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Immunochemical Studies of Human Fibrinopeptide A Using Synthetic Peptide Homologues†

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ABSTRACT: Previous studies have indicated that rabbit antisera R2 and R33 to human fibrinopeptide A differ markedly in terms of cross-reactivity with fibrinogen and fibrinopeptide A-containing fragments of the fibrinogen molecule. Antiserum specificity was characterized by comparison of inhibition of binding to radiolabeled tyrosyl fibrinopeptide A produced by synthetic fragments and enzymatic digests of the fibrinopeptide A molecule vs. the complete fibrinopeptide sequence (Aα 1–16). Synthetic COOH-terminal homologues through the dodecapeptide (Aα 5–16) exhibited less than 16% immunoreactivity with R33 antiserum, which cross-reacts extensively with fibrinogen and fibrinopeptide A-containing fibrinogen fragments. In contrast, the synthetic COOH-terminal decapeptide (Aα 7–16) gave 100% immunoreactivity with R2 antiserum, which cross-reacts minimally with fibrinogen and fibrinopeptide A-containing fibrinogen fragments. Synthetic homologues smaller than Aα 7–16, such as Aα 9–16 and Aα 7–11, reacted only minimally with R2 antiserum. Carboxypeptidase B digests of fibrinopeptide A retained less than 25% of their initial immunoreactivity with R2 antiserum. It is concluded that the antigenic determinants of R2 immunoreactivity reside entirely within the COOH-terminal ten-residue sequence of fibrinopeptide A, and that Phe-8, Asp-7, and Arg-16 contribute significantly to R2 immunoreactivity. The R2 antigenic determinants appear to be significantly less accessible to reaction with antibody than the R33 determinants when the fibrinopeptide is attached to its parent α chain (Canfield et al., 1976). A possible mechanism for the sequestration is discussed.

The conversion of fibrinogen to fibrin is directly related to the cleavage of fibrinopeptide A, representing the NH_2-terminal 16-residue segment of the fibrinogen α chain, by the enzyme thrombin (Blombäck et al., 1967). In order to specifically measure the action of this enzyme, a radioimmunoassay for human fibrinopeptide A (Aα 1–16) has been developed (Nossel et al., 1971) and applied to the study of clinical blood samples (Nossel et al., 1974; Gerrits et al., 1974). A number of antifibrinopeptide A sera have been prepared, and two, R2 and R33, have been used in clinical studies (Nossel et al., 1974).

Previous studies have shown that dialysates of thrombin-treated normal human plasma demonstrate equivalent immunoreactivity with R2 and R33 antisera, while dialysates of streptokinase-treated plasma showed significantly higher immunoreactivity with R33 antiserum than with R2 antiserum (Nossel et al., 1974). Following thrombin treatment of the dialysates, similar immunoreactivity was found with both R2 and R33 antisera. To explain these findings, it was postulated that fibrinopeptide A gave comparable results with the two antisera, but that larger fragments of the fibrinogen molecule which included the A peptide, as would be produced by plasmin cleavage, gave relatively lower reactivity with R2 antiserum (Canfield et al., 1976; Nossel et al., 1974). The hypothesis was advanced that R2, but not R33, antigenic determinants are immunologically hidden in the larger fibrinopeptide A-containing fragments of the fibrinogen molecule, thus permitting distinction between thrombin and plasmin proteolysis products in clinical plasma samples. This postulate has assumed a crucial role in the interpretation of fibrinopeptide A measurements in patients with various clinical disorders (Nossel et al., 1974). Detailed immunochemical studies of these antisera were therefore undertaken using synthetic fibrinopeptide homologues to define the antigenic sites of fibrinopeptide A which react with the R2 and R33 antisera.

Materials and Methods

Reagents. All chemicals were of reagent grade. N^{α}-*tert*-Butyloxycarbonyl (BOC[1]) amino acids were purchased from Fox Chemical Co., Los Angeles, California. BOC-amino acids with protected side chains were γ-benzylglutamic acid, β-benzylaspartic acid, *O*-benzylserine, and N^{g}-tosylarginine. All BOC-amino acids were of the L configuration with the exception of glycine. Purity of the BOC-

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[1] Abbreviations used: BOC, N^{α}-*tert*-butyloxycarbonyl; DFP, diisopropylfluorophosphate.

