Review

In Vivo Level of Poly(ADP-ribose)

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Abstract: PolyADP-ribosylation is a post-translational modification that plays key roles in cellular physiological functions and DNA damage responses. PolyADP-ribosylation is finely and dynamically regulated by various enzymes and factors involved in the synthesis and degradation of poly(ADP-ribose) (PAR). To better understand the function of polyADP-ribosylation, it is necessary to quantify and monitor the change of the in vivo level of PAR, the product of polyADP-ribosylation, which is rapidly turning over and kept in quite low level in cells or in organs. Recent developments of potent inhibitors of polyADP-ribosylation is expected to kill BRCA1/2-mutated breast cancer cells and ovarian cancer cells (synthetic lethality). To know the efficacy of these inhibitors in vivo, it is necessary to develop highly sensitive and reproducible methods to know PAR levels within cells or organs. However there have been several difficulties in measuring the physiologically low level of PAR without artefacts. Our experiments recently clarified that the method of sample preparation is very important in addition to the sensitivity and specificity. From reviewing the literature, including ours, we would like to emphasize the importance of the procedures of sample preparation for the assay, in addition to the sensitivity by comparing the reported PAR levels in vivo.

Keywords: polyADP-ribosylation; poly(ADP-ribose); PARP; PARG; PARP inhibitor; radioimmunoassay; ELISA; LC-MS/MS

1. Introduction

1.1. What Is PolyADP-Ribosylation?

PolyADP-ribosylation is a post-translational modification that adds a long polymer of poly(ADP-ribose) (PAR) chain to the acceptor proteins [1,2]. The enzymes, PAR polymerases (PARPs, also named ADP-ribosyltransferases-diphtheria toxin-like (ARTDs) [3]) and ADP-ribosyl transferase 2 (ART2), are responsible for synthesis of PAR attached to acceptor proteins using βNAD+ as the substrate [4–7]. On the other hand, PAR glycohydrolase (PARG) and ADP-ribosyl hydrolase 3 (ARH3) can degrade the PAR polymer from acceptor proteins [8–10]. Finally, ADP-ribosyl protein lyase, macrodomain-containing proteins, terminal ADP-ribose protein glycohydrolase 1 (TARG 1) and ARH3 can remove the proximal ADP-ribose residue from the acceptor protein [11–14] (Figure 1). There are monoADP-ribosylation reaction and polyADP-ribosylation reaction, and they are collectively termed...
“ADP-ribosylation reaction”, catalyzed by enzymes widely found in animals, plants and bacteria using NAD⁺ [15]. However, this article is intended to emphasize the importance of measuring the actual amount of PAR found in vivo and to discuss the methods and the resulting PAR levels in vivo.

1.2. Proposed Functions of PolyADP-Ribosylation

There have been many excellent reviews, including historical ones, on polyADP-ribosylation [1,2,16–22]. On the biological functions of polyADP-ribosylation, various works in vitro and in vivo have been reported using inhibitors of polyADP-ribosylation, gene-knockout animals of responsible enzymes or in vitro experiments with purified PARPs or PARG [23–27]. They include base-excision DNA repair [28], DNA replication [29,30], transcription [20,31,32], chromatin remodeling [33,34], sister chromatid exchanges [35,36], centrosome number regulation [37], telomere regulation [38], cell signaling [39–42], cell proliferation [23], carcinogenesis [43], neurodegeneration [12,26], immunological defense [23,44], hypoxia and reperfusion damage [45], and cell death [44,46].

There are two types of functions played by polyADP-ribosylation. One is that since polyADP-ribosylation is a modification of the acceptor proteins, the function of the acceptor proteins could be changed significantly in their enzyme activities and the interaction with other proteins. The acceptor proteins in vitro reaction include PARP1, histone H1, histone H2b, RNase and TP53 [40,47–51] and the number of acceptor molecules is still increasing.

The other is that the attached PAR polymer has a big size with large negative charges. Chemical analysis showed that PAR has branched structure [52]. Consistent with this finding, electron microscopy showed that the synthesized PAR had a branched shape [53,54]. The diameter of some of the PAR reached 200–300 nm in length. Althaus and his colleagues noticed and proposed that a free PAR chain is bound non-covalently by specified proteins including histones, and involved in the regulation of biological functions [55,56]. In fact, following modules or motifs that recognize specific
structures of PAR have been found including PAR binding motif (PBM), macrodomain motif, PAR binding zinc finger (PBZ), WWE domain and OB fold domain [21,57–62]. It is likely that PAR functions as a scaffold for macromolecules to support various biological activities.

