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Involvement of PARPs in cell death

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1. ABSTRACT

Poly(ADP-ribosylation), an NAD dependent reaction culminating in the formation of ADP-ribose monomers, and their following polymerization, is activated as an emergency process in crucial situations such as DNA damage and cellular stress; due to this crucial function, the modulation of poly(ADP-ribosylation) during cell death has been investigated. This review will describe the properties of poly(ADP-ribose) as a signalling molecule in different paradigms of cell death, *i.e.* apoptosis, parthanatos, necroptosis and autophagy.

2. INTRODUCTION

Poly(ADP-ribosylation) is a post-translational modification of proteins playing a crucial role in many processes, including DNA repair, transcription, and cell death (1). It is regulated by a superfamily of 17 poly(ADP-ribose) polymerases (PARPs), which synthesize a polymer of ADP-ribose (PAR) from NAD, releasing nicotinamide and protons (2-3). The polymer is bound (on the aa Glu, Asp and Lys) to several acceptor proteins including PARPs, thus promoting their association with several factors in order to build a scaffold and form functional

complexes. To strictly regulate poly(ADP-ribose) turnover, and ensure complex release, PAR undergoes degradation mainly by the enzyme poly(ADP-ribose) glycohydrolase (PARG) (4).

The involvement of poly(ADP-ribosylation) in cell death has been originally demonstrated with respect to apoptosis, which is an active, energy-dependent process characterized by a number of morphological hallmarks, such as membrane blebbing, chromatin condensation, cell shrinkage, and finely regulated biochemical reactions leading to internucleosomal DNA fragmentation and protein cleavage (5). Due to the many steps required to activate and execute apoptosis, the whole process is energy-consuming; accordingly, ATP intracellular level is considered the crucial discriminant to make decision of activating the apoptotic pathway or of dying through a less scheduled mechanims, *e.g.* necrosis (6). In this scenario, it is evident that all the NAD-consuming metabolic reactions, including poly(ADP-ribosylation), could be essential in modulating cell death (7-10). The trend of poly(ADP-ribosylation) during apoptosis is described in more detail in the paragraph 3.

Searching for further paradigms of programmed cell death, a form of caspase-independent death characterized by an active role of PAR in driving the translocation of mitochondrial factors, including AIF (Apoptosis Inducing Factors), to the nucleus has been recently described (11). This type of death, called parthanatos (PAR and θάνατος, the greek word of death), occurs mainly in neuronal cells (12-13), but can be activated also in drug treated cancer cells where PAR is synthesized in response to drug induced damage. As detailed in the paragraph 4, parthanatos is considered a genuine PAR-dependent death, governed by a number of precise steps merging in cell dismantling (11).

Remarkably, a Janus process that could be considered both pro-survival and pro-death, *i.e.* autophagy (14), proved to be influenced by poly(ADP-ribosylation) through a specific effect on the apical regulator mTOR (15). This kinase has the job to keep autophagy "off", but its role is reversed by poly(ADP-ribosylation), which is decisive in this essential switch. The downstream autophagic events governed by mTOR are illustrated in the paragraph 6.

Recently, a further route leading to death, named necroptosis (resembling necrosis but governed in a programmed way, has been identified (16) and described as a process involving PARPs, given that PAR represents a signaling molecule which convince cells to activate sequential steps leading to necrosis, which is no more considered as accidental but, instead, programmed (paragraph 5).

The common denominator of the involvement of poly(ADP-ribosylation) in different paradigms of cell death is represented by PAR (17), which can drive cells into diverse (and sometimes opposite) manners to die (18-22). This polymer, neglected for many decades, is now celebrated as a pivotal molecule; this innovative consideration has an obvious effect, that is a renewed interest towards the strategies for either increasing or suppressing PAR synthesis, depending on the situation. In fact, in some cases cell death has to be promoted, for instance to improve the efficacy of drug treatments in cancer cells, while the excessive discard of cells occurring in neurodegenerative disorders is worth contrasting by blocking cell death.

PAR synthesis can be monitored by a monoclonal antibody against PAR raised years ago and still used in many laboratories (23) to obtain the direct evidence for an involvement of poly(ADP-ribosylation) in damage/stress response (24-25). The visualization of this biochemical reaction can be performed by immunofluorescence experiments (Figure 1A) and western blot (Figure 1C); furthermore, commercially available kits allow the quantification of PAR in cell extracts.

