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Poly (ADP-ribose) glycohydrolase (PARG) and its therapeutic potential

WooKee Min[1], Zhao-Qi Wang[1,2]

[1]Leibniz Institute of Age Research - Fritz Lipmann Institute (FLI), 07745 Jena, Germany, [2] Faculty of Biology and Pharmacy, Friedrich Schiller University Jena, 07743 Jena, Germany

TABLE OF CONTENTS

1. ABSTRACT

Poly (ADP-robose) glycohydrolase (PARG) is a catabolic enzyme that cleaves ADP-ribose polymers synthesized by members of the poly (ADP-ribose) polymerase (PARP) family of enzymes. The growing evidence supports the importance of a tight control of poly (ADP-ribose) metabolism by the two major enzymes, PARP-1 and PARG. Recent studies have advanced the understanding of PARPs' and PARG's functions in various cellular and physiological processes. In the last 10 years, homeostasis of poly (ADP-ribosyl)ation has been a target of pharmaceutical interventions for various pathologies. Although the polymer synthesizing enzyme PARP-1 has been well studied, the function of PARG remains largely unknown. However, a great effort has been made in recent years to delineate biological functions of PARG and to explore the therapeutical potentials of PARG inhibition in pathophysiological conditions such as inflammation, ischemia, stroke, and cancer chemotherapy.

2. INTRODUCTION

Poly (ADP-ribosyl)ation is a post-translational modification of proteins with a poly (ADP-ribose) (pADPr) polymeric chain composed of linear and branched repeats. Homeostasis of poly (ADP-ribosyl)ation is known to play multiple roles in cellular and physiological processes. This is tightly controlled by two steps. First, pADPr is synthesized by poly (ADP-ribose) polymerases (PARPs), e.g., PARP-1 and -2. This process uses cellular NAD^+ leading to the depletion of the ATP pool and causing cell dysfunction or necrosis. pADPr attaches onto nuclear acceptor proteins including histones, transcription factors, PARP-1 itself, and thereby transiently modifies the functions of the acceptor proteins. At the second step, poly (ADP-ribose) glycohydrolase (PARG) degrades or cleaves the polymers with high specificity at the glycosidic bonds to generate mono (ADP-ribose) which can be re-used as a substrate for the ATP production ((1), see Figure 1). PARG is the biochemical and physiological counterpart of