1.3. Importance to Understand the In Vivo Level of PAR that is Rapidly Turning-Over under Various Biological Conditions

As shown in Figure 1, polyADP-ribosylation is regulated by enzymes involved in the synthesis and degradation of PAR. In addition, as shown by many investigations, the activities of PARP1 or PARP2 is greatly enhanced by the presence of DNA strand breaks [19]. The interaction of PARP1 or PARP2 with other proteins could also regulate the activities of the proteins. For example, the phosphorylated ERK1/2 protein, one of the important cellular signal transduction factors, could stimulate PARP1 enzyme activity without any involvement of DNA [39]. It is also noticed that PAR is rapidly degraded by PARG. Thus, measuring the amount of the specified enzymes or their activities are not enough to understand the polyADP-ribosylation status, and the measurement of the level of PAR, rapidly turning over in physiological conditions, should be measured as a more accurate marker. As are the characteristics of most of the post translational modifications, the speed of changes of the PAR level is quite rapid, being less than 1 min (unpublished). There are about 10^6 molecules of PARP1, the most abundant and well-studied member of PARP family present in a cell [32]. However, the level of PAR, the reaction product of PARP1, is considerably low, suggesting the necessity of tight and efficient method of regulation of PAR in vivo. For determining the PAR levels in vivo, under unstressed conditions (i.e., without exogenous DNA damaging agents), western blot analysis is not sensitive enough. Many researchers have frequently experienced the situation that the physiological level of PAR is difficult to show by western blot. Artificial synthesis of PAR could occur presumably due to uncontrollable DNA damages occurring during the procedures of cell lysis. Therefore, in addition to developing highly sensitive and specific assay methods, it is necessary to control the lysis conditions keeping the level of DNA damages that might occur during cell lysis and fixation processes before extraction of PAR minimal.

We will review efforts by various researchers including us to quantitate physiological levels of PAR without apparent exogenous DNA damaging agents to better understand the nature of polyADP-ribosylation and for clarification of its possible biological functions thus far unknown.

1.4. Measurement of the Amount of PAR In Vivo (i.e., in Cells or Tissues)

Although PAR is clearly observable as a smear by western blot analysis after treatment of exogenous DNA damaging agents, the level of PAR in unstressed conditions is quite low and difficult to show by western blot as stated in the previous section. Considering that PARP1 could be activated in the absence of DNA [39], it seems quite interesting to know other physiological functions of polyADP-ribosylation that are unrelated to the exogenous DNA damage and repair. There have been many works suggesting the physiological functions of polyADP-ribosylation using various kinds of PARP inhibitors. However, we do not know exactly how efficient these inhibitors worked in the cell. If the inhibition was not complete, it will cause confusions on the function of PARP1.

One of the difficulties of measuring the physiologically low level of PAR is due to the fact that the substrate of PARPs, NAD^+, is impermeable through cell membranes and radioactive NAD^+ could not be used for efficient and sensitive labeling of the intracellular PAR. Therefor the precursors of NAD^+, e.g., phosphate or adenine, have been used to prove the natural occurrence of PAR. Doly et al. injected [32^P]H_3PO_4 into chicken and isolated liver nuclei. Then, according to the procedure of purifying PAR, already known in vitro, they isolated and purified the PAR fraction and hydrolyzed it with snake venom phosphodiesterase (pyrophosphatase) and found Ado(P)-Rib-P, which is the specific hydrolysis product of PAR (Figure 2). This is the first indication that PAR is synthesized in vivo [63]. The other researchers used radiolabeled adenine to show the natural occurrence of PAR [64]. Subsequently, there have been several reports on the natural occurrence of PAR. Interestingly, naturally occurring antibody
Challenges 2018, 9, 23

against PAR was found in the sera of patients with systemic lupus erythematosus, an autoimmune disease [65]. However, accurate quantitation of PAR has been facing various problems.

Figure 2. Specific degradation products of polyADP-ribosylation.

As shown in Table 1, Smith and Stocken reported that histone F1 purified from rat liver contained adenine, ribose, phosphate, and serine as 0.89, 1.73, 1.83 and 0.31 nmol per mg protein, respectively. They suggested that ADP-ribose was bound to histone F1 via serine or serine phosphate [66].

