Glucose Deprivation Converts Poly(ADP-ribose) Polymerase-1 Hyperactivation into a Transient Energy-producing Process*

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Massive poly(ADP-ribose) formation by poly(ADP-ribose) polymerase-1 (PARP-1) triggers NAD depletion and cell death. These events have been invariably related to cellular energy failure due to ATP shortage. The latter occurs because of both ATP consumption for NAD resynthesis and impairment of mitochondrial ATP formation caused by an increase of the AMP/ADP ratio. ATP depletion is therefore thought to be an inevitable consequence of NAD loss and a hallmark of PARP-1 activation. Here, we challenge this scenario by showing that PARP-1 hyperactivation in cells cultured in the absence of glucose is followed by NAD depletion and an unexpected PARP-1 activity-dependent ATP increase. We found increased ATP content in resting Glu− cells, a condition that counteracts the increase of the AMP/ADP ratio during hyper-poly(ADP-ribosyl)ation and preserves mitochondrial coupling. We also show that the increase of ATP in Glu− cells is due to adenylate kinase activity, transforming AMP into ADP which, in turn, is converted into ATP by coupled mitochondria. Interestingly, PARP-1-dependent mitochondrial release of apoptosis-inducing factor (AIF) and cytochrome complex (Cyt c) is reduced in Glu− cells, even though cell death eventually occurs. Overall, the present study identifies basal ATP content and adenylate kinase as key determinants of bioenergetics during PARP-1 hyperactivation and unequivocally demonstrates that ATP loss is not metabolically related to NAD depletion.

Poly(ADP-ribosyl)ation is a post-translational modification of proteins operated by poly(ADP-ribose) polymerases (PARPs) (1). PARP-1, the oldest and best characterized member of the PARP family, is a nuclear enzyme converting NAD into polymers of poly(ADP-ribose) (PAR) that regulate chromatin-interacting proteins through steric hindrance and electrostatic repulsion (2). By so doing, the enzyme plays a key role in various nuclear process involved in maintenance of nuclear homeostasis such as DNA repair and epigenetic regulation of gene expression (3–5). Somehow paradoxically, besides this pleiotypic physiological role, PARP-1 is a powerful trigger of cell death (6, 7). This occurs when the enzymes undergo hyper-activation because of extensive DNA damage. PARP-1-dependent cell death was originally described as a necrotic process (8, 9), but an involvement of PARP-1 in apoptosis (10) and autophagy (11) has also been reported. In keeping with the central role of the enzyme in cell demise, chemical inhibitors of PARP-1 exert widespread cytoprotection in disparate in vitro and in vivo disease models (12).

It has been proposed that intracellular NAD depletion and continuous resynthesis are the main triggers of necrotic cell death upon hyperactivation of PARP-1. This is because NAD resynthesis through the NAD rescue pathway is an ATP-dependent process that eventually leads to energy failure. This death route, the so-called “suicide hypothesis” (13), has been validated by numerous studies showing NAD and ATP depletion in cells undergoing DNA damage-dependent PARP-1 activation (6). The suicide hypothesis, however, appeared too basic to explain the complex signaling pathways operating in cells undergoing hyper-poly(ADP-ribosyl)ation. In 2005, we reported that mitochondria readily sense nuclear PARP-1 activation, and failure of ATP formation occurs earlier in the organelles than in the cytosol (14). This information, on the one hand, provided the first hint that impairment of mitochondrial bioenergetics is causal in energy failure by PARP-1, and on the other, that mechanisms leading to ATP loss during massive PAR formation are more complex than previously envisaged.

In keeping with this, the suicide hypothesis has been recently complemented by the so called “Nudix hypothesis” (15). According to this theory, hydrolysis of PAR into ADP-ribose monomers by poly(ADP-ribose) glycohydrolase (PARG) and subsequent transformation of ADP-ribose into AMP by Nudix hydrolases (16) is the cause of energy collapse. In particular,
failure of ATP synthesis occurs because AMP can fit the cytosolic binding ADP site of the mitochondrial ATP/ADP translocator (ANT) with equal affinity to ADP but without sufficient energy to trigger mechanical rearrangement of the translocator and nucleotide internalization. This allows AMP, accumulated in large amounts because of rapid PAR degradation, to outcompete ADP binding, thereby precluding mitochondrial ADP entrance and ATP formation (15). Whether the Nudix hypothesis complements or replaces the suicide hypothesis is not known, and it is likely that the two death pathways, plus additional ones such as death signaling by PAR (17) or mTOR inhibition (18), may play different roles in PARP-1-dependent cell death according to the specific cell type and/or stressor under investigation.