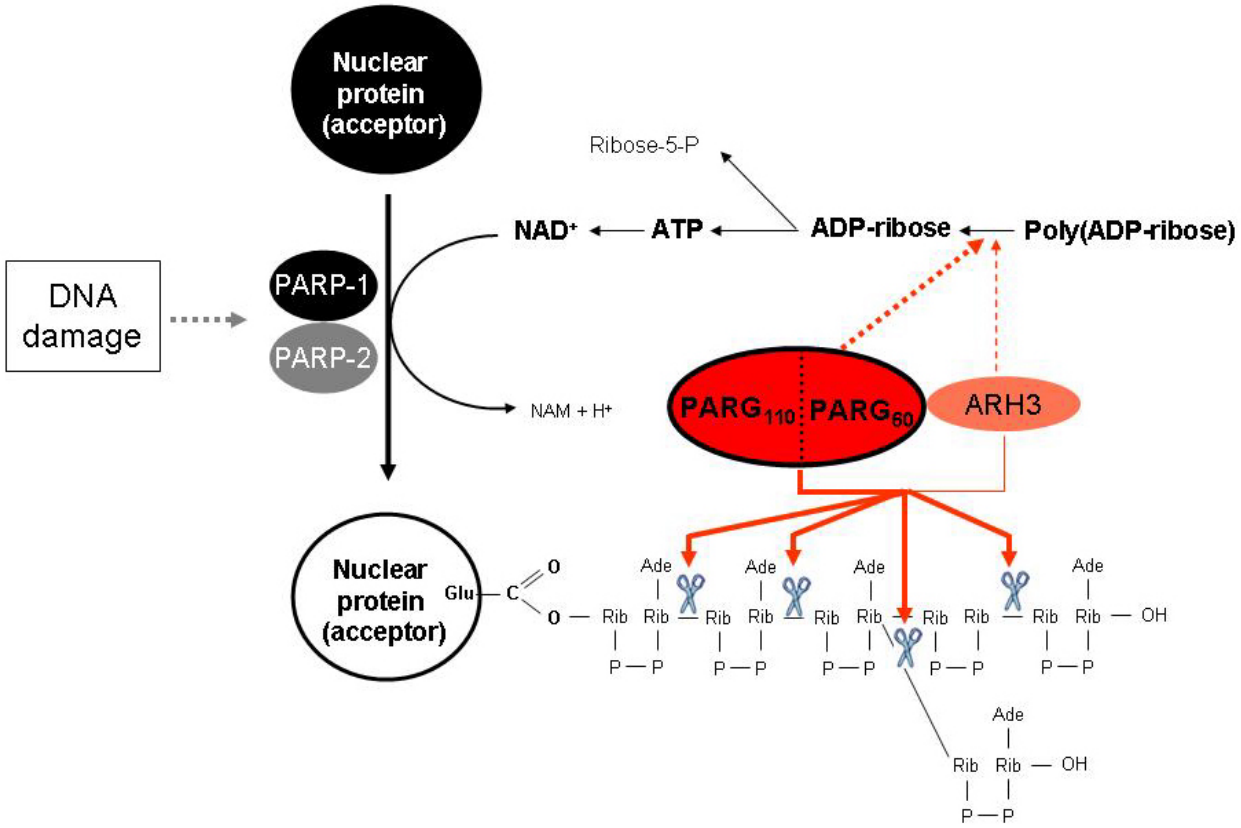

Figure 1. Homeostasis of poly (ADP-ribose). In response to DNA damage, PARP-1 (and perhaps also PARP-2) binds to DNA breaks to catalyze the pADPr formation onto acceptor proteins using NAD+ as a substrate. After the pADPr accumulation, PARG (e.g., $PARG_{110}$, $PARG_{60}$, and ARH3) cut either linear or branched glycosidic bonds between the (ADP-ribose) units into mono-ADP-ribose. This mono (ADP-ribose) is recycled as the ATP precursor, an important substrate to generate NAD^+.

PARP enzymes and thus involved in the poly (ADP-ribose) metabolism, which is speculated to regulate a large number of different biochemical machineries. Amounting evidence implicate that the regulation of poly (ADP-ribosyl)ation mediated by multiple PARPs and PARG contributes to a wide range of cellular functions. However, PARG has not been intensively studied due to the technical difficulty of protein purification, and the lack of specific chemical inhibition and genetic models.

Interfering with the pADPr metabolism using chemical inhibitors and genetic mutations has generated much insights into the biological roles of poly (ADP-ribosyl)ation and its potential therapeutical applications. PARPs have been well studied and many reviews were recently published focusing on the biochemical and biological properties of PARPs as well as the pharmacological applications of PARPs' inhibition. This article will summarize the recent development by studying the functions of PARG in physiological processes, and the attempt at testing PARG as a novel pharmaceutical target.

3. PARG ISOFORMS AND RELATED MEMBERS

The full size of the PARG protein is about 110-kD and it is encoded by 18 exons. The protein consists of the regulatory domain (exons 1-3, 1-426 amino acids) at the N-terminus, and the catalytic domain (exons 9-14, 486-838 amino acids) at the C-terminus ((2-4), see Figure 2). The catalytic domain and functionally important residues are evolutionarily conserved among all organisms, whereas putative regulatory domain varies among species (reviewed in (5)). Although putative N-glycosylation sites and/or the phosphorylation site (ser316) spanning the whole PARG sequence can be detected using the Protoscan program, no significant posttranslational modification site has yet been identified ((1, 5, 6), see Figure 2). PARG possesses at least four potential nuclear localization signals (NLS, aa10-16, aa32-38, aa421-446, and aa838-844), three nuclear export signals (NES, aa126-134, aa11421-446 and aa881-888) and one mitochondrial targeting signal (MTS, aa461-486) (Figure 2). However, the functional significance of these subcellular localization sites has not been experimentally studied.

