Rapid Degradation of Poly(ADP-ribose) after Injection into the Mouse Bloodstream

Yasuhisa Okajima, a,b# Tomoko Yoshida, b,c,d,# Hiroaki Fujimori, a Junhui Wang, a Hiromi Harada, a Ylenia Suzuki, b,c Hisanori Suzuki, c and Mitsuko Masutani*, a,b,c

a Division of Genome Stability Research, National Cancer Center Research Institute; b Biochemistry Division, National Cancer Center Research Institute; c ADP-ribosylation in Oncology Project, National Cancer Center Research Institute; d 1–3–1 Tsukiji, Chuo-ku, Tokyo 104–0045, Japan; e Kyoritsu University of Pharmacy; 2–15–45 Mita, Minato-ku, Tokyo 108–8345, Japan; * and # Section of Biochemistry, Department of Life and Reproduction Sciences, University of Verona Strada Le Grazie; 83713 Verona, Italy.

Received August 26, 2012; accepted December 11, 2012

Extensive DNA damage leads to the activation of poly(ADP-ribose) polymerase and subsequently to the formation of poly(ADP-ribose). When the damage is severe or leads to cell death, poly(ADP-ribose) may leak into the blood circulation. The metabolism of poly(ADP-ribose) in the bloodstream is not well understood. Thus, in the present study, the metabolism of 32P-labeled poly(ADP-ribose) was followed in mice after injection of this labeled compound into the tail vein. The results showed that 5 min after injection more than half of the radioactivity was concentrated in acid-soluble fractions, namely in low molecular weight compounds in the blood, liver, and kidneys. Most of this radioactivity was in the form of inorganic phosphate, detected 5 min post-injection in the blood, kidneys, and urine. By contrast, the metabolites ADP-ribose and phospho-riboisyl-AMP were not detected in any of the tissues nor in blood or urine. Taken together, these findings suggest that once poly(ADP-ribose) enters the bloodstream it is rapidly degraded, thereby preventing its accumulation in the blood.

Key words poly(ADP-ribose); poly(ADP-ribose) polymerase; blood; urine; mouse

Proteins belonging to the poly(ADP-ribose) polymerase (PARP) family1 as well as its product, poly(ADP-ribose) (PAR),2 are present almost ubiquitously in mammals.3 PAR is mostly synthesized in the nuclei by PARP1 and PARP2 after DNA damage but also on the extracellular surface by ADP-ribosyl transferase 4,5 while its degradation is catalyzed by the enzymes poly(ADP-ribose) glycohydrolase (PARG),5–8 ADP-ribosyl hydrolase-3 (ARH3),9 and phosphodiesterase.10 The localization of PARG is reported to be mainly in the nucleus, but it is also present in cytoplasm, centrosome11 and mitochondria in the cells.12,13 Its localization in the outside of cells has not been extensively studied. ARH3 is localized in mitochondria11 but its detailed localization is not reported. Following DNA damage, the poly(ADP-ribosylation) of cellular proteins profoundly increases.15 During necrotic cell death, some cellular components, including DNA, peroxiredoxin, and HMGB1 are reported to leak out in the bloodstream.16 It is thus speculated that in necrotic cell death or severe cell damage, the accumulated PAR may also leak into the bloodstream. However, there is not enough evidence that PAR leaks into the bloodstream. PAR is also suggested to be a death signal in pathological condition.17,18 If PAR is stably in the bloodstream, PAR level in the blood can be used as a biomarker for PARP and PARG inhibitor efficacy. Thus far, little is known about PAR metabolism in the body, including its degradation in the bloodstream. An understanding of the PAR metabolism in the bloodstream is important from both the therapeutic and the pathophysiological viewpoints. In the present study, to clarify the PAR stability in the bloodstream, the fate of PAR injected into the mouse tail vein was investigated.