Then Stone et al. initially used orthodox isotope dilution methods to quantitate endogenous PAR as 5.59 and 6.32 nmol/mg DNA, for adult and neonatal rat liver, respectively [67] (Table 1). However, the amount of endogenous PAR seems significantly higher than that was reported in the later publication by the same research group [68,69] (Table 1). This might be partly due to the procedure of calculating the specific activity of standard [3H]PAR. [3H]PAR was synthesized from [3H]NAD+ using the sonicated whole cell lysate of Ehrlich ascites carcinoma cells as the enzyme source that might have had already contained significant amount of unlabeled endogenous NAD+. These endogenous NAD+ in the cell lysate might dilute the specific activity of the originally used [3H]NAD+, making the specific activity of the standard [3H]PAR significantly lower than that of the originally used [3H]NAD+. This should cause overestimation of endogenous PAR level.

Kidwell and Mage made rabbit polyclonal antibody against PAR with a mean chain length of 24 after the method by Kanai et al. [70] and developed a radioimmunoassay system to determine PAR levels in HeLa cells [71] (Table 1). They lysed cultured HeLa cells in 0.1 N NaOH for 2 h at 37 °C, stored overnight at 4 °C, neutralized, treated with DNase I and micrococcal nuclease, digested with Pronase and extracted PAR with phenol to be used for the assay. To confirm the specificity of their radioimmunoassay, they showed that the binding of [14C]PAR to the antibody was inhibited with equal amount of unlabeled PAR by 50%, but 25,000-fold excess (w/w) amount of poly(A), native DNA or denatured DNA did not show any appreciable inhibition. Neither NAD+, NADP+, NADH nor NADPH appreciably inhibited the binding when present in the binding assay with a 100,000-fold excess amount of radiolabeled PAR. With ADP-ribose present at a 10,000-fold excess, the binding of labeled PAR was inhibited by 30% and, at a 100,000-fold excess, the inhibition was nearly total. They also labeled cells with [14C] ribose and the materials which bound to the antibody was digested with snake venom phosphodiesterase and separated by Dowex-formate column chromatography. Then they isolated the radioactive compound, which was identified as Ado(P)-Rib-P by Dowex-formate column chromatography as well as PEI-cellulose thin layer chromatography. Using the above specificity of the antibody, they reported the endogenous level of PAR as 3.7 pmol/10⁶ HeLa cells (200 ng/10⁶ cells) (Table 1). Interestingly, there was a small peak of PAR at
S phase and a large peak at G2 phase. However, the amount of endogenous PAR was about 100 times higher as compared to the report on HeLa cells by Martello et al. [72] or Ida et al. [73]. The reason for this discrepancy is not clear but might partly be due to the experimental condition to prepare the samples before the assay (discussed later).

Sakura et al. measured PAR with radioimmunoassay using calf thymus nuclei [74] (Table 1). They made rabbit polyclonal antibody against PAR with a mean chain length of 28 (it was later found that this PAR contained much higher molecular weight PAR with branches). In addition, using this polyclonal antibody they showed that there was an immunoreactive principle in the extract of calf thymus nuclei and its reactivity was lost by prior incubation with PARG or snake venom phosphodiesterase (pyrophosphatase), but not with DNase I, pancreatic RNase, nuclease P1, pronase E or 0.5 N NaOH treatment for 18 h at 37 °C. This was consistent with the character of in vitro synthesized PAR, indicating that there is PAR in calf thymus. They also showed that calf thymus contained PAR with various chain length (molecular size) and that PAR with shorter chain length reacted less efficiently to the antibody, as was expected with authentic PAR with shorter chain length. However, by normalizing the immunoreactivity of PAR with shorter chain length, the total amount of PAR in calf thymus was estimated as 740 pmol (0.4 µg)/g tissue or 37 pmol (0.02 µg)/mg DNA. Similar recovery of immunoreactivity was obtained when frozen calf thymus was homogenized directly in 5% trichloroacetic acid (TCA), without isolating nuclei, and processed similarly. This result suggested that there was no significant degradation or synthesis of PAR in vitro during the step of isolation of nuclei.

In 1979, Juarez-Salinas et al. treated cultured cells with 20% TCA, extracted PAR fraction and hydrolyzed PAR fraction with snake venom phosphodiesterase and alkaline phosphatase to isolate Ado-Rib (ribosyl adenosine), which is the dephosphorylated monomer form of PAR (Figure 2). Then they reacted this fraction with chloroacetaldehyde to make adenine residue to etheno adenine residue that is highly fluorescent. They separated these products with high performance liquid chromatography (HPLC) to show the presence of fluorescence-labeled etheno Ado-Rib, confirming the presence of PAR in vivo. Although the chain length distribution pattern of PAR could not be evaluated, this method could measure total amount of ADP-ribose residues constituting PAR quantitatively. In addition, they also showed that by treating the cultured cells with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), a DNA damaging agent, the PAR level increased dramatically but rapidly decreased [75] (Table 1).

