. Interestingly, PARP-1 activators such as Fas, staurosporin or H2O2 can increase \( \Delta \Psi_m \) in intact cells (48). Further, the finding that Complex I is active and supports \( \Delta \Psi_m \) during pyridine nucleotide depletion suggests that small amounts of NAD(H) suffices to provide electrons to counteract mitochondrial inner membrane proton leakage during PARP-1 hyperactivity. Evidence that Complex II activity contributes to \( \Delta \Psi_m \) maintenance only 2 h after MNNG exposure also indicates that nuclear poly(ADP-ribosyl)ation changes the contribution of single respiratory complexes to mitochondrial electron flow.

Detailed analysis of ATP contents in cells undergoing PARP-1-dependent apoptosis is lacking. Also, how much energy is required for apoptosis, and whether low energy may prompt the apoptotic machinery still waits to be unequivocally answered (49). We report here that drastic and permanent PARP-1-dependent loss of ATP precedes appearance of classical apoptotic hallmarks in HeLa cells. Although 10% of control ATP is present up to 6 h after PARP-1 activation, lack of caspase-3 activation suggests that this ATP does not fuel apoptosis. Of note, irreversible death commitment begins 2 h after MNNG exposure (Fig. 7B), i.e. at least 1h before appreciable \( \Delta \Psi_m \) loss. Although increased mitochondrial ROS production could have a role in triggering this form of cell death [Fig 4B and Ref (50)], radical scavengers such as vitamin E, Tempol or Tiron (0.1-1 mM) did not affect cell demise following MNNG exposure (not shown). These findings indicate that mitochondrial depolarization and oxidative stress are not always causally involved in triggering PARP-1-dependent apoptotic demise.

Despite their pathophysiological relevance, strategies adopted by cells to rescue energy dynamics once hyper-poly(ADP-ribosyl)ation is inhibited are largely unknown. We show here that upon inhibition of PARP-1 hyperactivation ATP but not NAD contents promptly recover. Evidence that glycolysis is instrumental in ATP rescue, together with its well known dependence on NAD, indicates that low levels of NAD (Fig. 6A) suffice to maintain key cellular functions under stress. Also, the finding that cells survive in this scenario implies that they can tolerate prolonged NAD shortage. As for the lack of rapid NAD rescue, several pathways leading to NAD (re)synthesis (51;52) and destruction (29) are known, but their involvement in stressful conditions remains to be investigated. Finally, evidence that mitochondria consume and produce ATP respectively in the early and late phase of energy recover indicates that the organelles rapidly sense the rate of nuclear PAR formation and regulate their functions accordingly. Although the underlying molecular mechanisms are unknown, data corroborates the tight crosstalk between nuclear PAR formation and mitochondria.

In conclusion, these results taken together provide new clues on how poly(ADP-ribosyl)ation affects the complex spatio-temporal signaling among subcellular organelles during cell death and survival.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. PARP-1 hyperactivation leads to caspase independent apoptotic cell death. (A) 12 h after MNNG I00µM/1h cells detach, join in clusters and appear round-shaped. At the same time point, chromatin condenses and nuclei appear pyknotic and fragmented (arrows). Annexin V-positive cells are evident 4 h after exposure to MNNG (1h/100 µM). (B) Caspase-3 and PARP-1 cleavage occurs in cells exposed to staurosporin (2 µM) but not MNNG (100 µM). (C) Under control conditions, AIF immunoreactivity appears punctate indicating mitochondrial localization. AIF distribution is not altered up to 3 h after MNNG exposure. Cytoplasmic and nuclear AIF redistribution becomes evident 4 h after exposure to the alkylating agent and is prevented by PHE (30 µM). (D) In keeping with immunocytochemistry, Western blotting of the cytosolic fraction of HeLa cells exposed to MNNG reveals that mitochondrial AIF and Cyt-c release occurs 3 h after exposure to the alkylating agent and further increases at 4 and 5 h. One experiment/blot representative of 3 is shown A-D. Scale bar = 10 µm (A) and 5 µm (B).

Fig. 2. Irreversible NAD, NADH and ATP depletion in HeLa cells exposed to MNNG is PARP-1-dependent. (A) PAR immunoreactivity, barely detectable in the cytoplasm of control cell, selectively increases in the nucleus 5 min after MNNG exposure. Polymer levels are significantly reduced at 10 min and return to control values 15 min after MNNG challenge. (B) Exposure to 100 µM MNNG decreases NAD and ATP contents to ~10% of control after 15 and 30 min, respectively. MNNG wash out after 1 h does not affect nucleotide depletion. Both cellular NAD and ATP are almost undetectable at 8 h from MNNG exposure. (Ca) Exposure to MNNG/1h drastically reduces cellular NADH contents. The presence of PHE (30µM) during MNNG exposure abolishes NADH depletion. This latter is also partially prevented by the presence of the mitochondrial complex I inhibitor rotenone (10µM). (Cb) Tri-dimensional visualization of HeLa autofluorescence. Autofluorescence, taken as an index of NADH content, is mainly localized in subcellular structures resembling mitochondria and strongly reduced by 100µM MNNG/1h. One experiment representative of 3 is shown in A and Cb. The mean ± S.E.M. of 5 (B) or 3 (Ca) experiments is shown. Scale bar = 10µm.