3. PARPs AND APOPTOSIS

During apoptosis, the most widely described form of programmed cell death (PCD type I), precocious and transient activation of PARPs (mainly PARP-1) by DNA damage or cellular stress (26-27) stimulates PAR synthesis in early apoptotic cells (28), thus causing an excessive NAD consumption. In this context, PARPs act as sensors of a dangerous situation and attempt to face it by triggering DNA repair (8, 28); however, when the critical situation persists, the overactivation of PARPs could be dangerous itself because it exhausts the intracellular energetic pool.

To face this deleterious (if prolonged) event, PAR production has to be limited; this job is carried out essentially by caspases (cysteine proteases cleaving at an aspartic acid) (29), which cleave PARP-1 (113 kDa) into an 89 kDa C-terminal fragment (containing the catalytic domain) and a 24 kDa N-terminal fragment with the DNA binding domain, the former retaining only the basal enzymatic activity and the latter being exclusively able to bind DNA breaks (9). Given that the peptides are unable to recognize DNA (89 kDa), or to synthesize PAR (24 kDa), they are completely inactive; this feature prevents a further NAD consumption.

Remarkably, p89 migrates from the nucleus into the cytoplasm in late apoptotic cells with advanced nuclear fragmentation (30), concomitantly with the extrusion of other nuclear proteins into the HERDS (Heterogeneous Ectopic RNP-derived structures) (31). These structures are made of factors outside their normal nuclear locations, reach the cytoplasm, pass into the cytoplasm and are finally extruded from the cell. The destiny of p89 is in fact reminiscent of the fate of different nuclear proteins during apoptosis, which can be responsible for autoimmune disorders (32).

After years of active research in this field, it is now clear that PARP-1 cleavage is a prominent biochemical hallmark of caspase-dependent apoptosis,