Wielkens et al. prepared rabbit polyclonal antibody against Ado(P)-Rib-P (phosphoribosyl AMP, or pseudoADP-ribose), the specific component of PAR (Figure 2). In addition, they made [3H]PAR of high specific activity from [3H]NAD + synthesized from [3H]ATP and nicotinamide mononucleotide using Ehrlich ascites tumor cell nuclei, which did not seem to contain significant amount of ATP. By combining radioimmunoassay and tracer [3H]PAR, they extracted PAR by first freeze-clamped normal rat liver. They reported the PAR level as 72.6–128.1 pmol/g rat liver tissue [69] (Table 1). Brederhorst et al. in the same research group reported the PAR level as 32 ± 9, 10 ± 2, 39 ± 5 pmol/mg DNA of neonatal (1 day), neonatal (17 days) and adult (>150 days) rat liver, respectively. They also reported the PAR level in hepatoma cells as 61 ± 11, 25 ± 3 and 60 ± 17 pmol/mg DNA for AH130, AH 794 and Reuber H35 hepatomas, respectively [68] (Table 1).

In 1982, Juarez-Salinas et al. using their fluorescence labeling technique [75] reported that 14, 18, and 8.0 pmol of Ado-Rib/mg DNA from the liver, the kidney and the spleen of rats, respectively (Table 1). They also reported the content of Ado-Rib-Rib (derived from branch point, Figure 2) as 0.8–1.6% of total PAR [76]. Jacobson et al. reported PAR level as 12 pmol Ado-Rib/10^6 normal human diploid fibroblasts (CF-3) and it increased to 200 pmol/10^6 cells after UV irradiation [77]. They also measured PAR level in SV40-transformed Balb/3T3 fibroblasts and reported as 0.09 or 0.14 pmol Ado-Rib/10^6 cells and it increased by hyperthermia at 43 °C up to 5.25 pmol/10^6 cells after 8 h [78] (Table 1). A review on various methods for biochemical study of PAR metabolism in vitro and in vivo was published in 1995 [79]. Malanga et al. using polyacrylamide gel electrophoresis (PAGE), silver staining and computer-aided scanning densitometry reported PAR level in human keratinocyte cell line (HaKaT) as 0.554 pmol/10^6 cells (6.65 pmol/1.2 × 10^7 cells) and it increased to 11.1 pmol/10^6 cells (133 pmol/1.2 × 10^7 cells) after MNNG treatment [80]
The interesting point is that although the total amount of PAR (the sum of ADP-ribose residues) is 20 times larger in the MNNG-treated cells, the chain length distribution pattern of PAR in the control (intact) cells seems not so different from that of MNNG-treated cells.

In 2008, Kinders et al. reported a sandwich ELISA of protein-bound PAR in tumor biopsies of human melanoma xenografts in mice after in vivo administration of PARP inhibitors (Table 1). This was a Phase 0 clinical trial of pharmacodynamic assay of PAR, leading to the current human clinical Phase 1 and 2 studies of PARP inhibitor in combination with anti-cancer drugs [81]. To invent a non-invasive procedure to monitor the effects of PARP inhibitor as a surrogate marker of anti-cancer drugs, they used the above method of Kinders et al., reported PAR level in human peripheral blood mononuclear cells (PBMC) as 0.024 pmol/10^6 PBMC (13.17 ag/cell) from healthy volunteer and 0.028 pmol/10^6 PBMC (14.92 ag/cell) from patients with cancer. The intra- and inter-individual variation over 3 weeks in healthy volunteers was 0.008–0.198 pmol/10^6 PBMC (4.4–107.3 ag/cell) [46] (Table 1). The reason there was relatively big variation of intra- and inter-individual variation of PAR levels in PBMC, as compared to the later report by another researcher group [72] (Table 1), is not known at present. However, it might be necessary to compare precisely the conditions of preparation of PBMC samples before analyzing the actual PAR levels.

In 2013, Martello et al. reported PAR levels using a modern technique of stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Table 1). They synthesized ^13^C,^15^N-labeled PAR from ^13^C,^15^N-labeled NAD^+ and added to the sample as tracer for estimating the recovery of PAR during purification. They reported PAR levels as 0.03 pmol/10^6 PBMC and 0.05–0.1 pmol/10^6 HeLa cells. It is interesting that although there was little variation of basal PAR levels in PBMC from different donors, there was considerable interdonor variation of increased PAR levels after H_2O_2 stress. They also reported the PAR levels of mouse tissues as 21.5 ± 11.4, 10.3 ± 3.4, 7.5 ± 1.7, 6.2 ± 2.3, 5.9 ± 1.5, 4.6 ± 1.6 and 2.7 ± 1.0 pmol/mg DNA for the heart, the kidney, the liver, the thymus, the testis, the spleen, and the lung, respectively [72].