The human PARG, encoded by a single gene locus, is reported to have five isoforms being 111-kD (localized in nuclear), 102-kD (in cytoplasm), 99-kD (in cytoplasm), 60-kD (in nuclear and cytoplasm), 55-kD (in mitochondria), respectively (3, 7-10). Haince *et al.* using the fluorescence recovery after the photobleaching (FRAP) technique found that the cytoplasmic 102-kD PARG

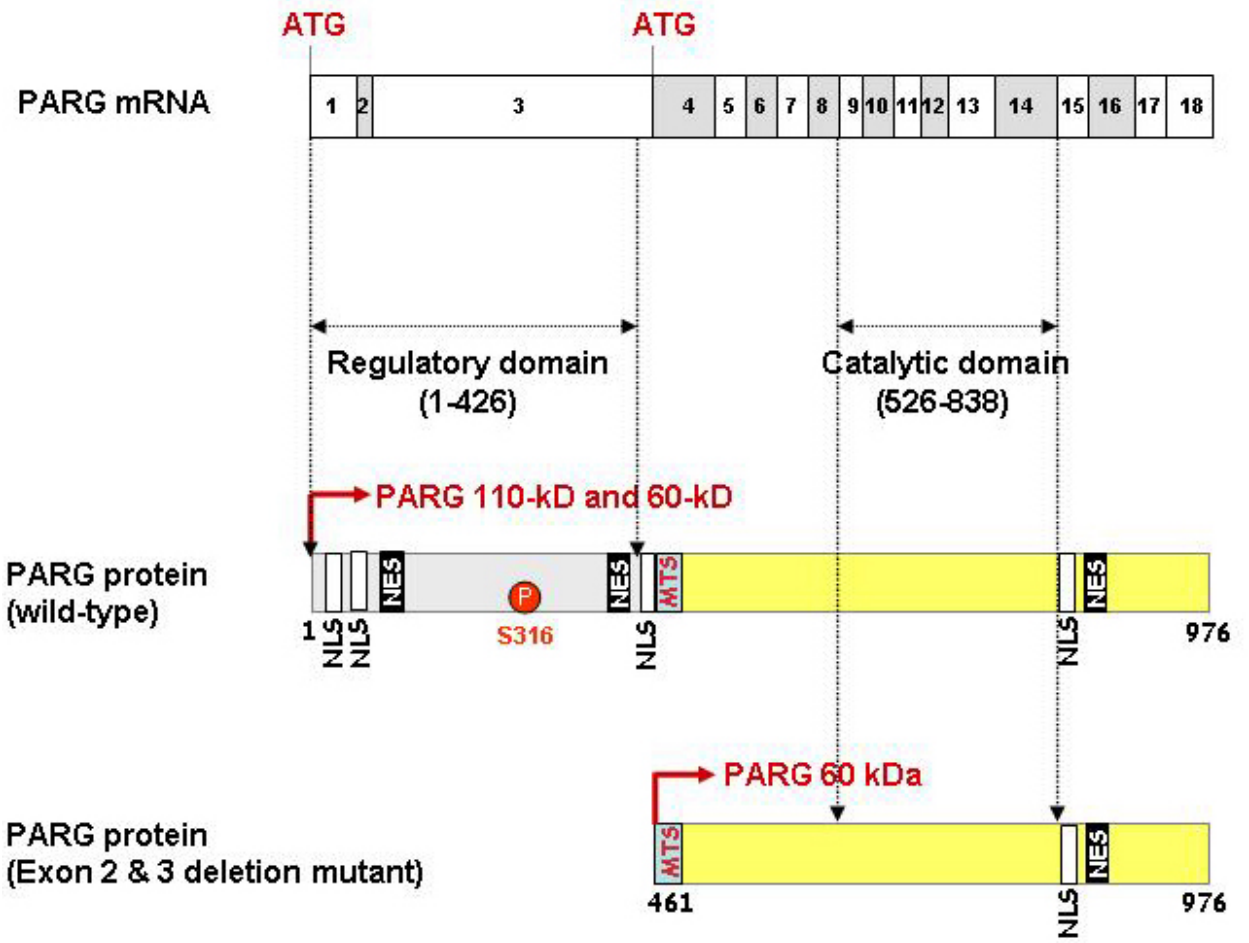

Figure 2. The mouse PARG isoforms and their functional domains. Translation begins from ATGs produces the 110- and 60-kD PARG proteins in wild-type mice. The N-terminal putative regulatory domain (aa1-426) contains two nuclear localization signals (NLS), and two nuclear export signals (NES). The serine-316 phosphorylation site is also predicted in the regulatory domain. Deletion of exons 2 and 3 resulted in production of the PARG 60-kD isoform in mice due to the reinitiation of the translation signal (ATG) encoded in exon 4. The catalytic C-terminal domain also contains one mitochondria targeting sequence (MTS), as well as one NLS and one NES.

isoform translocated into the nucleus in response to DNA damage induced by gamma-irradiation (11), which demonstrates a high mobility of major PARG isoforms and their dynamic distribution between the cytoplasm and nucleoplasm.

In the mouse, there are two PARG mRNA species that may both produce the 110-kD PARG isoform and one of them can also produce the 60-kD isoform (3, 4, 12). mRNA mapping and sequencing have predicted alternative and putative translation reinitiation of the PARG protein being standard 110-kD and perhaps also a short form of 60-kD in mice (3, 12). Furthermore, disruption of exons 2 and 3 in cells depletes the 110-kD isoform and discovered the reinitiation of PARG translation at exon 4 leading to the production of only the 60-kD isoform in mouse tissues, which possesses PARG activity (3, 12). Interestingly, $PARG_{110}$-deficient cells exhibited a 3-fold increase of PARG activity in mitochondria ((12), see Table 1). Although the mechanism by which PARG targets to mitochondria is not known, exon 4 contains the mitochondria targeting sequence (MRRMPRCGIRLPLLRP) that may generate the mitochondrially targeted PARG isoform (Figure 2). Indeed, mitochondrial localization of PARG was formed (13). Thus, the subcellular localization and the distinct functions of PARG isoforms require further investigation.

