Poly(ADP-ribose) polymerase (PARP) enzymes catalyze the conversion of NAD⁺ to polymers of poly(ADP-ribose) (PAR). Although its role in the DNA-damage response has long been recognized, recent work indicates that PAR itself acts at the mitochondria to directly induce cell death through stimulation of apoptosis-inducing factor (AIF) release. This review discusses PAR synthesis and degradation, and the role of PAR misregulation in various disease states. Attention is given to opportunities for therapeutic intervention with small molecules that are involved in PAR signaling, with specific focus on poly(ADP-ribose) glycohydrolase (PARG) and AIF.

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Introduction
Poly(ADP-ribose) (PAR) polymers have been under scrutiny for many decades, as they are influential in mitotic progression, DNA repair mechanisms, transcriptional control, and caspase-independent cell death. Traditionally, PAR is thought to be produced in response to DNA damage, but its role in cell death suggests that alterations to the synthesis or metabolism of PAR could be intriguing therapeutic options for a variety of disease states. The direct role of PAR in cell death is complex; cells that produce massive amounts of PAR were initially believed to die through energy depletion, but recently it was discovered that PAR itself is a death-inducing signal. This review will focus on discoveries made related to PAR in the past three years, with an emphasis on the relationship of PAR to cellular demise, diseases associated with aberrant PAR signaling, and the potential for pharmacological intervention through the proteins and cell death signals associated with PAR.

Biosynthesis and biological functions of PAR
Poly(ADP-ribose) polymerase-1 (PARP-1) is the main enzyme responsible for producing PAR polymers, accounting for >99% of PAR synthesis in the cell during genotoxic stress [1,2]. PAR is synthesized directly from NAD⁺ (thus indirectly from ATP), initially generating free nicotinamide, and protein-bound mono(ADP-ribose). Elongation of the polymeric chain occurs at the 2′-OH of the mono(ADP-ribose) and subsequent branching of the polymer occurs at the 2′-OH of the ribose moiety (Figure 1). PARP-1 is potently activated by single and double stranded DNA breaks, leading to a 10–500-fold increase in PAR levels [3]. PAR is degraded by the enzyme poly(ADP-ribose) glycohydrolase (PARG) in an endoglycosidic and exoglycosidic manner, with $K_M$ values of 0.1–0.4 μM and ~10 μM for branched and short unbranched polymers, respectively [4]. This $K_M$ difference of PARG for its substrate will become more important later as the discussion shifts to the inhibition of PARG in different disease states. PAR polymers can reach lengths of hundreds of ADP-ribose units in vivo, with a half-life on the order of 1–10 min depending on the extent and type of DNA damage [2].

The nuclear accumulation of PAR leads to the recruitment of DNA repair proteins such as XRCC1, DNA ligase III, and the Ku70 subunit of the DNA-dependent protein kinase [5,6]. More recently, the DNA-damage responsive kinase ATM was added to the list of PAR-binding proteins [7]. Additionally, PARP-1 can modulate transcription by poly(ADP-ribose)lysylating corehistones and transcription factors, and can recruit PAR-binding proteins to ribosylated proteins; PARP-1 itself can also act as a cofactor in transactivation. The reader is referred to a recent review [8] that delves much more deeply into the subject of PAR synthesis and its effect on transcription.

The importance of PAR in mitosis was recently suggested by Mitchison and coworkers [9*]. In these experiments, the addition of exogenous PARG into cycled Xenopus egg extracts rapidly led to misalignment of chromosomes and disruption of bipolar spindle assembly. The requirement of PAR for proper mitosis is further supported by the involvement of tankyrase-1 in metaphase progression [10,11]. Tankyrase-1 is a member of the PARP family that can bind to and poly(ADP-ribose)ate NuMA, whose function is to crosslink microtubules to spindle poles during mitosis [12]. The siRNA-mediated knockdown of tankyrase-1 leads to metaphase arrest because of the inhibition of sister telomere separation [10]. Interestingly, tankyrase-1 also positively regulates telomere length...
through its poly(ADP-ribosyl)ation and subsequent inhibition of TRF1 DNA binding [13].