Notwithstanding the mechanisms responsible for impairment of mitochondrial energy production, information currently available indicates that PARP-1 hyperactivity invariably leads to cellular energy depletion. In the present study, to gather further insight into PARP-1 and energy dynamics, we investigated the role of glycolysis in cells undergoing hyper-poly-(ADP-ribosyl)ation. We found that PARP-1 activation unexpectedly leads to a burst of energy production in cells acutely starved from glucose.

EXPERIMENTAL PROCEDURES

**Cells and Culture Conditions**—HeLa cells or 3T3 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mm glucose and supplemented with 2 mm glutamine, 1 mm pyruvate, 10% fetal bovine serum, and antibiotics. Cultures were brought to 50–70% confluence and exposed to DMEM containing 2 mM glutamine and 1 mM K2HPO4, 1% acetonitrile, 10 mM tetrabutylammonium borate (pH 6.9), and UV detection at 260 nm. The cellular ATP content was measured by means of an ATPlite kit (PerkinElmer Life Sciences) as described previously (14).

**Oxygen Consumption Analysis**—Quantitation of oxygen consumption was conducted by means of the Oxygraph system (Hansatech Instruments, Norfolk, UK). Cells (250,000) were loaded in the chamber containing 400 μl of DMEM with or without glucose, and oxygen consumption monitored for 10 min at 37 °C.

**Adenylate Kinase Activity**—Adenylate kinase (AK) activity has been measured in cytosolic extracts of HeLa cells deprived of glucose for 30 min (to eliminate ATP formation by glycolysis) measuring ATP formation from ADP. The reaction mixture consisted of 10 mm Tris-HCl (pH 7.4), 150 mm NaCl, 1 mm MgCl2, 0.25 mm NAD+, 2 mm glucose. The reaction was initiated by the addition of 1 mm ADP and stopped within 2 min according to Ref. 21.

**siRNA and RT-PCR**—siRNA and RT-PCR were conducted as described (22, 23). siRNA was purchased from Qiagen. The following primers were used: AK1, 5′-TGAGCGACGGATTG-GACAGCCC-3′ (sense) and 5′-CTTGGCAGACATGCCAC- GTTTCTC-3′ (antisense); AK2, 5′-TGTTTCTACACCAAGA- GGAGTCCAACC-3′ (sense) and 5′-GATGTTGCGAAC- ACGACATCGG-3′ (antisense).

**Evaluation of Mitochondrial Membrane Potential**—Mitochondrial membrane potential (ΔΨm) was evaluated by means of flow cytometry. Cells seeded in 48-well plates were incubated with 2.5 nm TMRE in DMEM with or without glucose, exposed or not to 100 μM MNNG, and analyzed. Briefly, cells were detached with trypsin and then diluted in DMEM with or without glucose. After gentle pipetting, 200 μl of the cell suspension was further diluted with PBS and analyzed by the flow cytometer Coulter EPICS XL (Beckman Coulter) equipped with the EXPO32 Flow Cytometry ADC software (Beckman Coulter). 2.5 nm TMRE was present in all of the solutions used for cell preparation and measurement.

**Cell Death Analysis**—Cell death was quantified by means of cytofluorometric analysis. Briefly, cells were detached with trypsin and EDTA, washed, resuspended in DMEM containing 3 μg/ml propidium iodide, incubated at 37 °C for 10 min, and then analyzed by the flow cytometer Coulter EPICS XL equipped with the EXPO32 Flow Cytometry ADC software.

**Statistical Analysis**—Evaluation of significant differences among groups was performed using ANOVA followed by Tukey’s w test.