Although these PARG isoforms are encoded by a single gene in the mammalian genome, an additional mammalian gene carrying a residual PARG catalytic activity has been recently reported (14). The ARH3, a mammalian 39-kD ADP-ribose hydrolase-like protein, also possesses the catalytic activity of pADPr glycohydrolase albeit at a much low level ((14), see Figure 1). PARG and ARH3 show only 19% amino acid identity and the essential glutamate residues are not conserved suggesting the distinction of both proteins. However, the contribution of endogenous ARH3 in pADPr turnover is not known.

Table 1. PARG activity in $PARG_{110}$–deficient MEFs

Subcellular fractions	Genotype	pADPr degrading activity (pmol/min/mg)	Ratio of $PARG_{110}^{-/-}$ / Wild type
Cytosol	Wild type	69	3%
	$PARG_{110}^{-/-}$	2	
Nucleus	Wild type	32	28%
	$PARG_{110}^{-/-}$	9	
Mitochondria	Wild type	16	331%
	$PARG_{110}^{-/-}$	53	

PARG specific activity in subcellular fractions of $PARG_{110}$ mouse embryonic fibroblast (MEF) cells. While the PARG activity is almost abolished in the nucleus and cytosol compartments, mitochondrial PARG activity show 331% higher in $PARG_{110}^{-/-}$ cells compared to the wild-type cells, suggesting an important function for PARG in cell life.

4. THE BIOLOGICAL FUNCTION OF PARG

4.1. Embryonic development and cell death

Complete deletion of the PARG protein causes embryonic lethality in the *Drosophila* and mice due to increased apoptosis (15, 16). It has been shown that the excess of pADPr by inhibition of PARG activity resulted in the release of the apoptosis inducing factor (AIF) from the mitochondria, which is a caspase-independent cell death signal (17). It is thus believed that PARG activity is essential for the embryonic development by preventing massive cell death. Moreover, hypomorphic mutation of PARG, by deleting the PARG 110-kD isoform, is compatible with the life of mice probably because the remaining 60-kD isoform can compensate for at least partially the PARG function (12). However, the mutant mice were hypersensitive to genotoxic stress. Given its 3-fold increased activity in $PARG_{110}$-deficient mitochondria (Table 1), it is speculated that the mitochondrial PARG activity is necessary to cope with the cell death signal triggered by excessive pADPr in mitochondria, and to recycle pADPr to compensate for the ATP and NAD^+ depletion in response to oxidative stress.