Up until now, for measurement of physiological low level of PAR, the importance of the sensitivity and the specificity have been greatly emphasized. However there have been less attention on the artificial synthesis or degradation of PAR during the extraction procedures. We recently reported a sandwich ELISA system to detect protein free PAR level with special attention to use TCA and not ordinary cell lysis buffer to extract PAR to avoid any interference of the assay. In addition, to ensure the recovery of PAR, we did not use any columns such as DHB-Sepharose. With this method it became possible to measure the basal PAR level using 10^6 cells. The PAR levels were 0.039 pmol/10^6 HeLa cells (20.8 ag/cell), 0.038 pmol/10^6 HEK293T cells (20.5 ag/cell), and 0.002 pmol/10^6 HepG2 cells (1.0 ag/cell). The PAR level of HeLa cells increased 160 times to 6.39 pmol/10^6 HeLa cells (3460 ag/cell) after MNNG treatment [73]. The amount of PAR in HeLa cells is consistent with the value reported by Martello et al. [72] (Table 1). If the chain length distribution pattern of PAR did not change significantly in physiological condition, it is understandable that both ELISA system and LC-MS/MS system give similar results on the total amount of PAR. Yamashita et al. in the same research group reported PAR level in HeLa cells as 0.025 pmol/10^6 cells (13.3 ag/cell) and 0.074 pmol/10^6 cells (39.3 ag/cell) at 37 °C; 0.023 pmol/10^6 cells (12.5 ag/cell) at 33.5 °C; 0.085 pmol/10^6 cells (45.4 ag/cell) and 0.155 pmol/10^6 cells (82.9 ag/cell) at 40.5 °C. The PAR level in CHO-K1 cells was 0.063 pmol/10^6 cells (33.7 ag/cell) at 37 °C and 0.128 pmol/10^6 cells (68.2 ag/cell) at 40.5 °C (Table 1). With western blot analysis, γH2AX protein band was detected when HeLa cells or CHO-K1 cells were cultured at 40.5 °C and it further increased in the presence of 7 mM 3AB, a PARP inhibitor. Immunocytochemistry also showed similar results [83]. These results suggested the occurrence of double strand DNA breaks occurring at relatively mild shifted-up temperatures such as 40.5 °C, during which we might sometimes experience a microbial infection in daily life.
<table>
<thead>
<tr>
<th>Name of Methods</th>
<th>Target</th>
<th>Reagents</th>
<th>Tissues or Cells</th>
<th>Fixation</th>
<th>Extraction, Purification</th>
<th>Sensitivity *</th>
<th>Level of PAR *</th>
<th>Comments by Authors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical analysis</td>
<td>Histone F1</td>
<td>Rat liver nuclei</td>
<td>Histone F1 extracted with 5% HClO₂ and precipitated with 20% TCA</td>
<td>DEAE-cellulose column chromatography to separate from non-histone proteins</td>
<td>0.89–1.73 nmol/mg F1 histone protein</td>
<td>Smith and Stocken (1973) [66]</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Isotope dilution</td>
<td>Ado(P)-Rib-P</td>
<td>[³H]PAR as indicator, prepared using sonicated Ehrlich carcinoma cell lysate as enzyme source</td>
<td>Rat liver (adult and neonatal), Zajdela hepatoma</td>
<td>Freeze-clamped (rat liver)</td>
<td>Homogenized in 0.25 N KOH, sonicated, 1 M NH₄OH; venom PDE; anion exchange column chromatography; paper chromatography; phosphatase; paper chromatography</td>
<td>5.59 (adult), 6.32 (neonatal), 1.24 (hepatoma) nmol/mg DNA</td>
<td>Stone et al. (1976) [67]</td>
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<td>RIA</td>
<td>PAR</td>
<td>[¹⁴C]PAR, polyclonal antibody against PAR</td>
<td>HeLa cells</td>
<td>0.1 N NaOH, 2 h, 37 °C; 4 °C overnight</td>
<td>DNase I, micrococcal nuclease; Pronase; phenol extraction</td>
<td>3.7 pmol/10⁶ HeLa cells; small peak of PAR at S phase and large peak at G2 phase</td>
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<td>RIA</td>
<td>PAR</td>
<td>[¹⁴C]PAR, polyclonal antibody against PAR</td>
<td>Calf tissues</td>
<td>Frozen after slaughterhouse</td>
<td>Homogenized to isolate nuclei and ethanol precipitation; or homogenized in 5% TCA without isolating nuclei; Pronase; phenol extraction; DNase I, RNase, nuclease P1; Pronase; phenol extraction; hydroxyapatite column chromatography</td>
<td>37 pmol/mg DNA or 740 pmol/g calf thymus</td>
<td>Chain length distribution shown with hydroxyapatite column chromatography</td>
<td>Sakurai et al. (1977) [74]</td>
<td></td>
</tr>
<tr>
<td>Fluorescence method</td>
<td>Etheno Ado-Rib</td>
<td>Fluorescent labeling of Ado-Rib</td>
<td>SV40-transformed 3T3 cells</td>
<td>20% TCA</td>
<td>0.1 M potassium phosphate (pH 5) in 8 M guanidine hydrochloride, sonication; dihydroxyboryl-Sepharose column chromatography; alkaline phosphatase and venom PDE; chloroacetaldehyde; HPLC</td>
<td>5 pmol of Ado-Rib</td>
<td>0.05 pmol/10⁶ SV40 virus–transformed 3T3 cells, increased to 7.50 pmol/10⁶ cells after 20 min of MNNG</td>
<td>Juanes-Salinas et al. (1979) [75]</td>
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<td>RIA: Method A</td>
<td>Ado(P)-Rib-P</td>
<td>[³H]PAR as tracer prepared using Ehrlich carcinoma cell nuclei, [¹⁴H]Ado(P)-Rib-P, antibody against Ado(P)-Rib-P</td>
<td>Rat liver (adult)</td>
<td>Freeze-clamped and frozen</td>
<td>Homogenized in 20% TCA; dissolved in 6 M guanidine hydrochloride and morpholine buffer; 0.3 M NaOH, 1 h, 56 °C; boronate column chromatography; venom PDE; HPLC</td>
<td>1 pmol Ado(P)-Rib-P</td>
<td>88.6 ± 20.7 and 72.6 pmol/g tissue</td>
<td>Weilkers et al. (1981) [69]</td>
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<td>RIA: Method B</td>
<td>Ado(P)-Rib-P</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Homogenized in 20% TCA; dissolved in cold water; 0.33 M NaOH, 3 h, 56 °C; alkaline phosphatase; proteinase K; heat inactivation; venom PDE; 5% TCA; anion exchange column chromatography, HPLC</td>
<td>1 pmol Ado(P)-Rib-P</td>
<td>89.0 ± 10.7 and 128.1 ± 7.3 pmol/g tissue</td>
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<td>Freeze-clamped and frozen (rat liver) or washed and frozen in liquid nitrogen (hepatoma cells)</td>
<td>RIA Method A (Wielkens et al., 1981) [69]</td>
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<td>14, 18, and 8.0 pmol of Ado-Rib/mg DNA of liver, kidney and spleen of rat, respectively; Ado-Rib-Rib constituted 0.8-1.8% of total PAR</td>
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<td>No significant DNA strand breaks upon alkaline sucrose gradient centrifugation</td>
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<td></td>
</tr>
<tr>
<td>PAGE, silver staining and computer-aided scanning, densitometry</td>
<td>PAR</td>
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<td>Jacobson et al. (1983) [77]</td>
<td>0.09 or 0.14 pmol Ado-Rib/10⁶ cells; increased by hyperthermia at 43 °C up to 2.0 pmol/10⁶ cells after 8 h</td>
<td>Chain length distribution shown with PAGE</td>
<td>Malanga et al. (1995) [80]</td>
<td></td>
</tr>
<tr>
<td>Sandwich ELISA</td>
<td>Protein-bound PAR</td>
<td>PAR standard; mouse monoclonal (10H) and rabbit polyclonal antibody against PAR</td>
<td>Xenograft tumors of human melanoma cell lines (A375 and Colo829)</td>
<td>Fresh frozen</td>
<td>Sanderson et al. (2008)</td>
<td>5044 units PAR (large tumors); 4146 units PAR (small tumors) [All units are pg PAR/mL per 100 mg protein]; Higher amount of protein and DNA caused interference</td>
<td>Protein and DNA were not digested. Protein concentration should be diluted to 0.1-1 mg/mL before assay. Effect of dose of PARP inhibitor and time course of PAR levels in xenograft were presented</td>
<td>Sanderson et al. (2008) [81]</td>
<td></td>
</tr>
</tbody>
</table>
Table 1. Cont.