MATERIALS AND METHODS

Preparation of 32P-Poly(ADP-ribose) PAR was prepared using recombinant human PARP-1 expressed in Escherichia coli, as described elsewhere.23–25 A pellet prepared from these bacteria was suspended in lysis buffer [50 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 10 µg/ml] and treated with of egg-white lysozyme at 200 µg/ml at 0 °C for 15 min. NaCl was then added to the sample to a final concentration of 0.6 M and NP-40 to 1.0%, followed by centrifugation for 45 min at 25000×g and 4 °C. The supernatant was treated with 50 mM Tris–Cl (pH7.5), 1 mM dithiothreitol, 30 mM MgCl2, histone (Sigma-Aldrich Corp.) at 20 µg/ml, activated calf thymus DNA (Sigma-Aldrich Corp.) at 10 µg/ml, 10–20 µM [adenylate-32P] NAD (0.46 MBq/nmol, PerkinElmer), and proteinase inhibitor cocktail (Complete, Roche Applied Science) and incubated at 25 °C for 60 min. 32P-poly(ADP-ribose) was released from proteins with a 1/50 volume of 5% NaOH and incubation for 60 min at 37 °C. After proteinase K treatment, phenol extraction, and ethanol precipitation, the sample was further treated with DNase I, nuclease P1, and RNase A and then with pronase.
The fraction containing 32P-poly(ADP-ribose) was purified with QIAGen-tip (QIAGen) and dialyzed against water.

Preparation of the Standards 32P-ortho-Phosphate was prepared by labeling oligonucleotide DNA with T4 polynucleotide kinase (TaKaRa, Japan) and γ-32P-ATP (Amersham) at 16°C for 1 h, followed by purification on a Sephadex G-50 column. The resulting 32P-oligonucleotide DNA was treated with bacterial alkaline phosphatase (Toyobo, Japan) at 60°C for 1 h and the released 32P-ortho-phosphate thereby recovered.

Thin-Layer Chromatography (TLC) Two-dimensional TLC was carried out with solvent buffers of isobutyric acid–25% NH4OH–H2O (50 : 1.1 : 28.9) in the first dimension and 0.1 M sodium-phosphate (pH 6.3)–ammonium sulfate–n-propanol (100 : 60 : 2) in the second dimension, as described elsewhere.26) The radioactivity was analyzed using BAS2500 (FUJI FILM, Japan).

Degradation of PAR Injected into the Mouse Tail Vein Male ICR mice 11–13 weeks old (Clea Japan) were anesthetized with an intraperitoneal injection of nembutal (Dainippon Laboratories, Japan). Ten micrograms/approximately 6×10^4 cpm/100 µL of 32P-poly(ADP-ribose) was then injected from tail vein. Blood plasma, urine, and the liver, kidneys, spleen, and lungs were obtained after euthanization and autopsy of the mice (four to eight mice for each group). The tissues were weighed and then homogenized in phosphate buffered saline (PBS) with a Polytron homogenizer. Equal amounts of Solvable tissue lysis buffer (Packard) were added to the homogenate, followed by a 50°C overnight incubation. Next, 0.1 M ethylenediamine-N,N,N’,N’-tetraacetic acid (EDTA) (pH 8.0) and 31% (w/v) hydrogen peroxide (Mitsubishi Chemical Company, Inc., Japan) were added to 20% (v/v) and 6% (v/v), respectively, and allowed to stand for 1 d at room temperature to quench fluorescence. Then, 10–15 mL of Instagel Plus (Parkin-Elmer) liquid scintillator cocktail was added and the radioactivity was measured in a liquid scintillation counter (Beckman).

32P-Poly(ADP-ribose) degradation was analyzed by extracting the mouse tissues with 0.5 N HClO4 for 20 min, followed by neutralization with 3 N KOH/0.7 M Gly–Gly. After a 10-min centrifugation at 15000×g, an aliquot was subjected to acid-soluble and acid-insoluble fractionation. PAR was recovered in the insoluble fraction while the soluble fraction contained degraded small molecules from PAR. The insoluble fraction was dissolved in 1 N NaOH and neutralized with 6 N HCl. Sodium dodecyl sulfate (SDS) and proteinase K were added to 0.1% and 100 µg/mL, respectively, and the sample then incubated at 37°C overnight. After phenol and ethanol precipitations, this fraction containing PAR was dissolved in water and an aliquot was spotted onto DE81 paper (Whatman) to measure the radioactivity with BAS2500.