4.2. DNA repair

PARG is expected to be important in DNA repair given its role in degrading pADPr synthesized by PARPs. The catalytic domain of PARG interacts with the automodification domain of PARP-1 (18). PARG also interacts with XRCC1, a DNA repair scaffolding molecule that is recruited by DNA damage-activated PARP-1 (18). The production of ATP from pADPr is required to efficiently repair damaged DNA (19). The PARG activity participates in the ATP production from pADPr that is necessary for the DNA replication activity (20). PARG is also found to be a novel and critical component of single strand break repair in concert with PARP-1 (21). We found that mouse embryonic fibroblast (MEF) cells from $PARG_{110}^{-/-}$ mice were hypersensitive to various DNA damage treatments such as adriamycin or alkylating agent, which is likely due to impaired DNA repair efficiency in the absence of PARG (Min *et al.*, unpublished data). Moreover, following MNNG treatment, $PARG_{110}^{-/-}$ cells showed less XRCC1 foci, which delayed H2AX phosphorylation, decreased DNA break intermediates during repair, and increased cell death (10). This is consistent with the observation that PARP-1 deficient cells and mice are hypersensitive to DNA damage treatments due to repair defects (see review by (22-24). Deletion of the PARG 110-kD isoform indeed down-regulated PARP-1 automodification (12). These data support the notion that poly (ADP-ribosyl)ation is critical for efficient DNA repair.

4.3. Tissue damage and disease models

4.3.1. Pharmacological inhibition

The chemical inhibition of PARPs has been a major target in the development of pharmaceutical strategies to benefit patients with various pathological conditions (25). Similarly, there has been a great effort to test whether PARG inhibition using tannin-based synthetic compounds could be an alternative solution. However, unspecific inhibition of tannin-derived compounds is the hurdle for the therapeutic applications (26). Recently, potent specific PARG inhibitors, GPI 16552 and GPI 18214 based on the tilorone family, have been developed for studying various diseases including inflammation, stroke, ischemia-reperfusion and chemosensitive effects.

For example, PARG inhibition by GPI 16552 and GPI 18214 significantly reduced mortality and peritonitis development, as well as decreased peroxinitrite formation in mice (27). In addition, PARG inhibition in mouse models by these drugs prevented tissue injury and neutrophil infiltration judged by myeloperoxidase (MPO) evaluation in the lungs, small intestine and liver (27). In the gut ischemia and ischemia-reperfusion experimental rat and mouse models, PARG activity was responsible for the fall of the blood pressure, the development of splanchnic artery occlusion (SAO) shock, and tissue injury, which were most likely caused by massive neutrophil infiltration, and the up-regulation of P-selectin and intercellular adhesion molecule 1 (ICAM1) (28).

The pharmacological treatment with GPI 16552 and 18214 protected mice from dinitrobenzene sulfonic acid (DNBS)-induced colitis. The mucosa of colon tissues showed a reduction of MPO activity and attenuated staining for ICAM-1and vascular cell adhesion molecule 1 (VCAM1). Moreover, overproduction of proinflammatory factors (TNF-alpha and IL-1beta) and the activation of the cell death signaling pathway (the FAS ligand) were inhibited in these PARG inhibitor-treated mice (29). These results demonstrate that PARG modulates the inflammatory response and tissue injury events associated with colitis. PARG inhibition significantly ameliorated the degree of spinal cord inflammation, tissue injury and at the same time facilitated the recovery of limb function. The level of neutrophil infiltration, cytokine production (TNF-alpha and IL-1beta) and apoptosis was also significantly reduced (30).