<table>
<thead>
<tr>
<th>Name of Methods</th>
<th>Target</th>
<th>Reagents</th>
<th>Tissues or Cells</th>
<th>Fixation</th>
<th>Extraction, Purification</th>
<th>Sensitivity *</th>
<th>Level of PAR *</th>
<th>Comments by Authors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandwich ELISA</td>
<td>Protein-bound PAR</td>
<td>PAR standard, mouse monoclonal (10H) and rabbit polyclonal antibody against PAR</td>
<td>Human PBMC</td>
<td>Fresh frozen</td>
<td>Suspended in cell extraction buffer (Invitrogen) with protease inhibitors, 30 min on ice; 1% SDS; boiled 5 min; supernatant</td>
<td>0.024 pmol/10⁶ PBMC (healthy volunteers); 0.028 pmol/10⁶ PBMC (patients with cancer); 0.008-0.198 pmol/10⁶ PBMC (intra- and inter-individual variation over 3 weeks in healthy volunteers)</td>
<td>Protein and DNA were not digested</td>
<td>Ji et al. (2011) [46]</td>
<td></td>
</tr>
<tr>
<td>Stable isotope dilution LC-MS/MS</td>
<td>PAR</td>
<td>¹³C,¹⁵N-labeled PAR</td>
<td>PBMC, COPFS cells, HeLa cells, mouse tissues</td>
<td>20% TCA</td>
<td>0.5 M KOH, 37 °C, 45 min; ¹³C, ¹⁵N labelled PAR added; DNase I, RNase A, proteinase K; solid phase extraction of PAR; venom PDE, alkaline phosphatase; HPLC; mass spectrometry</td>
<td>50 fmol of Ado-Rib</td>
<td>0.03 pmol/10⁶ PBMC; 0.05-0.1 pmol/10⁶ HeLa cells</td>
<td>Considerable heterogeneity in the stress-induced PARylation response in human population</td>
<td>Martello et al. (2013) [72]</td>
</tr>
<tr>
<td>Sandwich ELISA</td>
<td>PAR</td>
<td>PAR standard, mouse monoclonal (10H) and rabbit polyclonal antibody against PAR</td>
<td>HeLa cells, HEK293T cells, HepG2 cells</td>
<td>20% TCA</td>
<td>Sonication; 0.1 N NaOH, 37 °C 1 h; DNase I, RNase A, Nuclease P1, Proteinase K;</td>
<td>15 fmol of PAR</td>
<td>0.039 pmol/10⁶ HeLa cells; 0.038 pmol/10⁶ HEK293T cells; 0.002 pmol/10⁶ HepG2 cells; increased to 6.49 pmol/10⁶ HeLa cells after MNNG.</td>
<td>Big difference of PAR level depending the isolation procedures and cell types</td>
<td>Ida et al. (2016) [73]</td>
</tr>
<tr>
<td>Sandwich ELISA</td>
<td>PAR</td>
<td>PAR standard, mouse monoclonal (10H) and rabbit polyclonal antibody against PAR</td>
<td>HeLa cells, CHO-K1 cells</td>
<td>20% TCA</td>
<td>Ida et al. (2016) [73]</td>
<td>15 fmol of PAR</td>
<td>0.025 and 0.074 pmol/10⁶ HeLa cells at 37 °C; 0.023 pmol/10⁶ cells at 35.5 °C; 0.085 and 0.155 pmol/10⁶ cells at 40.5 °C; 0.063 pmol/10⁶ CHO-K1 cells at 37 °C; 0.128 pmol/10⁶ cells at 40.5 °C</td>
<td>Phosphorylation of histone H2AX was found at 40.5 °C and further increased by PARP inhibitor.</td>
<td>Yamashita et al. (2016) [83]</td>
</tr>
</tbody>
</table>