Table I: Analysis of Synthetic Human Fibrinopeptide A Homologues.

Residue[a]	Peptide Sequence				
	Aα 5–16	Aα 7–16	Aα 9–16	Aα 1–8	Aα 7–11
Asp	0.99 (1)	0.98 (1)		1.95 (2)	0.99 (1)
Ser				0.90 (1)	
Glu	2.11 (2)	0.99 (1)	1.00 (1)	1.02 (1)	0.95 (1)
Gly	4.19 (4)	2.98 (3)	3.16 (3)	2.03 (2)	
Ala	1.03 (1)	0.99 (1)	0.97 (1)	1.06 (1)	1.03 (1)
Val	1.02 (1)	0.97 (1)	1.07 (1)		
Leu	0.91 (1)	1.02 (1)	0.89 (1)		1.01 (1)
Phe	1.00 (1)	1.02 (1)		1.03 (1)	1.01 (1)
Arg	1.00 (1)	1.06 (1)	1.00 (1)		
$R_f^{(1)}$	0.70	0.68	0.57	0.66	0.72
$R_f^{(2)}$	0.54	0.55	0.40	0.68	0.40

[a] Values shown represent average molar ratios. Theoretical values are shown within parentheses.

amino acids was assessed by melting points (uncorrected) and thin-layer chromatography (Stewart and Young, 1969). Sephadex G-15, CM Sephadex C-25, and SP Sephadex C-25 were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Human fibrinogen solutions, native and synthetic human fibrinopeptide A (Aα 1–16), *N*-tyrosyl fibrinopeptide A, and ^{125}I-*N*-tyrosyl fibrinopeptide A were prepared as previously described (Nossel et al., 1971, 1974). The preparations of rabbit antihuman fibrinopeptide A antisera R2 and R33 have been described in detail (Nossel et al., 1971, 1974). Protein-coated charcoal suspensions and buffered saline–ovalbumin solutions were prepared as previously described (Nossel et al., 1974). Solutions containing inhibitor or tracer were prepared in Tris-buffered saline, pH 8.6, containing 0.1% ovalbumin. ^{125}I-Labeled tracer dilutions were adjusted to 10 000 cpm and contained about 9.0×10^{-3} μCi of ^{125}I per 50 μl. Specific activity of the tracer varied from 40 to 100 μCi/μg. Styrene–divinylbenzene beads (1% cross-linked, 200–400 mesh) were purchased from Bio-Rad Laboratories, Richmond, Calif., and chloromethylated (1.11 mmol of Cl^-/g) as described by Gutte and Merrifield (1971).

Synthesis of Homologues of Human Fibrinopeptide A (Johnson and May, 1969; Blombäck et al., 1969; Nossel et al., 1971; Andreatta et al., 1971; Dorman et al., 1972; Budzynski and Marder, 1973).