Fig. 3. Effects of PARP-1 activation on ∆Ψm. (A) Evaluation of ∆Ψm by means of TMRE and digital imaging microscopy. (a) ∆Ψm increases 1 h after MNNG exposure and then decreases to values slightly lower than control ones. Such values are maintained up to 3 h after MNNG exposure. ∆Ψm starts collapsing 4 h post MNNG. (b) Tri-dimensional visualization of TMRE fluorescence in mitochondria of control cells or exposed 1 h to 100 µM MNNG. (c) FCCP (1µM) leads to prompt and complete loss of TMRE fluorescence in cells incubated under control conditions or exposed 1 h to MNNG 100 µM. (d) PHE (30 µM) prevents increase of ∆Ψm at 30 and 60 min after MNNG exposure. (B) Flow cytometric analysis confirms increase of ∆Ψm 1h after MNNG exposure. After 2-3 h mitochondria are still hyperpolarized even if ∆Ψm start collapsing in a small fraction of cells. Almost complete ∆Ψm loss occurs 4 h after MNNG exposure. (C) Rotenone (ROT, 10 µM) decreases ∆Ψm of cells at 1 and 2 h after MNNG (1h/100 µM), whereas 3-nitropropionic acid (NP, 1 mM) reduces ∆Ψm only 2 h after MNNG treatment. *p<0.05, **p<0.01 vs control (A) or MNNG (C) at the
respective time points. The mean ± S.E.M. of 3 experiments is shown in Aa, c, d and C. One experiment representative of 3 is shown in Ab and B. Scale bar = 5µm.

Fig. 4. Effects of PARP-1 hyperactivation on cellular ADP contents and O$_2^-$ production. (A) ADP contents decreases time dependently in cells exposed to MNNG 100 µM. The presence of 30 µM PHE in the incubating medium prevents such a decrease. (B) 30 µM PHE abrogates increased O$_2^-$ production in cells exposed to 100 µM MNNG. The effect of antimycin (10 µM, inhibitor of respiratory complex III) is shown as positive control. *p<0.05 vs control. The mean ± S.E.M. of 4 (A) and 3 (B) experiments is shown.

Fig. 5. Effect of PARP-1 hyperactivation on cytosolic- or mitochondrial-luciferase activity. PARP-1, AIF and Cyt-c by immunocytochemistry. (A) Effects of 100 µM MNNG on photon emission from cytosolic- or mitochondrial-luciferase transfected HeLa cells. Note that decrease of photon emission is quicker and larger in mitochondria than in cytosol. (B) Western blotting reveals PARP-1 localized in the nuclear (Nucl) fraction but absent in the cytosolic (Cyt) and mitochondrial (Mit) ones. (C) PARP-1 immunoreactivity revealed by a polyclonal or monoclonal anti-PARP antibody is localized in the nucleus and does not colocalize with AIF- or Cyt-c-positive mitochondria, respectively. Mitochondrial colocalization is evident for AIF and Cyt c immunoreactivity. One experiment representative of 3 and 4 is shown in (A, B) and (C), respectively. Scale bar = 2 µm.

Fig. 6. Energy dynamics following inhibition of PARP-1 hyperactivation. (A) Exposure to PHE (30 µM) after MNNG (100 µM) wash out leads to complete ATP rescue without affecting NAD depletion. (B) ATP rescue also occurs in the presence of oligomycin, rotenone and antimycin (ORA, all at 10 µM). Note that ATP contents are higher in cells exposed to ORA at 5 and 10 min after MNNG wash out and PHE exposure (W+PHE+ORA) with respect to cells only subjected to wash out and PHE exposure (W+PHE). Conversely, cells treated with W+PHE+ORA have lower ATP content respect to those receiving W+PHE at 60 min from wash out. (C) Photon emission linearly increases from cytosolic luciferase-transfected cells after MNNG wash out and PHE exposure. Photon emission also increases from mitochondrial luciferase-transfected cells but reaches a plateau after 5-10 min. The mean ± S.E.M. of 5 and 3 experiments is shown in A and b, respectively. One experiment representative of 2 is shown in C. Scale bar = 10 µm.

Fig. 7. Effect of delayed PARP-1 inhibition on energy rescue and cell survival. (A) Full rescue of ATP occurs in cells exposed to 30 µM PHE up to 3 h after MNNG wash out (ATP was measured 1h after PHE exposure). PHE added to the medium 4 h after MNNG wash out prompts only a partial ATP rescue. **p<0.01 vs MNNG. (B) Exposure to PHE 2-4 h after MNNG wash out provides only partial protection from cell death. *p<0.01, **p<0.01, ***p<0.001 vs control; #p<0.01, ###p<0.01, vs PHE at t=0. The mean ± S.E.M. of 3 (A) and 4 (B) experiments is shown.
Figure 1
Figure 2

A

B

C

MNNG 100µM

- NAD
- ATP

% of control

NADH (% of control)

MNNG 100µM

Control

MNNG

Figure 2
Figure 3
Figure 4
Figure 5

A

% of photon emission

Cytosol
Mitochondria

B

Mit. Cyt. Nucl.

-PARP-1

C

Anti PARP-1 polyclonal
Anti Cyt c
Merged

Anti PARP-1 monoclon.
Anti AIF
Merged

Anti AIF
Anti Cyt c
Merged
Figure 6
Figure 7
Nuclear poly (ADP-ribose) polymerase-1 rapidly triggers mitochondrial dysfunction

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