**Death signaling by PAR**

As mentioned above, mild DNA damage leads to the stimulation of PARP-1 activity and the nuclear accumulation of PAR; this allows for the recruitment of DNA repair proteins and confers cytoprotection against genotoxic stress. However, under conditions of excessive DNA damage, PARP-1 overactivation can be detrimental to cell viability. Proposed by Berger and coworkers [14], the ‘suicide hypothesis’ connects PARP activity to necrotic cell death by suggesting that the PARP-dependent depletion of cellular NAD\(^+\)/ATP pools biases the cell toward necrosis [15]. This hypothesis is supported by the observed protective effects of the genetic ablation of PARP-1, and the use of PARP-1 inhibitors to hinder cell death mediated by oxidative insult (H\(_2\)O\(_2\), peroxynitrite), excitotoxic injury (NMDA, glutamate), complex I inhibition (MPTP/MPP\(^+\)), and ischemia/reperfusion [16,17]. But what role does energy depletion play in PARP-1-dependent cell death? Evidence now suggests that PAR itself could contribute to cell death induced by PARP-1 activation.

To investigate whether free PAR itself was toxic, Dawson and coworkers recently performed a study [18**] where the PARP-1-dependent decrease in cellular...
NAD\(^+\) and ATP levels was uncoupled from the production of PAR. They used a lipid-based delivery system to transport \textit{in vitro} synthesized PAR into living cells. The cell death observed after PAR delivery was strongly dependent on the size and dose of PAR; it was also caspase-independent, as cell death was not abrogated by the use of the pan-caspase inhibitor zVAD-fmk. As expected, pretreatment of the PAR polymer with PARG or phosphodiesterase 1 (PD1) before delivery prevented cell death, as the more toxic long-chain polymers were degraded.

To analyze the involvement of PAR in a cellular disease model, mouse cortical neurons were treated with the excitatory toxin \(N\)-methyl-\(d\)-aspartate (NMDA) [18**]. NMDA overstimulates NMDA receptors leading to a large increase in intracellular calcium, which leads to the production of reactive oxygen species (ROS). Intracellular ROS production and the subsequent DNA-damage results in PARP-dependent cell death. Using an ELISA-based assay, the authors found that in response to NMDA treatment (one hour) the levels of PAR reached ~80 nM causing ~60\% cell death. This concentration and toxicity was similar to the results obtained when PAR was \textit{in vitro} injected into HeLa cells. Due to the embryonic lethality of PARG\(^{-/-}\) mice, mice heterozygous for PARG were used in a middle cerebral artery occlusion (MCAO) study, in which NMDA receptor overstimulation is thought to mediate cell death after ischemia [19]. The authors found that the infarct volume was increased significantly in PARG\(^{+/−}\) mice, and decreased in transgenic mice over-expressing PARG. Given the enhancement of infarct volume observed in PARG\(^{+/−}\) mice, it would be interesting to study the ablation of different PARG isoforms (discussed below) and their involvement in MCAO. These studies indicate that PAR-dependent cell death may be due more to the direct toxicity of PAR and less to the energy depletion that may occur upon massive PAR synthesis.

**The duality of PAR**

The PAR polymer is thus a central player in both the life and death of the cell. PAR is required for proper mitosis, and PAR synthesis helps to stimulate the DNA-damage repair response. Conversely, too much PAR synthesis may deplete NAD\(^+\)/ATP stores and kill the cell, and long-chain PAR itself is a key player in caspase-independent cell death (Figure 2). Depending on the therapeutic goal, enhancement or reduction in cell death could be accomplished by modulating PAR levels; one way to do this would be the inhibition of PARG, as it is the key enzyme involved in the degradation of PAR. Keeping with this life/death duality of PAR, the evidence presented below indicates that under certain conditions PARG inhibition could be cytoprotective and under other conditions it could be cytotoxic.