Peptides Aα 5–16, Aα 7–16, Aα 9–16, Aα 1–8, and Aα 7–11 were synthesized manually using Merrifield's solid-phase method (Meienhofer, 1973). Esterification of the COOH-terminal residue to the insoluble support was accomplished by refluxing in ethanol–triethylamine (Merrifield, 1964). Amino group deprotection was accomplished using 25% trifluoroacetic acid in methylene chloride (Stewart and Matseuda, 1972), and neutralization was performed using 10% triethylamine in methylene chloride. All residue couplings were performed in methylene chloride using dicyclohexylcarbodiimide (4 molar excess of reagents), and couplings were terminated at each step using *N*-acetylimidazole (Markley and Dorman, 1970). The completed, fully protected peptide was cleaved from its insoluble support and deprotected by reaction with anhydrous hydrogen fluoride and anisole (Gutte and Merrifield, 1971). Following extraction with dry ethyl acetate, the crude peptide was dissolved with 50% acetic acid, desalted on a Sephadex G-15 column, and evaporated to dryness. Peptide Aα 9–16 was purified by chromatography on CM-Sephadex C-25 using a buffer system described by Coy et al. (1973). The remaining synthetic peptides were chromatographed on SP-Sephadex C-25 columns and eluted with linear pH gradients developed in a varigrad device (Buchler Instruments, Inc., Fort Lee, N.J.), using 0.05 M ammonium formate,[2] pH 2.8, as starting and 0.1 M ammonium acetate, pH 7.0, as limit buffers. Column dimensions varied between 1.6 × 23 cm and 1.6 × 46 cm depending on the quantity of material to be purified. Eluate fractions were analyzed by absorbance at 225 nm, followed by ninhydrin reaction following alkaline hydrolysis (Fruchter and Crestfield, 1965; Moore, 1968). Major peaks were pooled, evaporated, redissolved in small quantities of distilled water, and stored frozen. Aliquots were removed and hydrolyzed in 6 N HCl in sealed tubes for 24 h at 110 °C. Amino acid analysis was obtained (Spackman et al., 1958), using a Beckman Model 121 analyzer equipped with an Infotronics Model 110 A integrator. Eluate fractions containing the desired amino acid composition were tested for homogeneity by means of two-dimensional thin-layer chromatography and thin-layer electrophoresis, using precoated cellulose plates (Merck, Darmstadt, Germany) and buffer systems as described by Ritschard (1964). Ninhydrin and chlorine: *o*-tolidine sprays (Stewart and Young, 1969) were used for peptide identification. Thin-layer chromatography of the purified peptides was performed using glass plates precoated with silica gel G (Analtech, Inc., Newark, Del.) and developed under conditions of chamber saturation. Fluorescamine spray (Felix and Jiminez, 1974) was used for peptide visualization. R_f values on these plates refer to the following solvent systems: (1) 1-butanol–acetic acid–water (1:1:1, v/v/v); and (2) ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v/v/v).

Enzyme Digestion Studies. Synthetic human fibrinopeptide A (0.12 μmol) was dissolved in 100 μl of 0.2 M ammonium bicarbonate and digested for 2 h at 37 °C with 0.06 unit of DFP-treated porcine carboxypeptidase B (EC 3.4.12.3) (No. COBDFP, Worthington Biochemical Corp., Freehold, N.J.). The specific activity of this enzyme preparation was 13.2 units/mg, as determined by hippuryl-L-arginine substrate assay (Folk et al., 1960). Similar reaction mixtures without added enzyme or peptide served as controls. Following digestion, the reaction mixtures were immediately frozen and freeze-dried. Amino acid analysis showed that, under conditions of enzymatic digestion, 1.0 mol of arginine and 0.1 mol of valine were released per mol of fibrinopeptide.

Inhibition Studies. The displacement of binding of antisera to ^{125}I-*N*-tyrosylfibrinopeptide by various peptides was measured by a modification of the radioimmunoassay procedure previously described (Nossel et al., 1971, 1974). The final reaction volume was adjusted to 500 μl by varying the volume of buffer added. Following incubation (1 h at 4 °C)

[2] Molarity refers to initial concentration of acid used in preparing buffer solution.