Table 2. Effects of PARG Inhibitors for disease models with comparison of $PARG_{110}^{-/-}$ mice

PARG Inhibitors (IC_{50}[1])	Compounds	Effects and Disease Type	Comparison by studies of $PARG_{110}^{-/-}$ mice
GPI16552 (IC_{50}) GPI18214 (4.2μM)	Tilorone-base (N-bis- (3-phenyl-proryl)9-oxo-fluorene-2,7-diamide)	↓[2] Mortality, peritonitis development peroxinitrite production	Yes
		↓ Tissue injury, neutrophil infiltration in lung, small intestine and liver	Yes
		↓ Overproduction of pro-inflammatory responses (TNF-, IL-1, cell death)	Yes
		↓ Ischemia-reperfusion injuries in gut, spinal cord	Yes
		Protective against DNBS-induced colitis	Yes
		↑[3] Neuronal cell death (stroke)	Worsen the brain ischemia-reperfusion injury
		Together with temozolomide (TMZ), enhance the melanoma killing effect and reduce the lung metastasis	ND[4]
Gallotannin / Nobotanin B (1.0μM)	Tannin-base	↓ Oxidative-induced neuronal cell death in vivo	ND
		Protective effect (abolished AIF-induced cell death)	ND

[1] half maximal inhibitory concentration, [2] down-regulation, [3] up-regulation, [4] not done. The effect of PARG chemical inhibitors in various animal models is summarized. Some of the effects by chemical inhibitors are also confirmed using genetically inactivated PARG mouse models.

The chemical inhibition of PARG by GPI 16552 prior to middle cerebral artery occlusion (MCAO) significantly reduced the total infarct volume, suggesting that this PARG specific inhibitor can provide *in vivo* neuro-protection in the murine model of stroke (31). Moreover, the PARG inhibitors, gallotannin (GT) and nobotanin B, profoundly reduced oxidative-induced neuronal cell death *in vitro* (32). Intranasal delivery of GT after a focal brain ischemia markedly decreased the infarct formation and neurological deficits in rats. Molecularly, the GT treatment abolished the nuclear translocation of AIF in the ischemic brains, suggesting that the prevention of AIF translocation may contribute to the protective effects of GT (33). However, PARG activity showed a protective effect in stroke models using mice lacking the PARG 110-kD isoform (see below).

Inhibition of PARG has also been tested in killing tumor cells either alone, or in combination with other antitumor drugs. The PARG inhibitor GPI 16552 was administered in mice combination with temozolomide (TMZ), a specific drug for melanoma treatment; it was injected subcutaneously or intracranially with B16 melanoma cells. Combined treatment with GPI 16552 and TMZ significantly inhibited melanoma growth, and decreased the ability of melanoma cells to form lung metastases and to invade the extracellular matrix (34). Thus, PARG inhibition synergistically enhances TMZ efficacy against melanoma.

Taken together, pharmacological inhibition of the PARG activity seems to play an important role in inflammatory response, in tissue injury events associated with spinal cord trauma and in apoptosis. PARG may be considered as a novel target for pharmacological interventions for the pathogenesis of these diseases.

4.3.2. Genetic disruption of PARG in mouse models

PARG as a therapeutic target that has been studied in rat and mouse models using a number of PARG chemical inhibitors, is further explored by studying genetically PARG deleted mouse models ($PARG_{110}$-deleted mice; (12), see Table 2). Because null mutation of PARG is embryonic lethal, these hypomorphic mutant mice are thus useful to verify and confirm the effect of PARG activity in various disease models. For example, $PARG_{110}$-deleted mice showed hyper-sensitivity to endotoxic shock induced by lipopolysaccharide (LPS) and the serum levels of TNF-alpha in LPS-treated animals were also higher in $PARG_{110}$-deficient mice compared to their wild-type counterparts, indicating that PARG plays a protective role against LPS-induced septic shock (12).

However, the inhibition of the PARG activity seems to be beneficial against various pathological treatments. Genetic disruption of the PARG gene in mice significantly improved the histological status of the reperfused intestine associated with reduced expression of P-selectin and ICAM-1, the production of TNF- alpha, and neutrophil infiltration (28). In the renal injury model, renal dysfunction caused by ischemia reperfusion was significantly reduced in $PARG_{110}$-deficient mice when compared with their wild-type littermates. Thus, it is proposed that endogenous $PARG_{110}$ plays a pivotal role in the pathophysiology of ischemia reperfusion injury of the kidney (35).