RESULTS AND DISCUSSION

To follow the metabolism of PAR present in the bloodstream, 10 µg of 32P-labeled PAR (Fig. 1A) was injected into the tail vein of ICR mice and the time course of the...
Ten micrograms of $^{32}$P-Poly(ADP-ribose) were injected into the mouse tail vein and the distribution and degradation of the radioactive compound were monitored. (C) The left panel: The distribution of radioactivity 5 min post-injection in the high molecular weight (precipitate (ppt)) and low molecular weight (supernatant) fractions prepared from blood, liver, and kidneys of two mice. The right panel: $^{32}$P-Poly(ADP-ribose) present in blood 5 min post-injection (the right lane) analyzed with injected $^{32}$P-Poly(ADP-ribose) (the left lane) by 20% PAGE. The numbers next to the image correspond to the numbers of ADP-ribose residues. (D) Two-dimensional TLC analysis of the soluble fractions after acid extraction of the blood, kidneys, and urine 5 min after the injection of $^{32}$P-Poly(ADP-ribose). (E) Analysis of the soluble fraction of urine prepared 1 and 3 h after the injection of $^{32}$P-Poly(ADP-ribose).
distribution and metabolism of the labeled compound was analyzed. Figure 1B shows the distribution of the radioactivity among the tissues. Only a small proportion of the radioactivity was detected in the blood, accounting for approximately 2% of the injected amount, at 5 min and 1 h after injection, and its level became approximately 1% at 3 h. Approximately 25% of the total radioactivity was distributed in the liver and kidneys, with far less in the lungs and spleen. One hour after injection, approximately 5% of the radioactivity appeared in the urine.

The degradation of the injected PAR in tissues was analyzed as well. At 5 min post-injection, 64%, 84%, and 91% of the radioactivity was detected, respectively, in the blood, liver, and kidneys, in the acid-soluble fraction and thus in low molecular weight compounds in these tissues of mouse No.1 (Fig. 1C, left panel). The similar appearance of acid-soluble fraction was also observed with mouse No. 2 (Fig. 1C, left panel). The acid-insoluble fraction of the blood sample was subjected to 20% polyacrylamide gel electrophoresis (PAGE). This acid-insoluble fraction showed that the PAR remaining in the bloodstream became shorter than the injected PAR at 5 min post-injection (Fig. 1C, right panel). Also at this time-point, the major Rf value of the 32P radioactivity in the acid-soluble fraction corresponded to inorganic phosphate, present in the blood, kidneys, and urine (Figs. 1D, E). By contrast, no TLC spots corresponding to AMP, PR-AMP, or ADP-ribose were observed. The blood sample of 5 min post-injection showed a spot at the origin (the left panel of Fig. 1D). This spot could correspond to the dimer of ADP-ribose or other short oligo(ADP-ribose) molecules, because trichloroacetic acid can efficiently precipitate larger molecules than ten residues but the dimer or shorter oligo(ADP-ribose) are not precipitated efficiently. At 1 and 3 h post-injection, the 32P radioactivity migrated to a chromatographic position corresponding to inorganic phosphate in the urine (Fig. 1E). We could not analyze the 1 h or 3 h samples of blood by TLC, because of the decay of the radioactivity. Further analysis may be planned using mass-spectrometry or enzyme-linked immunosorbent assay for PAR using non-labeled PAR, which may provide a benefit for easier analysis.

These results suggested that, in vivo, PAR is rapidly degraded in the blood, accompanied by the production of inorganic phosphates. Phosphodiesterase activity may be involved in the degradation process like in the case with DNA. However, PARG or other enzymes could be also engaged in the rapid degradation process. Because rapid degradation of DNA in the bloodstream involves phosphodiesterase activity, it is a question whether phosphodiesterase is involved in the degradation of PAR. It is of note that we could detect inorganic phosphate, in contrast, other possible intermediate metabolites, such as ADP-ribose, phosphoribosyl-AMP or AMP, were not detected in the acid-soluble fractions of the tissues. This might suggest a rapid removal of phosphate residue from the intermediate metabolites.