Ado(P)-Rib-P: phosphoribosyl AMP; Ado-Rib: ribosyl adenosine; Ado-Rib-Rib: ribosyl-ribosyl adenosine; ag: attogram; amol: attomol; DEAE: diethylaminoethyl; ELISA: enzyme-linked immunosorbent assay; HPLC: high performance liquid chromatography; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MNNG: N-methyl-N′-nitro-N-nitrosoguanidine; MOPS: 3-(N-morpholino)propanesulfonic acid; PAGE: polyacrylamide gel electrophoresis; PAR: poly(ADP-ribose); PARylation: polyADP-riboseylation; PBMC: peripheral blood mononuclear cells; PDE: phosphodiesterase; RIA: radioimmunoassay; SDS: sodium dodecyl sulfate; TCA: trichloroacetic acid; UV: ultraviolet. * The amount of PAR was expressed as ADP-ribose residues and calculated to moles (mol)/mg DNA or 10⁶ cells, taking into consideration of the molecular weight of internal ADP-ribose residue as 541.
As described in the “Proposed functions of polyADP-ribosylation” section of this review, analysis of the amino acid modification sites of the acceptor proteins by polyADP-ribosylation are important, since they might directly modify the function of the acceptor proteins and also change the protein-protein interaction. For this purpose Chapman et al. hydrolyzed polyADP-ribosylated PARP1 with snake venom phosphodiesterase, and determined phospho-ribosylated amino acid residues using liquid chromatography-tandem mass spectrometry [84]. The quantification of the PAR attached to each acceptor amino acids will be expected direction in the future research. Since the structure of PAR with long chain length with many branches [52,53] is not studied well, it will be necessary not only to measure the amount of PAR, but also to analyze the role of the big PAR as a scaffold to support essential cellular functions. In this context, analysis of the proteomic approach to search protein motifs, which bind non-covalently to PAR, will give valuable information [56,85,86]. The information is important for analyzing the function of PAR in molecular levels. At the present time, we think our sandwich ELISA system will be useful in every research laboratory without expensive instrumentation, and is cost-effective. The catching monoclonal antibody (10H; anti poly(ADP-ribose) IgG3 kappa producing), which is necessary to coat the wells could be easily prepared by culturing 10H hybridoma cells [82], which is available from The RIKEN BioResource Research Center (BRC), a non-profit institution funded by the Japanese Government, under the code of (Cell No. RCB0705) (http://cell.brc.riken.jp/en/creditpay).