$PARG_{110}$-deleted mice were also protected from experimental spinal cord trauma (30). In wild-type mice, spinal cord injuries induced by the application of vascular clips, resulted in severe traumas characterized by edema, neutrophil infiltration, and the overproduction of cytokines (TNF-alpha and IL-1beta) followed by the recruitment of other inflammatory cells, the production of a range of inflammation mediators, apoptosis, and tissue damage. Depletion of the PARG 110-kD isoform in mice significantly improved these symptoms (30). Moreover, mice lacking the functional 110-kD isoform of PARG were resistant to colon injury induced by DNBS and showed reduction of MPO activity and attenuated staining for ICAM 1 and VCAM 1. Overproduction of TNF-alpha and IL-1beta, and activation of cell death signaling pathway, i.e., the FAS ligand, were also inhibited in these mutant mice (29).

In contrast to chemical inhibitors, PARG activity seems to play a protective role in the ischemic brain injury model using $PARG_{110}$-deleted mice. Distal middle cerebral artery occlusion caused higher levels of pADPr and larger infarct volumes in $PARG_{110}^{-/-}$ brain than in wild-type

counterparts, suggesting that the pADPr accumulation worsens ischemic brain injury (36). This is also in contrast to other studies mentioned above that showed protective effects of PARG inhibition. The explanation of the opposite results may be several-folds. First, pADPr accumulation and degradation is more critical in the brain. Second, the $PARG_{60}$ isoform in $PARG_{110}$-deleted mice may express and function differently in various tissues. Finally, compared to other tissues, neurons may use different cell death pathways that are modulated by pADPr accumulation. Interestingly, the level of pADPr is higher in the brain tissue of $PARG_{110}^{-/-}$ mice than wild-type mice, both at basal level and after PARP over-activation conditions (36). These apparent discrepancies in the potency of PARG inhibitors remain unclear and require further studies. Nevertheless, the effect of PARG inhibition by chemical inhibitors or PARG genetic inactivation demonstrates that PARG is a potential therapeutic target in the treatment of inflammation response in several pathological conditions.

5. PERSPECTIVES

Homeostasis of poly (ADP-ribosyl)ation has been used to develop pharmaceutical therapies for various pathologies including cancer. While PARP-1, the major pADPr synthesizing enzyme, has been the target of pharmacological interventions for the last 10 years, the research on PARG as a novel target starts to generate interesting data at the cellular and animal levels. In this review, we have summarized the recent development of the research on the poly (ADP-ribose) metabolism modulated by PARG. Because poly (ADP-ribosyl)ation has multiple functions depending on the tissues and cell types, it is challenging to develop drugs targeting PARPs or PARG that are not only cell-type specific, but also posses a physiological potency to modulate pADPr. We still do not know the impact of the pADPr formation in terms of size, turnover and branching properties *in vivo*. Similarly important we need to understand mechanistically the nature and function of various PARG isoforms and how they carry out pADPr degrading activities, which are concerted with PARPs to regulate the pADPr dynamics in cellular and physiological processes.

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Abbreviations: PARG: poly (ADP-ribose) glycohydrolase, PARP: poly (ADP-ribose) polymerase, pADPr: poly (ADP-ribose), ARH3: ADP-ribose hydrolase-like protein 3, AIF: apoptosis inducing factor, MPO: myeloperoxidase, DNBS: dinitrobenzene sulfonic acid, ICAM1: intercellular adhesion molecule 1, VCAM1: vascular cell adhesion molecule 1, MCAO: middle cerebral artery occlusion, SAO: splanchnic artery occlusion, GT: gallotannin, TMZ: temozolomide, LPS: lipopolysaccharide

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Send correspondence to: Zhao-Qi Wang, Leibniz Institute of Age Research - Fritz Lipmann Institute (FLI), Beutenbergstr 11, 07745 Jena, Germany, Tel: 0049 3641 656415, Fax: 0049 3641 656413, E-mail: zqwang@fli-leibniz.de

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