There is also a possibility that PAR is rapidly translocated and degraded in the tissues or cell surface. It is previously reported that PAR is degraded by PARG and then by phosphodiesterase and AMP is used to generate ATP. The current study showed that PAR is rapidly degraded in the bloodstream. Because PAR used in this study has mostly linear form of less than 100 residues. There is a possibility that poly(ADP-ribosylated) protein or long PAR of more than 100 residues may be more stable. The relationship of the structure of PAR and degradation potential should be also studied to evaluate the potential of PAR as a biomarker for monitoring the efficacy of PARP and PARG inhibitors. Further analysis of the degradation of PAR using in vitro reaction and structural analysis will be helpful for understanding the biological significance of PAR degradation in the bloodstream.

Acknowledgements We would like to thank Tomoko Okada and Naoyuki Uchiya for their technical assistance. We are grateful for Sei-ichi Tanuma, Shizuku Kobayashi, Hitoshi Nakagama, and Takashi Sugimura for discussion and suggestions. This work was supported in part by a Grant-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control, National Cancer Center Research and Development Fund; a Grant-in-Aid for A-STEP Feasibility Study from JST (AS22IZ03546F); and a Grant-in-Aid from the Foundation for Promotion of Cancer Research in Japan.

REFERENCES

1) Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. Nat. Rev. Mol. Cell Biol., 7, 517–528 (2006).
2) Sugimura T. Poly(adenosine diphosphate ribose). Prog. Nucleic Acid Res. Mol. Biol., 13, 127–151 (1973).
3) Miwa M, Masutani M. PolyADP-ribosylation and cancer. Cancer Sci., 98, 1528–1535 (2007).
4) Morrison AR, Moss J, Stevens LA, Evans JE, Farrell C, Merithew E, Lambright DG, Greiner DL, Morses JP, Rossini AA, Bortell R. ART2, a T cell surface mono-ADP-ribosyltransferase, generates extracellular poly(ADP-ribose). J. Biol. Chem., 281, 33363–33372 (2006).
5) Miwa M, Sugimura T. Splitting of the ribose–riboside linkage of poly(adenosine diphosphate-ribose) by a calf thymus extract. J. Biol. Chem., 246, 690–696 (1971).
6) Uchida K, Suzuki H, Maruta H, Abe H, Aoki K, Miwa M, Tanuma S. Preferential degradation of protein-bound (ADP-ribose)_{n} by nuclear poly(ADP-ribose) glycohydrolase from human placenta. J. Biol. Chem., 268, 3194–3200 (1993).
7) Lin W, Amé JC, Aboul-Ela N, Jacobson EL, Jacobson MK. Isolation and characterization of the cDNA encoding bovine poly(ADP-ribose) glycohydrolase. J. Biol. Chem., 272, 11895–11901 (1997).
8) Affar EB, Shah RG, Poirier GG. Poly(ADP-ribose) turnover in quail myoblast cells: relation between the polymer level and its catalysis by glycohydrolase. Mol. Cell. Biochem., 193, 127–135 (1999).
9) Oka S, Kato J, Moss J. Identification and characterization of a mammalian 39-kDa poly(ADP-ribose) glycohydrolase. J. Biol. Chem., 281, 705–713 (2006).
10) Futai M, Mizuno D, Sugimura T. Hydrolysis of the polymer formed from NAD with rat liver phosphodiesterase yielding nucleoside 5′-monophosphate. Biochem. Biophys. Res. Commun., 28, 395–399 (1967).
11) Ohashi S, Kanai M, Hanai S, Uchiimi F, Maruta H, Tanuma S, Miwa M. Subcellular localization of poly(ADP-ribose) glycohydrolase in mammalian cells. Biochem. Biophys. Res. Commun., 307, 915–921 (2003).
12) Meyer-Ficca ML, Meyer RG, Coyle DL, Jacobson EL, Jacobson MK. Human poly(ADP-ribose) glycohydrolase is expressed in alternative splice variants yielding isoforms that localize to different cell compartments. Exp. Cell Res., 297, 521–532 (2004).
13) Whatcott CJ, Meyer-Ficca ML, Meyer RG, Jacobson MK. A specific isoform of poly(ADP-ribose) glycohydrolase is targeted to the mitochondrial matrix by a N-terminal mitochondrial targeting
sequence. Exp. Cell Res., 315, 3477–3485 (2009).