**Modulating PAR levels: knockdowns, knockouts, and small molecule inhibition of PARG**

PARG isoforms present in mammalian cells are the full length PARG\(_{110}\) (nuclear), PARG\(_{103}\) and PARG\(_{99}\) (cytoplasmic), PARG\(_{60}\) (cytoplasmic/mitochondrial), and PARG\(_{85}\) and PARG\(_{73}\) (cytoplasmic, catalytically active fragments produced upon caspase-3 cleavage). While most of the PARG activity in healthy cells is contained in the cytoplasm, DNA damage causes the nuclear translocation of cytoplasmic PARG isoforms [20,21*].

In recent years, much work has been performed to identify the role of PARG in response to genotoxic stress and ischemia; most studies employ either total PARG depletion by RNAi or the genetic deletion of exons 2 and 3 which leaves only the 60 kDa isoform. RNAi-mediated reduction of PARG results in sensitivity to radiation in \textit{Caenorhabditis elegans} [22], as well as accumulation of PAR and increased infarct volume after MCAO in mice [23*]. Interestingly, conflicting results have been reported addressing the role of PARG in DNA repair. Genetic deletion of PARG exons 2 and 3 caused decreased XRCC1 foci formation, but an increase in DNA repair was observed [24*]. In contrast, RNAi-mediated knockdown of PARG resulted in increased XRCC1 foci formation and duration, yet a decrease in DNA repair was observed [25*]. It should be noted that two separate studies have reported the early embryonic lethality of PARG\(^{-/-}\) mice [26] or \textit{Drosophila} lacking a functional PARG gene [27]. PARG accumulation was observed in both studies, probably because of the inability to hydrolyze PAR. Phenotypically, PARG\(^{-/-}\) mouse trophoblast stem cell lines established before embryonic failure required the PARP inhibitor benzamide (0.5 mM) for growth and were more sensitive to the toxic action of MNNG and menadione. Surviving \textit{Drosophila} embryos that lacked a functional PARG gene had to be grown at a permissive temperature for proper embryo eclosion and exhibited progressive neurodegeneration during their brief adult life.

In PARG’s absence, PAR accumulates; at the same time PARP-1 automodification is increased, causing the inactivation of PARP-1 and its dissociation from DNA. In this way, the inhibition of PARG results in the indirect inhibition of PARP-1, at least in an acute insult or DNA repair scenario. As previously mentioned, the inhibition of PPAR or PARG in DNA repair scenarios generally results in the increased sensitivity of the cell to additional insults, thus the interest in combining PARP/PARG inhibitors with DNA-damaging agents for treating cancer [17,28,29]. In contrast to basal DNA repair in cancer, an alternative scenario emerges when the DNA damage is overwhelming, causing the overactivation of PARP-1 and a large and immediate reduction in the cytosolic pool of NAD\(^+\). Inhibition of PARP or PARG
in this scenario will halt the production of PAR and the consumption of NAD⁺, offering cytoprotective effects in models such as ischemia/reperfusion [30,31] and heart attack [32]. Additionally, Blenn et al. [33] report that RNAi-mediated silencing of PARG results in the accumulation of PAR, but confers resistance to H₂O₂. It thus appears that the absence of PARG can either sensitize cells to oxidative damage or protect them from injury during times of extreme stress. Thus, from a therapeutic perspective, modulating PAR levels could be an attractive strategy for either induction or prevention of cell death, depending on the disease. The obvious choice for altering cellular PAR levels would be through the inhibition of PARP or PARG. In vitro inhibition of PARG is possible with the substrate mimic ADP-HPD [34], but this compound is not useful in cell culture or in vivo as it is not cell-permeable. Ethacridine and other DNA intercalating agents inhibit PARG by binding to DNA and thus are not useful in studying PARG specifically [35]. A large body of work has been performed on the cytoprotective effects of tannins, however, their inhibition of PARG may be nonspecific and their protective effect may be because of their ROS scavenging abilities [36]. The most recent class of synthetic PARG inhibitors, typified by GPI 16552, initially did not show much in vitro PARG inhibition, but their recently reported in vivo effects against ischemia/reperfusion injury may warrant further investigation [37]. With few potent specific inhibitors of PARG, the modulation of PAR synthesis would seem to largely depend on PARP-1 inhibition. However, there is an additional protein that is strongly connected to PAR signaling and could be targeted by small molecules, apoptosis-inducing factor (AIF).
PAR stimulates AIF translocation