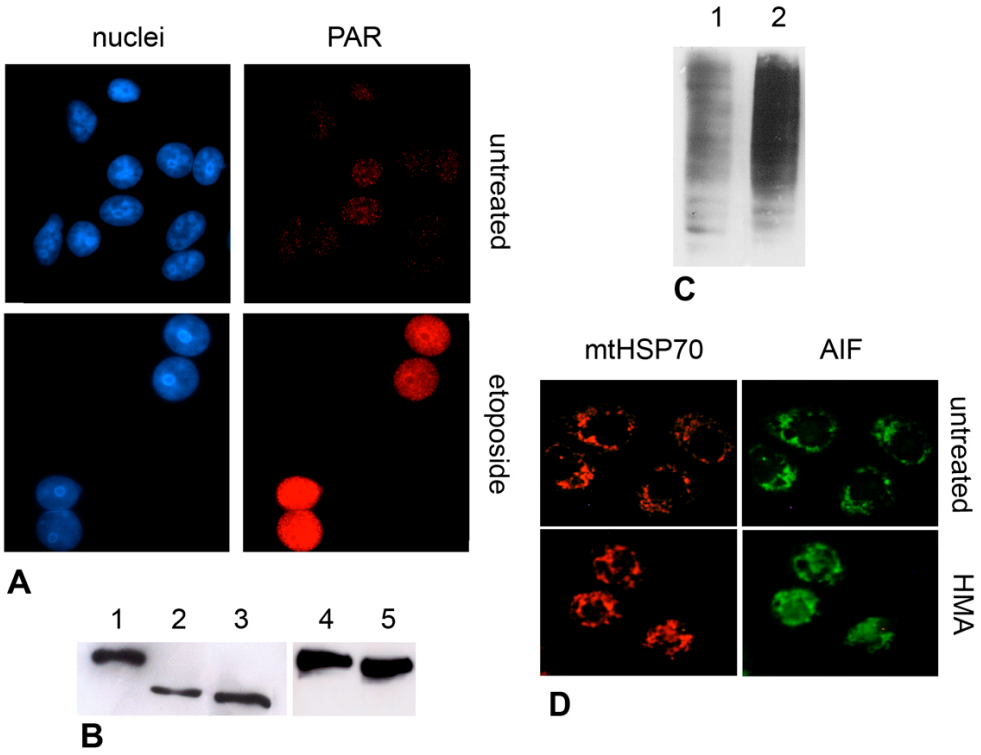

Figure 1. Monitoring of PAR synthesis, PARP-1 proteolysis and AIF relocation in cancer cells. A) Immunofluorescence analysis of PAR (97, 98); B) Western blot analysis of PARP-1 proteolysis in untreated human cancer cells (lanes 1 and 4) and cells made apoptotic with bleomycin (lane 2) or etoposide (lane 3), or autophagic by HMA (lane 5) (97, 98); C) Western blot analysis of PAR synthesis in untreated human cancer cells (lane 1) and samples made autophagic by HMA (lane 2, (97, 98); D) Double immunofluorescence analysis of the mitochondrial protein mtHSP70 (red fluorescence) and AIF (green fluorescence) which colocalize in untreated human cancer cells, whereas AIF enter the nucleus in cells treated with HMA as an effect of parthanatos (97, 98).

which can be visualized by western blot, immunofluorescence or flow cytometry. An example of PARP-1 proteolysis occurring during apoptosis is shown in Figure 1B. It has to be reminded that PARP-1 proteolysis is the final step of a cascade of events involving initiator and effector caspases; in fact, caspase 8 and 9 cleave (and activate) caspase 3 and 7, which act on a number of substrates, including PARP-1.

The discovery of a family of PARPs prompted many groups to extend the analysis of poly(ADP-ribosylation) during apoptosis. After the discovery of PARP-2, a DNA dependent PARP involved at a low extent in the damage response (33, 34), an active research aiming at depicting its role in cell death has been carried on. So far, it seems that PARP-2-is not a regulator of the expression of apoptotic genes, as revealed by array analyses (35-36). As for PARP-3, although this enzyme is involved in double strand break repair (37-40) possibly through its ability to modulate ATM activity (40), its possible role in apoptosis has not been addressed; intriguingly, it has been reported that PARP-3 has the feature of a mono(ADP-ribosyl) transferase and cooperates to activate PARP-1 (41).

A crucial role of the PARP family members tankyrases (TRF1-interacting, Ankyrin-related ADP-ribose polymerase), originally isolated through the binding to TRF1 (Telomeric Repeat binding Factor-1) was postulated. Tankyrases contain an ankirin domain that allows their interaction with other telomeric proteins, thus modulating telomere elongation and cancer development (42-44). It has been shown that tankyrase 1 interaction with the anti-apoptotic protein Mcl-1 (a member of Bcl-2 family) could regulate apoptosis (45-46), thus supporting an active function of this PARP family member.

To evaluate the global modulation of poly(ADP-ribosylation) during apoptosis not only in terms of activation/inactivation of PARPs, also the properties of PAR degrading enzyme(s), mainly PARG (47), have been investigated. Different isoforms of PARG have been identified, having a cytoplasmic/nuclear localization and being recruited to a DNA damage site in order to cooperate with PARPs (48-49). Due to its role in DNA repair (49) and to the dramatic impact of its inactivation in mammalian cells (50-51), it is widely accepted that PARG enzyme is an active player in cell death mechanisms (52-54).

During apoptosis, the activity of the enzymes responsible for PAR degradation has to be tightly regulated in order to avoid either PAR accumulation or inappropriate PAR degradation. In turn, the activity of PARG is controlled during apoptosis; in fact, PARG proved to be a substrate of executioner caspases (55), which are in charge for managing the intracellular localization and function of PARG proteolytic fragments (56).

4. PARPs AND PARTHANATOS

As a direct consequence of PARP activation, PAR is synthesized as long and branched chains in the nucleus, where it is bound to acceptors proteins and normally degraded by PARG and other accessory enzymatic activities that ensure its correct metabolism. However, accumulation of PAR could occur, as a consequence of either hyperactivation of PARPs under damage/stress conditions (57) or inefficient PARG (58). PAR accumulation has been observed during a peculiar caspase-independent paradigm of cell death involving the conversion of LEI (Leukocyte Elastase Inhibitor) into L-DNase II and its translocation to the nucleus, where PARP-1 is overactivated by the association to L-DNase II, thus producing excessive amounts of PAR (59-60).