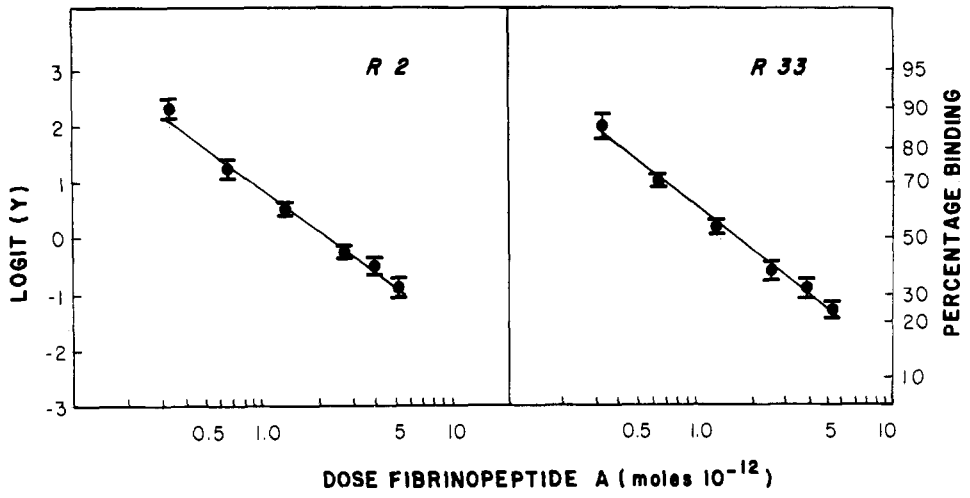

FIGURE 1: Standard dose-response curve demonstrating the reaction of human fibrinopeptide A (Aα 1-16) with R2 and R33 antisera. Response data have been linearized by means of logit transforms, as defined in the Materials and Methods section. The plots show the mean of ten replicate assays, with vertical bars indicating one standard deviation.

Table II: Quantity[a] of Inhibitors Required to Produce 50% Inhibition[b] of Binding of 2–4 × 10^{-3} μCi of ^{125}I-Labeled Human Fibrinopeptide A Analogue.

Inhibitor	Sequence (1) (2) (3) (4) (5) (6) (7) (8) (9) (10) (11) (12) (13) (14) (15) (16)	Antiserum R2	Antiserum R33
Fibrinogen[c] $(A\alpha B\beta\gamma)_2$	H-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-	1.3	240
Fibrinopeptide A Aα 1–16	H-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-OH	2.4	2.0
Aα 5–16	H-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-OH	2.0	13
Aα 7–16	H-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-OH	1.5	2 500
Aα 9–16	H-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-OH	980	>110 000 (28%)
Aα 1–8	H-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-OH	>129 000 (30%)	>129 000 (25%)
Aα 7–11	H-Asp-Phe-Leu-Ala-Glu-OH	12 000	22 000

[a] In mol × 10^{-12}. [b] Instances where 50% inhibition was not attained, largest quantity of inhibitor used is listed, and percent inhibition achieved by that amount of inhibitor is shown in parentheses. [c] NH_2-terminal sequence of the Aα chain is shown.

of a mixture containing inhibitor, radiolabeled tracer, and diluted antiserum, 500 μl of a 2.5% charcoal suspension was added. The tubes were mixed and immediately centrifuged, and the supernatant was counted in a gamma-ray spectrometer (Packard Instrument Company, Downers Grove, Ill.). The final dilution of antisera as used in this assay procedure was 1:8000 for R2 and 1:10 000 for R33. Antiserum binding, under the conditions of this assay procedure, varied between 25 and 40%. Dose-response curves were linearized by means of logit transforms as described by Wilner and Birken, (1975).

Results

Synthetic homologues of human fibrinopeptide A (Aα 1-16) gave the expected molar ratios on amino acid analysis and migrated as a single spot on thin-layer chromatography (Table I). Additional evidence of homogeneity was provided by demonstrating that each of the peptides yielded a single spot when subjected to two-dimensional, thin-layer chromatography and electrophoresis.

Both R2 and R33 antisera yielded radioimmunoassay inhibition curves of a similar degree of sensitivity, using human fibrinopeptide A (Aα 1-16) as the inhibiting antigen (Figure 1; Table II). Intraassay variability for ten replicate fibrinopeptide A determinations was similar for both antisera (Figure 1).

The ability of synthetic fragments of human fibrinopeptide A to competitively inhibit binding of ^{125}I-*N*-tyrosyl human fibrinopeptide A (Aα 1-16) to antisera is shown in Table II. Carboxy-terminal portions of the fibrinopeptide A sequence, including the dodecapeptide (Aα 5-16), manifested only partial immunoreactivity with antiserum R33 (Table II; Figure 2). The NH_2-terminal half of the fibrinopeptide A molecule (Aα 1-8) showed little or no immunoreactivity. In contrast, little or no difference was observed in the immunoreactivity of the COOH-terminal decapeptide (Aα 7-16), the COOH-terminal dodecapeptide (Aα 5-16), and the complete fibrinopeptide A sequence (Aα 1-16) with antiserum R2. The COOH-terminal octapeptide (Aα 9-16), however, showed only partial reactivity (Table II; Figure 2). Peptide Aα 7-11, which constitutes the NH_2-terminal half of peptide Aα 7-16, gave minimal displacement of labeled tracer binding to R2 antiserum (Table II).