First described in mammalian cells by Susin and coworkers in 1999, translocation of AIF from the mitochondria to the nucleus was found to induce initial early stage chromatin condensation, large-scale (~50 kb) DNA fragmentation, and cell death [38]. AIF is a flavoprotein normally anchored to the inner mitochondrial membrane where it acts as a NADH oxidase, and possibly contributes to the maintenance of complex I [39]. AIF is released from the mitochondria in response to a wide range of caspase-dependent and caspase-independent insults [40]. Once in the cytoplasm, AIF translocates to the nucleus where it is involved in chromatin condensation (although it itself is not a nuclease) and recruits nucleases such as cyclophilin A (cypA) [41] and endonuclease G [42]. The exact mechanism of AIF release
remains a question, though the requirement of Bax oligomerization and pore formation on the surface of the mitochondria, or mitochondrial outer membrane permeabilization (MOMP), has been well studied in the release of other mitochondrial proapoptotic factors [43]. The discovery of AIF helped bring into focus the process of caspase-independent cell death (Figure 3). AIF is required for embryonic development, as the only viable system for its study is the Hq mutation which results in a 80% reduction in AIF expression [44]; complete ablation of the AIF gene is embryonic lethal [45].

The connection between PARP-1 and AIF was first described in 2002 [46], wherein embryonic fibroblasts from PARP-1 KO mice failed to release AIF from mitochondria and cytoprotection was conferred against DNA-damaging agents. More recently and published simultaneously with the discovery of free PAR as a cytotoxin was another report by Dawson and coworkers describing AIF as the mediator of cell death in response to PAR delivery [47**]. Interestingly, AIF was released from isolated mitochondria in response to PAR-containing nuclear supernatants, as well as in response to purified PAR in a size-dependent and dose-dependent manner. Treatment of nuclear supernatants with proteinase K failed to prevent AIF release, suggesting that the factor crucial for the release of AIF may be nonproteinaceous in nature, consistent with the involvement of PAR. Indeed, purified PAR does induce release of AIF, but the possibility of a carrier protein not confined to the nucleus cannot be ruled out. Analogous to the cytoprotective effect of pretreating PAR polymers with PARG or PD1, AIF release was reduced when PAR polymer was degraded before addition to isolated mitochondria. Additionally, AIF nuclear translocation and chromatin condensation was induced by PAR delivery into living cells, and this effect was prevented by pretreatment with PARG or PD1. The cell death associated with PAR was shown to be AIF-dependent as cortical neurons harvested from Hq mice were resistant to both PAR and NMDA toxicity. However, the mechanism of AIF release by PAR is still unclear. Although PAR can elicit AIF release from isolated mitochondria in vitro, what other factors might be involved inside a cell?