In addition it might be mentioned here that monoADP-ribosylation reaction is as important as polyADP-ribosylation in biological reaction. However, there is no suitable method to quantify the number of ADP-ribose molecules attached to each amino acids of the acceptor proteins. It is expected to develop such methods by applying recent techniques including mass spectrometry.

1.5. Prospect: In Vivo PAR Level to Understand More Biological Functions and Its Application for Clinic

PARP1 has been known to function in base excision repair (BER). When a single-strand break of DNA occurs, PARP1 immediately binds to the sites of DNA breaks and is activated to synthesize PAR attached to PARP1 itself (automodification), serving as a scaffold for recruiting BER enzymes. However, when PARP1 is inhibited by chemical inhibitor, single strand break persists and could make double strand break when replication fork passes through during DNA replication. These double strand breaks are usually repaired by homologous recombination controlled by BRCA1/2 gene(s). Therefore BRCA1- and BRCA2-mutated human breast cancer cells are subjected to killing by PARP inhibitors. This idea and the data were presented by 2 important papers (synthetic lethality) [87,88]. Since various anticancer drugs target DNA to make strand breaks to kill cancer cells, inhibition of DNA repair system in cancer cells is utmost effective. For this reason, combination of PARP inhibitors to DNA damaging anticancer drugs have also been studied in Phase 1 and 2 clinical trials [89–92]. It is interesting that cisplatin resistant non-small cell lung carcinoma cells frequently show hyperactivation of PARP than original tumor cells and the elevated PAR levels could be more accurate marker than the expression levels of PARP protein to predict the susceptibility of the cancer cells to PARP inhibitors [93].

Besides cancer, treatment of neurodegenerative diseases including Alzheimer disease and Parkinson’s disease becomes important in developed countries. When PAR is not degraded by mutation of degradation enzyme, PARG, neurodegenerative changes occurs in the brain and caused neurological symptoms in experimental animal model [26]. Also when the enzyme, TARG1, is mutated severe neurological symptoms appeared in the patient [12]. In addition, other diseases such as inflammation and immunological diseases are also considered to be possible targets of PARP inhibitors [94].

2. Conclusions

Since various biological functions have been proposed as described in the first part of this review, if we know more about quantitative view such as that on PAR level, we will be much closer to inventing effective methods to control or prevent diseases. Precise genomic information in addition to BRCA
mutation might be required for classification of heterogeneous diseases to be treated. The important thing is that we will encourage more intimate information exchanges between bench and bedside.

**Author Contributions:** M.M. conceived the contents of this review; M.M., C.I., S.Y., Y.K., T.E., N.O., T.S. and M.Ta. had performed the cited experiments, which motivated to write this review; M.M., K.K., M.Ts. and M.Ta. collected the references and analyzed the data of sample preparation of the cited references on the in vivo level of PAR; M.M. wrote the paper.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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