Digestion of fibrinopeptide A with carboxypeptidase B under conditions where 1.0 mol of arginine and 0.1 mol of valine were released resulted in a fourfold decrease in R2

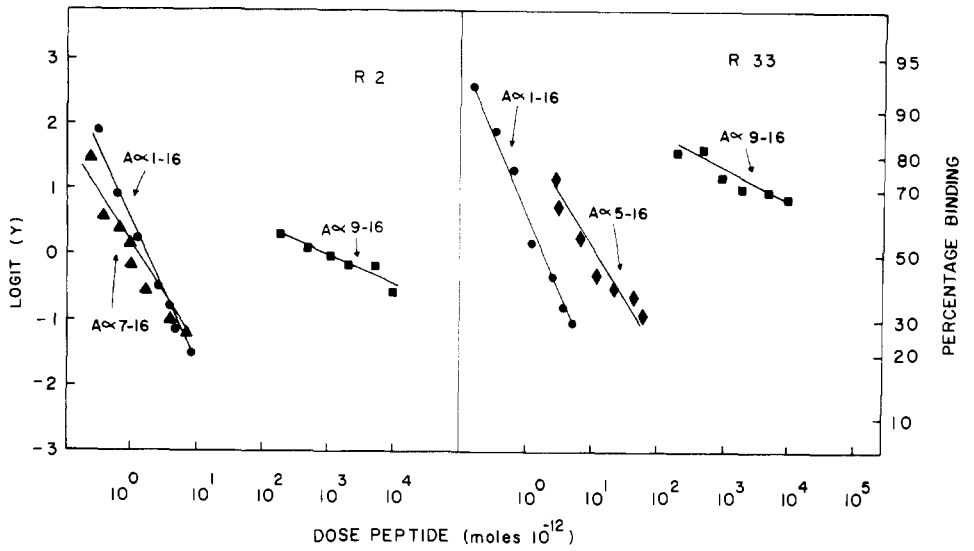

FIGURE 2: The relative capacities of different concentrations of synthetic human fibrinopeptide A homologues Aα 9-16 (■), Aα 7-16 (▲), Aα 5-16 (♦), and fibrinopeptide A (●) to inhibit the binding of 2-4 × 10^{-3} μCi of ^{125}I-labeled human fibrinopeptide A analogue with R2 and R33 antisera. Response data have been linearized by means of logit transforms, as defined in the Materials and Methods section.

Table III: Effect of Carboxypeptidase B Digestion of Fibrinopeptide A on Its Immunoreactivity.[a]

Antiserum	Antigen Tested	50% Inhibition Dose (mol × 10^{-12})
R2	Peptide control	2.4
R2	Enzyme-treated peptide	10.0
R2	Enzyme control[b]	(4.5%)
R33	Peptide control	2.0
R33	Enzyme-treated peptide	2.7
R33	Enzyme control[b]	(1.0%)

[a] Under conditions of enzymatic digestion, 1.0 mol of arginine and 0.1 mol of valine were removed. Results represent quantity of antigen required to produce 50% inhibition of binding 2-4 × 10^{-3} μCi of ^{125}I-labeled human fibrinopeptide A analogue. Where 50% inhibition was not attained, the percent inhibition achieved by the amount of inhibitor tested is shown in parenthesis. [b] Quantity of enzyme represents the maximum quantity of enzyme present in any of assay mixtures (≤4μg).

immunoreactivity, while R33 immunoreactivity showed little change (Table III).

Discussion

The present study has provided conclusive evidence for the presence of different and distinct antigenic determinants within the fibrinopeptide A molecule, for antisera R2 and R33. A complete and specific immunoreactive site within the fibrinopeptide A sequence has not been defined for R33. However, the studies with synthetic fibrinopeptide A fragments (Table II), as well as cross-reactivity studies with canine fibrinopeptide A,[3] suggest that residues amino-terminal to Asp-7 contribute significantly to R33 immunoreactivity. Carboxypeptidase B treatment of fibrinopeptide A produced only slight reduction in R33 immunoreactivity (Table III). It appears that Arg-16 contributes little to R33 immunoreactivity. The demonstrated cross-reactivity between fibrinogen and fibrinopeptide A-containing fragments of the fibrinogen molecule and R33 antiserum (Canfield et al., 1976; Nossel et al., 1974) indicates that the R33 antigenic determinants are relatively accessible when the fibrinopeptide A molecule is attached to its parent α chain.