14) Niere M, Kernstock S, Koch-Nolte F, Ziegler M. Functional localization of two poly(ADP-ribose)-degrading enzymes to the mitochondrial matrix. Mol. Cell. Biol., 28, 814–824 (2008).

15) Durkacz BW, Omidiji O, Gray DA, Shall S. (ADP-ribose) n participates in DNA excision repair. Nature, 283, 593–596 (1980).

16) Miyake Y, Yamasaki S. Sensing necrotic cells. Adv. Exp. Med. Biol., 738, 144–152 (2012).

17) Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM. Poly(ADP-ribose) (PAR) polymer is a death signal. Proc. Natl. Acad. Sci. U.S.A., 103, 18308–18313 (2006).

18) Yu SW, Wang H, Poitras MF, Coombs C, Bowers JW, Federoff HJ, Poirier GG, Dawson TM, Dawson VL. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science, 297, 259–263 (2002).

19) Mullen PM, Lollo CP, Phan QC, Amini A, Banaszczuk MG, Fabrycki JM, Wu D, Carlo AT, Pezzoli P, Coffin CC, Carlo DJ. Strength of conjugate binding to plasmid DNA affects degradation rate and expression level in vivo. Biochim. Biophys. Acta, 1523, 103–110 (2000).

20) Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, Mortimer P, Swaïsland H, Lau A, O’Connor MJ, Ashworth A, Carmichael J, Kaye SB, Schellens JH, de Bon J. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N. Engl. J. Med., 361, 123–134 (2009).

21) Steffen JD, Coyle DL, Damodaran K, Beroza P, Jacobson MK. Discovery and structure–activity relationships of modified salicylanilides as cell permeable inhibitors of poly(ADP-ribose) glycohydrolase (PARG). J. Med. Chem., 54, 5403–5413 (2011).

22) Fauzee NJ, Pan J, Wang YL. PARP and PARG inhibitors—new therapeutic targets in cancer treatment. Pathol. Oncol. Res., 16, 469–478 (2010).

23) Ikejima M, Noguchi S, Yamashita R, Suzuki H, Sugimura T, Miwa M. Expression of human poly(ADP-ribose) polymerase with DNA-dependent enzymatic activity in Escherichia coli. Biochem. Biophys. Res. Commun., 163, 739–745 (1989).

24) Shimokawa T, Ogno H, Maeda D, Nakagama H, Sugimura T, Masutani M. Poly(ADP-ribose) preparation using anion-exchange column chromatography. Organic Chemistry Insights, 2, 1–5 (2009).

25) Shimokawa T, Masutani M, Nagasawa S, Nozaki T, Ikota N, Aoki Y, Nakagama H, Sugimura T. Isolation and cloning of rat poly(ADP-ribose) glycohydrolase: presence of a potential nuclear export signal conserved in mammalian orthologs. J. Biochem., 126, 748–755 (1999).

26) Keith G, Desgrès J, de Murcia G. Use of two-dimensional thin-layer chromatography for the components study of poly(adenosine diphosphate ribose). Anal. Biochem., 191, 309–313 (1990).

27) Panzeter PL, Althaus FR. High resolution size analysis of ADP-ribose polymers using modified DNA sequencing gels. Nucleic Acids Res., 18, 2194 (1990).

28) Maruta H, Matsumura N, Tanuma S. Role of (ADP-ribose) catalysis in DNA repair. Biochem. Biophys. Res. Commun., 236, 265–269 (1997).