In a recent report, Moubarak et al. identify PARP-1, calpains, and Bax as requirements for AIF release and cell death in response to MNNG [48**]. Using a series of knockouts, they identify PARP-1 upstream of calpains, though a possible mechanism for calpain activation after PARP-1 activation is not proposed. Furthermore, active Bax was significantly reduced in calpain knockouts, suggesting that calpain may activate Bax in addition to the well-studied mechanism of caspase-8-dependent, Poly(ADP-ribose) makes a date with death

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Figure 4

Modulation of PARP-1, PARG, and AIF activity as a therapeutic strategy. The current applications of chemical and genetic inhibition of PARP-1, PARG, and AIF are shown. The reduction in PARP-1 activity (through inhibition, genetic knockout, or RNAi) has shown promise in all three disease states [16,17]. Both genetic and chemical studies have shown that PARG inhibition could sensitize cells to certain anticancer agents, and could be protective in stroke [28–33,54]. Studies utilizing RNAi or Hq mice (and cells derived from those mice) indicate that the reduction in AIF would be protective in neurodegenerative models and stroke [40,52,53,55,56]. Genetic and chemical inhibition of PARG activity in neurodegeneration, as well as chemical inhibition of AIF, remains to be explored.
Bid-mediated activation of Bax. Calpains have also been implicated as the enzyme responsible for AIF cleavage and release from the inner mitochondrial membrane [49].

**AIF and PARG: potential for small molecule inhibition?**

Given the broad scope of diseases associated with aberrant PAR metabolism, it is not surprising that there is significant interest in the development of potent and cell-permeable inhibitors of PARP, PARG, and AIF (Figure 4). In addition to stroke and cancer, PARP-1 inhibitors have been shown to be efficacious in chronic neurodegenerative models [50]; a more comprehensive description of the benefits of PARP inhibitors has been compiled elsewhere [17]. PARG inhibitors have been shown to be beneficial in the potentiation of the alkylating agent temozolomide [51], and against ischemia/reperfusion injury [37]. Currently, no small molecule inhibitors of AIF exist, so the evidence remains purely genetic in nature; reduction of AIF levels has been shown to be beneficial in models of neurodegenerative disease [52] and stroke [53].

Figure 5

Potential consequences of PARG inhibition. (A) The PARP–PARG cycle. PARP-1 binds to damaged DNA, catalyzes the addition of PAR onto itself and acceptor proteins, dissociates from DNA, and then has PAR removed from it by PARG and MARH activities, freeing it to bind to damaged DNA again. While the accumulation of PAR leads to beneficial consequences such as DNA repair, AIF translocation and NAD⁺ depletion also occur. (B) Addition of a PARG inhibitor (green hexagon) that would block the exoglycosidic activity of PARG. This would leave PARP-1 poly(ADP-ribosyl)ated, preventing NAD⁺ depletion. It would also result in the degradation of long-chain PAR, preventing the nuclear translocation of AIF.
What about the inhibition of PARG in neurodegenerative disease? The recent data from Dawson and coworkers indicate that PARG activity is probably cytoprotective in many contexts. These findings imply that the inhibition of PARG would enhance the effect of PAR-induced toxicity. When combined with other reports discussed in this review indicating that the reduction in PARG expression as a sensitizer to oxidative injury, it would appear on the surface that PARG inhibition would not be beneficial for neurons under constant oxidative assault.

However, if the longest PAR polymers are the most toxic, and there is at least a 40-fold difference in $K_M$ values for PARG against long-branched polymers versus short-chain polymers [4], it may be possible to primarily inhibit the slow reaction, thus allowing for the PARG-mediated degradation of toxic long-chain PAR (Figure 5), but still preventing NAD$^+$/ATP depletion. If in fact this were an attainable scenario, questions still exist: firstly, will the autoregulation of PARP-1 still retain its ability to recruit DNA repair proteins without long-complex PAR polymers attached to it? Secondly, are there benefits to inhibiting PARG versus AIF or PARP-1 if potent and specific inhibitors were developed for those proteins? Thirdly, how will inhibitors of the endoglycosidic activity of PARG be identified? As mentioned previously, caveats exist with most if not all of the PARG inhibitors identified so far [54].