When reaching high levels and consequent accumulation, PAR represents a potent signal for activating a form of caspase-independent cell death called parthanatos, executed through the translocation of the protein AIF (Apoptosis Inducing Factor) from the mitochondria to the nucleus (11, 57, 61). AIF is a flavoprotein synthesized in the cytosol as a precursor of 67 kDa, then imported as a mature form (62 kDa) into the mitochondrial intermembrane space, where it regulates important mitochondrial biochemical reactions. Upon an emergency signal, AIF is further converted into the 57 kDa soluble form tAIF and translocates to the nucleus where it promotes chromatin condensation and high molecular weight DNA degradation (62-63). DNA degradation promoted by tAIF requires the interaction with the histone H2AX to further recruit endonucleases/DNases (64).

The basic reactions characterizing parthanatos are PARP-1 activation, PAR formation, mitochondrial AIF release/nuclear translocation, AIF-mediated chromatin condensation/DNA fragmentation. In particular, the relocation of AIF is considered the marker of parthanatos (61) and can be visualized by immunofluorescence experiments, often coupled to the detection of a specific mitochondrial marker (Figure 1D). During parthanatos, PARP-1 remains intact (Figure 1B).

The signal triggering AIF release from mitochondria is in fact represented by PAR, which promotes the communication between the nuclear and the mitochondrial compartment (11). The precise mechanism of action of PAR in parthanatos has not been completely elucidated; of note, it has been shown that AIF contains a PAR-binding motif favoring a direct association between PARP-1 and AIF (65-66). In this respect, an active metabolism of PAR within the mitochondria has been described, possibly explaining the observed direct interaction between PAR and AIF (67-70). This evidence stimulated a controversial about the dogma according to which PAR is synthesized exclusively in the nucleus and does not leave this intracellular compartment; according to this assumption, PAR has been never been visualized within mitochondria. However, it has to be noticed that 10H antibody recognizes polymers of ADP-ribose longer than 10 units, thus being unable to detect short oligomers (23).

Parthanatos is generally considered as a causative event of neurodegenerative disorders characterized by a decreased number of neuronal cells (as an example, see 71). Taking into account the active role of PAR in this process, the impact of PARP inhibitors has been investigated, showing that pharmacological PARP inhibition could rescue neuronal cell death (11, 72-73).

5. PARPs AND NECROPTOSIS

The notion of Programmed Cell Death (PCD) was usually coping with the idea that the main PCD is apoptosis; in the second wave of research in PCD field, the concept of "programmed necrosis" was developed, based on the evidence that the passive features of necrosis are in fact well regulated. Thus, the word necroptosis was coined (74-75) and the idea of necrosis as a passive, accidental and uncontrolled process was no more taken into account. The crucial players of necroptosis are RIPs (Receptor-Interacting Proteins), *i.e.* death domain-containing Ser/Thr kinases, which are activated upon stress and promote death through the stimulation of the NF-kB pathway (76-79).

Of note, RIP activity could be negatively regulated by caspase 8, pointing the interplay between different death pathways (74, 79). According to the hypothesis of Hitomi et al. (74), it is possible to conceive that in the absence of caspase activation, which is a requirement to carry on apoptosis, and under conditions leading to cell death, necroptosis takes place. The investigation of the impact of necroptosis on cell metabolism under different conditions greatly was benefiting from the discovery that RIPs can be specifically inhibited by necrostatins, which affect TNF-α-induced necroptosis without interfering with the apoptotic pathway(s) (80).

The first evidence of an involvement of PARP-1 in necroptosis originated from the observation that overactivation of PARP-1 leads to a necrotic cell death controlled by TNF-α (Tumor Necrosis Factor) within a regulated pathway (81). Also TRAIL (TNF-Related Apoptosis Inducing Ligand) is able to induce necroptosis by triggering early intracellular ATP depletion paralleled by PARP-1 activation, and dependent on the action of RIPs, thus stimulating the hypothesis that an interaction between PARP-1 and necroptotic kinases exists (82-84). An active role for PARPs has been postulated on the basis of the evidence that PARP inhibitors affect the entire process (84, 85), even in a not univocal way (86). Moreover, PARP-2 was found among the many genes identified through a screening required for necroptosis (74).

As a matter of debate, controversial data reported by Sosna et al. (86) indicate that TNF-induced necroptosis and PARP pathway represent distinct and independent routes to programmed necrosis, given that the inhibition of TNF pathway did not affect/prevent the activation of necroptosis by PARP-1 signalling, and conversely PARP inactivation had no effect the execution of necroptosis TNF-dependent. Further work is required to better define the real impact of PARP on necroptosis. The identification of the molecular bases of necroptosis and the definition of the signals governing the shift between apoptosis and necroptosis could provide useful information on how to facilitate one process at the expense of the other.