**RESULTS**

**Effect of Glucose on PARP-1-dependent NAD and ATP Depletion**—We first evaluated the effect of the glycolytic flux on depletion of NAD and ATP in cells undergoing PARP-1 hyperactivation. To this end, we exposed the highly glycolytic HeLa cells to the alkylating agent MNNG in culture media containing 3 μg/ml propidium iodide, incubated at 37 °C for 10 min, and then analyzed by the flow cytometer Coulter EPICS XL containing the mitochondrial energetic substrates glutamine and pyruvate in the presence or absence of glucose. It is well established that MNNG leads to NAD and ATP depletion as well as to cell death in a manner entirely dependent on PARP-1 hyperactivity (14). As shown in Fig. 1A, 5 min after MNNG exposure, nuclear PAR accumulation was similar in cells exposed to a medium containing (Glu+ cells) or lacking (Glu− cells) glucose. When PAR
contents were analyzed over time by Western blotting, we found that accumulation of polymers in Glu\(^+\)/H11001 cells lasted up to 10 min after MNNG exposure, was highly reduced after 15 min, and completely disappeared at 30 min (Fig. 1B). This is consistent with prior work, indicating rapid degradation of PAR by PARG (15, 24). The same kinetic of PAR accumulation and degradation was detected in Glu\(^-\)/H11002 cells (Fig. 1B). In keeping with this, MNNG-triggered PARP-1-dependent NAD depletion was similar in Glu\(^+\)/H11001 and Glu\(^-\)/H11002 cells (Fig. 1C). Unexpectedly, however, drastic differences were found when ATP depletion was analyzed. Specifically, whereas an almost complete loss of ATP content occurred 1 h after MNNG exposure and remained constant up to 5 h in Glu\(^+\) cells, a surprising and substantial ATP increase was found in Glu\(^-\) cells exposed to MNNG. After this initial burst, ATP contents linearly decreased over time and reached control levels 4 h after PARP-1 activation. An abrupt reduction of energy levels occurred in Glu\(^-\) cells 1 h later (Fig. 1D). Glucose deprivation also did not affect NAD depletion in mouse fibroblasts exposed to MNNG for 1 h. Similar to HeLa cells, however, in these cells ATP contents were reduced in the presence of glucose and increased in its absence (Fig. 1E). To ascertain that the ATP increase in MNNG-exposed Glu\(^-\) cells was due to PARP-1 activity, we evaluated the effect of chemical inhibitors of the enzyme. Fig. 1F shows that PARP-1 inhibitors phenanthridinone (PHE, 30 \(\mu\)M) and PJ34 (20 \(\mu\)M) on MNNG-induced ATP changes in Glu\(^+\) and Glu\(^-\) cells. In A and B one experiment representative of four is shown. In C–F each point represents the mean \pm S.E. (error bars) of at least five experiments conducted in duplicate.

**Effects of glucose deprivation on PAR formation as well as NAD and ATP depletion in HeLa cells exposed to MNNG.** A, visualization of PAR distribution in control or MNNG-exposed cells (100 \(\mu\)M/5 min) in the presence or absence of glucose. B, time course of PAR content evaluated by Western blotting in HeLa cells exposed for different times to 100 \(\mu\)M MNNG in the presence or absence of glucose. Tubulin is shown as a loading control. C and D, effect of glucose deprivation on NAD (C) or ATP (D) content in HeLa cells exposed for different times to MNNG. E, effect of glucose deprivation on NAD and ATP content of mouse fibroblasts after MNNG exposure (100 \(\mu\)M/1 h). Basal contents expressed as nmol/mg of protein were: 7.8 \pm 5.2 (NAD in Glu\(^+\) and Glu\(^-\) cells), 51 \pm 3.7 (ATP in Glu\(^+\) cells), and 41.3 \pm 3.2 (ATP in Glu\(^-\) cells). F, effect of PARP-1 inhibitors phenanthridinone (PHE, 30 \(\mu\)M) and PJ34 (20 \(\mu\)M) on MNNG-induced ATP changes in Glu\(^+\) and Glu\(^-\) cells. In A and B one experiment representative of four is shown. In C–F each point represents the mean \pm S.E. (error bars) of at least five experiments conducted in duplicate.

**Effect of Glucose on Derangement of Mitochondrial Bioenergetics by PARP-1 Hyperactivation**—A large body of evidence indicates that PARP-1 hyperactivation impairs mitochondrial bioenergetics (10, 14, 25, 26). We previously reported that, upon massive PAR formation, mitochondria undergo decoupling because of both cytosolic ADP shortage and ANT blockade (15). Consistent with mitochondrial uncoupling, an early increase of mitochondrial membrane potential takes place upon PARP-1 activation (14). On this basis, we next evaluated whether mitochondrial derangement during hyper-poly(ADP-ribosyl)ation also occurs in Glu\(^-\) cells. Interestingly, in these cells we found that PARP-1 hyperactivation did not trigger
Glycolysis Dictates ATP Levels during PARP-1 Hyperactivation