The antigenic determinants for R2 immunoreactivity are completely contained within the COOH-terminal decapeptide of the fibrinopeptide molecule (Aα 7-16). The data in Table II indicate that Phe-8 and Asp-7 of the fibrinopeptide sequence contribute significantly to R2 immunoreactivity. Carboxypeptidase B digestion of fibrinopeptide A produced a fourfold reduction in R2 immunoreactivity (Table III), suggesting that Arg-16 may be of critical importance as well.

The limited cross-reactivity observed between Aα chain segment 1-51 and other fibrinopeptide A-containing fibrinogen fragments (Canfield et al., 1976; Nossel et al., 1974) indicates that the R2 antigenic determinants are immunologically hindered by the attachment of fibrinopeptide A to its parent α chain. It is postulated that the interaction of R2 antigenic determinants with antibody is directly related to the assumption by these determinants of a specific stable conformation. The relative selectivity of R2 antiserum for free fibrinopeptide A may be explained by the inability of the R2 antigenic determinants to achieve the particular conformation specified by the antibody combining sites when these determinants are attached to their parent α chain (Canfield et al., 1976). The suggestion by Haber and associates (1967) that peptide antigens such as bradykinin have a favored conformation, either in solution or within the antibody combining site which determines their antigenic identity, implies that a favored conformation might exist for Aα 7-16. Circular dichroic spectral and statistical studies reported by Huseby (1973) suggesting the existence of an ordered conformation within the COOH-terminal half of the fibrinopeptide A molecule tend to support this hypothesis.

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Changes in Poly(adenosine diphosphate-ribose) and Poly(adenosine diphosphate-ribose) Polymerase in Synchronous HeLa Cells†

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ABSTRACT: An antibody has been prepared which is highly specific for poly(adenosine diphosphate-ribose). Neither poly(A), DNA, nor a variety of adenine-containing nucleosides or nucleotides were effective in competing with poly(ADP-ribose) for binding to the antibody. Of all compounds tested, only adenosine diphosphate-ribose competed for binding to the antibody. Unlabeled poly(adenosine diphosphate-ribose) was about 10 000 times more effective in competing with labeled polymer for antibody binding than was adenosine diphosphate-ribose. Using the antibody, the amount of poly(adenosine diphosphate-ribose) was found to increase from early S phase to a peak at mid S with a second, even larger increase seen at the S-G_2 transition point in synchronously dividing HeLa cells. Pulse labeling of the polymer with [2-^{3}H]adenosine was also maximal at the same time points. Changes in the levels of poly(adenosine diphosphate-ribose) polymerase activity measured in isolated nuclei coincided with the changes in amounts of polymer present in intact cells during progression from S phase into G_2.

The homopolymer, poly(ADP-ribose)[1] is synthesized from NAD by an enzyme, poly(ADP-ribose) polymerase, which is tightly associated with the chromatin of eukaryotic cells (Chambon et al., 1966; Hasegawa et al., 1967; Reeder et al., 1967). Although the function of the polymer is not known, the fact that it appears in part to be covalently joined to f_1 histones in vitro (Nishizuka et al., 1969) and in vivo (Ueda et al., 1975) may indicate that it plays an important role in modifying chromatin structure.

Two lines of evidence suggest that the polymer plays some role at or near the time of the S-G_2 transition point in

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[1] Abbreviations used: ADP-ribose, adenosine diphosphate-ribose; phosphoribosyl-AMP, 2′-(5″-phosphoribosyl)-5′-AMP; poly(ADP-ribose), poly(adenosine diphosphate-ribose), a polymer of ADP-ribose subunits joined in a 1′-2′ ribose-ribose linkage.