6. PARPs AND AUTOPHAGY

Autophagy is a self-degradative process that ensures cell homeostasis by orchestrating the cellular components turnover; this orchestra is conducted by a limited number of highly conserved genes called ATG (AuTophaGy related genes). In general, autophagy is considered a survival mechanism, having protective functions in many cellular stress conditions, like starvation, through the ability to recycle energy originated from macromolecule degradation, useful for the *de novo* synthesis of macromolecules (87). The "dark side" of autophagy concerns its action as type II PCD, which occurs in response to a prolonged exposure to stress conditions which cannot be rescued, in order to ensure a safe survival.

The role of autophagy as energy sensor is mainly regulated by the action of serine/threonine kinases like mTOR (mammalian Target Of Rapamycin) and AMPK (AMP-activated Protein Kinase). Both enzymes are able to detect metabolic alterations and to face them in an opposite way: mTOR, which is the apical negative autophagy regulator, is kept active in case of high nutrient supply in order to keep autophagy "off" and inactivated during starvation, when autophagy needs to be switched on. Conversely, AMPK activation is required when new energy has to be produced through the autophagic pathway (88-89).

From this portrait describing the Janus face of autophagy (90), the key role of this process in metabolic balancing appears to be clear and suggests a possible role of PARPs as sensors of metabolic stress and in general of cellular stress conditions. Indeed, PARP-1 can modulate cell metabolism, being able to bind to DNA structures, nucleosomes, or protein partners, even in the absence of DNA damage (91-92), which is the most immediate signal for PARP activity.

The possible interaction between PARPs and the autophagy is of interest especially in cancer biology. Several studies have demonstrated that anticancer drugs that mainly induce DNA damage promote the interaction between PARP-1 and mTOR and/or AMPK. Moreover, in case of DNA damage and oxidative stress, PARP-1 is strictly required for the induction of autophagy, given that when PARP-1 is absent or inhibited the autophagic pathway is unable to proceed or even is not triggered because of the activation of an upstream activator of mTOR through Akt phosphorylation (93-94).

During autophagy, PARP-1 is not cleaved (Figure 1B) and remains so active that it can synthesize a huge amount of PAR (Figure 1C).

7. CONCLUSIONS

The active role of poly(ADP-ribosylation) in different paradigms of cell death, including apoptosis, parthanatos, necroptosis and autophagy, has been above described. According to the schematic representation shown in Figure 2, the only condition where PARP activity is unnecessary (because dangerous) is canonical apoptosis, which accomplishes the final steps after PARP-1 cleavage (and consequent inactivation) by caspases. In fact, although poly(ADP-ribosylation) represents a cellular emergency reaction, excessive PARP activity can be detrimental, as it occurs in several disorders such as inflammation, diabetes, nervous diseases, shock and cardiac failures. In this respect, it has been demonstrated that the use of PARP inhibitors to block poly(ADP-ribosylation) could exert a protective effect towards a number of pathological conditions (95-96).

The situation is less defined in the other types of cell death that are characterized by an active synthesis of PAR, whose accumulation represents a death signal able to activate a cascade of events converging to caspase-independent cell death pathways, including parthanatos and necroptosis (Figure 2). In this respect, the detection of PAR within the cells could be a marker not only of persistent DNA damage but also of prolonged stress conditions and a prerequisite for cell death induction.

Special attention is actually paid to the elucidation of the role of poly(ADP-ribosylation) in autophagy. Despite the important progress made in the disclosure of the molecular mechanisms governing when and how autophagy is activated, it is hard to define an unidirectional function for it. In fact, autophagy is a housekeeping survival mechanism protecting cells against stress conditions; however, when an unfavorable status persists, it may act as cell death (90). Intra/extracellular signals, including those governing cancer development and drug resistance, are instrumental for the dual role of autophagy; however, the precise signalling upstream the decision is largely unknown. By consequence, the elaboration of a strategy to manage poly(ADP-ribosylation) in this context appears to be arduous.