Unaltered PAR degradation concomitant to lack of AMP increase in Glu− cells suggested that glucose deprivation conferred the ability to degrade the nucleotide. Key enzymes converting AMP back to ADP are AKs (27) that catalyze the \( \text{AMP} + \text{ATP} \leftrightarrow \text{ADP} + \text{ADP} \) reaction in a forward or reverse direction based on the relative concentrations of the nucleotides. AKs are key enzymes involved in intracellular AMP signaling and metabolic regulation (27). We speculated, therefore, that AK activity prevents AMP increase in Glu− cells given their maintained ATP content during PARP-1 hyperactivation. We also reasoned that the involvement of AK could explain the paradoxical increase of ATP in these cells. Indeed, AK activity might allow the formation of an extra pool of ADP from AMP that, in turn, could be readily converted into ATP by coupled mitochondria. To corroborate this hypothesis, given that selective AK inhibitors are not available, we attempted to suppress cellular AK activity by silencing AK1 and AK2, the AK isoforms responsible for the majority of cellular AK activity. Remarkably, silencing of the enzymes (Fig. 3E) reduced cytosolic AK activity (Fig. 3F) and prevented the increase of ATP in Glu− cells exposed to MNNG (Fig. 3F). Notably, we found that MNNG exposure increased the adenylate pool of Glu− cells from 97 ± 12 to 120 ± 13 nmol/mg of protein, in keeping with the hypothesis of a net transformation of NAD into ATP via PARP→PARG→Nudix→AK. We also measured the mass action ratio of AK (i.e. \([\text{ATP}]\times[\text{AMP}]\)/[ADP]^2) under the different conditions and found values of 0.7 ± 0.13 (Glu− cells), 0.58 ± 0.16 (Glu+ cells plus 30 min of MNNG), 0.4 ± 0.19 (Glu− cells), 0.59 ± 0.09 (Glu− cells plus 30 min of MNNG). Evidence that all of these ratios are in the range of the equilibrium constant (from 0.44 to 0.77) (28) indicates that the enzyme works close to equilibrium under the different conditions and, therefore, is not regulatory.

Effect of Glucose Deprivation on PARP-1-dependent Cell Death—We next wondered whether the remarkable difference in bioenergetics of cells undergoing hyper-poly(ADP-ribosyl)ation in the presence or absence of glucose could affect execution of PARP-1-dependent cell death. It is well known that release of AIF from mitochondria readily occurs upon PARP-1 activation (29). Mitochondrial release of Cyt c also occurs in conditions of massive PAR formation (10, 14). We therefore evaluated by Western blotting the cytoplasmic levels of these two mitochondrial proteins in Glu− and Glu+ cells undergoing PARP-1 hyperactivation. Of note, we found that they accumulated to a lower extent in Glu− cells (Fig. 4, A and B). Accordingly, nuclear redistribution of mitochondrial AIF after MNNG exposure was less evident in Glu− than Glu+ cells (Fig. 4C). Importantly, when cell death was evaluated over time, we found that it was significantly delayed in Glu− cells. Their resistance to MNNG tended to reduce with time and disappeared 16 h after exposure to the alkylating agent (Fig. 4, D and E).