AIF has been implicated in the premature cell death observed in many different disease states [40,52,53,55,56], and given the connection recently made between PARP-1, PAR, and AIF, it would seem that inhibitors of the AIF–DNA interaction would be cytoprotective. Although originally identified as being more sensitive to oxidative stress [44], the Hq mutation (with an 80% reduction in levels of AIF) has proved a useful tool in studying the role of AIF in neuronal cell death. When cortical neurons from the Hq mouse are treated with camptothecin or excitatory toxins (glutamate, NMDA, AMPA, and kainic acid), they show increased viability relative to WT neurons [57]. Recently, mice carrying the Hq mutation also displayed a reduced infarct volume after hypoxia/ischemia [58]. Additionally, siRNA-mediated knockdown of AIF attenuated MPTP/MPP$^+$ toxicity in dopaminergic neurons [52]. All of these studies imply that inhibition of AIF could be cytoprotective.

Unlike PARP-1 and PARG, AIF does not have an enzymatic activity directly associated with its cell death function, thus complicating the search for AIF-specific inhibitors. However, key residues have been identified for AIF–DNA binding, and the ablation of these residues results in decreased DNA binding, decreased chromatin condensation, and improved viability versus overexpressed AIF alone [59]. These points offer hope that small molecule inhibitors of the AIF–DNA interaction can be identified.

Conclusion
Significant progress in our understanding of PAR, its regulation, and its role in cell death has been made in the last three years. Highlights include the discovery of diverse localizations of PARG isoforms, steps toward the mechanisms of AIF release from mitochondria, and the identification of a direct proapoptotic role for PAR, an additional responsibility for an already busy biopolymer.

Conflict of interest statement
The authors state that they have no conflicts of interest.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


First explicit description of the toxic action of PAR by itself. Isolated PAR and PAR-containing supernatants were found to cause the release of AIF from mitochondria. Pretreatment of PAR with PARP or PDI prevents AIF translocation, chromatin condensation, and cell death associated with PAR. The Hq mutation protects against NMDA and PAR-mediated cell death.


Interesting report on the localization of the different PARG isoforms during genotoxic stress. PARG103 relocates from the nucleus to the cytoplasm after gamma irradiation, PARP103,PARP96, and PARP25 translocate from the cytoplasm to the nucleus in this model of DNA damage.


This study reports that the genetic deletion of PARG exons 2 and 3 results in increased PAR accumulation, and an increase in infarct volume after MCAO.


This report combined with [25°] give conflicting results, though differing methods of PARG depletion and genotoxins used could account for the difference. PARG depletion by RNAi results in sustained PAR accumulation, an increase in the number and duration of XRCC1 foci, and decreased DNA repair as measured by the Comet assay.


This study reports that the genetic deletion of PARG exons 2 and 3 results in greater PARP and PARG activity, less PARP-1 autophosphorylation, reduced XRCC1 foci formation and H2AX phosphorylation, and increased DNA repair as measured by the Comet assay. Relocalization of remaining 60 kDa isofrom of PARG may account for increased DNA repair.


This study reports that the silencing of PARG leads to an increase in PAR levels, but unexpectedly a reduced sensitivity to H2O2, as well as a reduction in energy loss associated with PARP-1 overactivation. Interestingly, no protection was observed against MNNG.


Although no insight into the mechanism of AIF release induced by PAR is given, this work is a large step forward in the understanding of PARP-1-dependent AIF-mediated cell death. PAR is delivered to intact cells and results in caspase-independent cell death. Cell death is prevented by transient overexpression of PARP or treatment with a PAR antibody. PARG- cells are sensitized to NMDA and have a larger infarct volume after MCAO, while PARG overexpressing cells have a smaller infarct volume.


Knockout study showing that PARP-1 activation, calpain activity, and active Bax are required for AIF release and sensitivity to MNNG. It would be interesting to see whether or not free PAR (as demonstrated in [46]) would cause the release of AIF in Bax- cells.