Finally, it has to be reminded that the impact of PAR on cell death does not follow an univocal scheme given that different death subroutines can be interconnected, as recently demonstrated by our studies on the effect of a drug affecting tumor microenvironment (97, 98). For example, an intricate

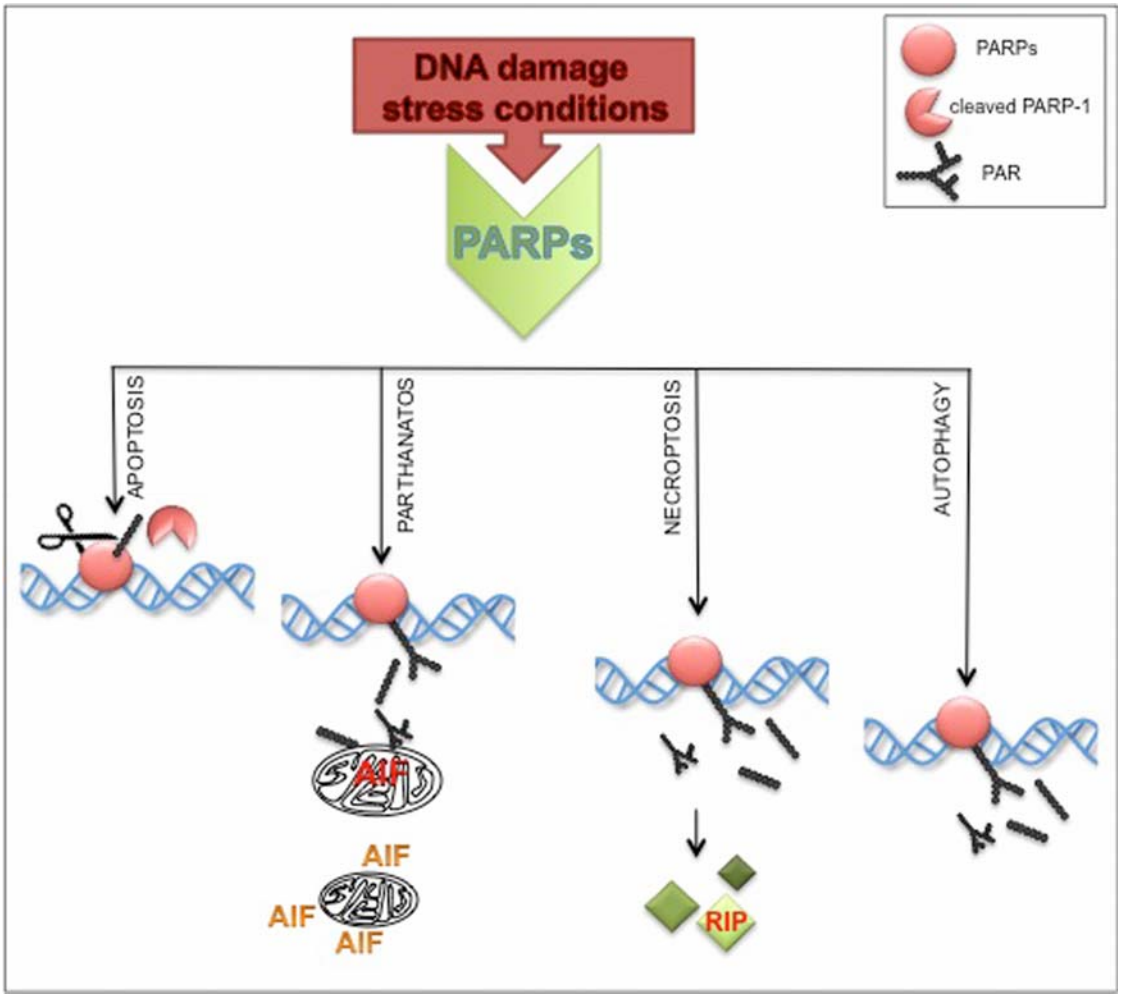

Figure 2. Schematic representation of PARP involvement in different death paradigms. AIF: Apoptosis Inducing Factor; RIP: Receptor-Interacting Protein.

interplay between apoptosis and autophagy exists (99-101), often leading to a critical situation when exogenous suppression of apoptosis induces autophagy, while autophagy inhibition causes apoptosis (102, 103). Thus, the modulation of poly(ADP-ribosylation) could be beneficial towards one process and noxious with respect to the other.

In conclusion, the evidence of a modulation of poly(ADP-ribosylation) during cell death pinpoints an additional role of this post-translational modification of proteins, thus expanding the notion of its functional relevance and stimulating new fields of research.

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