**DISCUSSION**

For the first time, our study reports that massive PAR formation is not invariably related to ATP depletion, and availability of glucose is a key determinant of PARP-1-dependent energy failure. This information significantly changes the scenario of the biochemical events responsible for loss of ATP in cells...
undergoing hyper-poly(ADP-ribosyl)ation. Indeed, by showing that NAD loss is completely unaffected in Glu−/H11002 cells despite a concomitant increase of ATP content, we demonstrate that NAD loss does not consequentially trigger ATP depletion. Rather, we propose that the key parameter that dictates energy failure is the AMP/ADP ratio present in the cytosol at the beginning of PAR degradation. Whereas our prior work demonstrates that the rate and extent of PAR degradation into AMP via Nudix hydrolases is a crucial element of the AMP/ADP ratio (15), the present data indicate that glucose availability is an additional determinant of the ratio. We reason that although the Nudix pathway obviously regulates the numerator of the ratio, the presence of a glycolytic flux is a key determinant of the denominator. This is because in the absence of glucose ADP accumulates in the cytoplasm given that it is no longer converted into ATP through glycolysis. Accordingly, ADP contents promptly increase upon exposure to a medium lacking glucose (Fig. 3A). Increased ADP contents, in turn, competes with accumulating AMP on the ADP-binding site of ANT, thereby preventing failure of mitochondrial ADP/ATP exchange. Under these conditions, mitochondrial ATP extruded in the cytosol allows AK to transform AMP into ADP that can be readily converted into extra ATP in the mitochondria. In keeping with this interpretation, silencing of the main cellular AK isoforms AK1 and AK2 (27, 30) prevents the increase of ATP content above basal levels in Glu− cells undergoing PARP-1 activation (Fig. 3G). The present study therefore identifies cytoplasmic ADP contents during the first minutes of PAR degradation as the key determinant of PARP-1-dependent energy failure. An increase of ADP sufficient to outcompete AMP binding to the ANT from the very first phase of its accumulation warrants mitochondrial ATP production and ATP availability to AKs to convert AMP into ADP thereby preventing ANT failure and energy depletion. Of course, AKs do not play this role during PARP-1 activation in Glu−/H11001 cells because, despite massive AMP availability, ATP is lacking. Remarkably, the extremely rapid (within 5 min, Fig. 1B) PARG-dependent degradation of PAR causes an immediate burst of AMP accumulation that acutely alters adenine nucleotide homeostasis and renders even more critical the amount of ADP necessary to counteract the detrimental effect of accumulating AMP on ANT. Data showing that upon PAR degradation ADP content is lower than that of AMP in Glu−/H11001 cells but higher in those Glu+/H11002 is in agreement with our hypothesis. Prior work also demonstrates that PAR can be a converted into ATP (31, 32). However, this is a different biochemical pathway in which ADP-ribose pyrophosphorylase transforms ADP-ribose and pyrophosphate into ATP that, in turn, assists DNA repair.
We show here that ATP synthesis is maintained despite significant reduction of respiration in Glu$^+$ and Glu$^-$ cells. This may be ascribed to the loss of cytosolic NAD pool with ensuing failure of the NADH-transhydrogenase shuttle that provides respiratory equivalents (i.e. NADH) to mitochondria. As an additional hypothesis, decrease of respiration might be due to PARP-1-dependent mitochondrial NAD depletion. This might occur either because of a possible existence of a mitochondrial FIGURE 4. Effects of glucose deprivation on PARP-1-dependent cell death of HeLa cells. A, Western blotting evaluation of cytoplasmic AIF and Cyt c in Glu$^+$ and Glu$^-$ cells at different times after MNNG exposure. B, densitometric evaluation of three Western blots as shown in A, C, visualization of AIF localization in Glu$^+$ and Glu$^-$ cells exposed to MNNG for 2 h. D and E, representative (D) and total (E) cytofluorometric analysis of propidium iodide (PI)-positive cells at the indicated time points after MNNG treatment in Glu$^+$ and Glu$^-$ cells. In A and C an experiment representative of four is shown. In E, columns represent the mean ± S.E. (error bars) of three experiments conducted in duplicate. *, p < 0.05; **, p < 0.01 versus control. ANOVA and Tukey’s post hoc test were used.
PARP-1 or because of mitochondrial NAD efflux driven by reduced cytoplasmic content. Whereas the presence of PARP-1 within mitochondria is a highly controversial issue (14, 33–35), NAD fluxes across the mitochondrial membranes have been reported (36), as well as bidirectional mitochondrial NAD transporters in plants (37) and yeasts (38). A recent contribution, however, is at odds with the hypothesis that mitochondrial NAD content changes during PARP-1 hyperactivity (20). As a final note, reduced oxygen consumption in Glu− cells might be due to MNNG-dependent alkylation of respiratory complexes. Notwithstanding the underlying biochemical events, the fact that respiration diminishes to a lower extent in Glu− than in Glu+ cells and is paralleled by maintained mitochondrial coupling and ATP production suggests that it occurs through different mechanisms.

A key finding of our study is that PARP-1-dependent AIF and Cyt c release is reduced in Glu− cells. Of note, this finding indicates that bioenergetic homeostasis of mitochondria affects release of mitochondrial death factors during hyper-poly(ADP-ribosyl)ation. Also, data showing that cell death delay is lost 16 h after PARP-1 hyperactivation have important biochemical implications. Specifically, they suggest that the sole maintenance of ANT function is not sufficient to prevent PARP-1-dependent cell death. Indeed, AIF and Cyt c are released 3 h after PARP-1 activation even in Glu− cells that, at this time point, have unaltered ATP contents. Overall, data suggest that signals in addition to impairment of energy dynamic alter mitochondrial functioning during PARP-1-dependent cell death. Such signals may well be PAR polymer per se (29).

The present study also has potential pathophysiological implications. For instance, the sensitivity to agents leading to PARP-1-dependent cell death should depend on the glycolytic rate of a given tissue. More specifically, it will be its metabolic milieu and the resulting cytoplasmic ADP content that will determine the rate and extent of bioenergetic failure that follows PARP-1 activation. In conclusion, data corroborate the relevance of the Nudix hypothesis to PARP-1-dependent energy depletion, identifying glycolytic flux, ADP availability, and AK as new players in the detrimental events that follow cellular hyper-poly(ADP-ribosyl)ation.

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