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An enzyme-linked immunosorbent assay-based system for determining the physiological level of poly(ADP-ribose) in cultured cells

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Abstract

PolyADP-ribosylation is mediated by poly(ADP-ribose) (PAR) polymerases (PARPs) and may be involved in various cellular events, including chromosomal stability, DNA repair, transcription, cell death, and differentiation. The physiological level of PAR is difficult to determine in intact cells because of the rapid synthesis of PAR by PARPs and the breakdown of PAR by PAR-degrading enzymes, including poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase 3. Artifacts synthesis and/or degradation of PAR likely occurs during lysis of cells in culture. We developed a sensitive enzyme-linked immunosorbent assay (ELISA) to measure the physiological levels of PAR in cultured cells. We immediately inactivated enzymes that catalyze the synthesis and degradation of PAR. We validated that trichloroacetic acid is suitable for inactivating PARPs, PARG, and other enzymes involved in metabolizing PAR in cultured cells during cell lysis. The PAR level in cells harvested with the standard radioimmunoprecipitation assay buffer was increased by 450-fold compared with trichloroacetic acid for lysis, presumably because of activation of PARPs by DNA damage that occurred during cell lysis. This ELISA can be used to analyze the biological functions of polyADP-ribosylation under various physiological conditions in cultured cells.

Keywords

Poly(ADP-ribose) polymerase; Poly(ADP-ribose) glycohydrolase; Antibody; ELISA; HeLa cells

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PolyADP-ribosylation, a post-translational modification of proteins in eukaryotic cells, is mediated by poly(ADP-ribose) (PAR) polymerases (PARPs) and ADP-ribosyltransferase 2 (ART2). Using NAD⁺ as the substrate, these enzymes catalyze formation of linear and branched ADP-ribose polymers that are attached to acceptor proteins. PolyADP-ribosylation is involved in various biological functions, including carcinogenesis, differentiation, and cell death [1]. Among 17 PARPs that have been identified and characterized, PARP-1 and PARP-2 are activated by DNA strand breaks [2–5]. The roles of PARPs in DNA repair have been studied extensively [1,6].

Many proteins that are polyADP-ribosylated by PARPs have been identified, including histones [7], p53 [8], and PARP-1 itself [9]. These acceptor proteins contain a PAR-binding consensus motif [10]. PARP-1 (EC 2.4.2.30), one of the major PARPs, is found mainly in nuclei and is activated by binding to DNA strand breaks via zinc-finger motifs [1,6,11]. However, polyADP-ribosylation also occurs in the absence of intracellular DNA damage [12–15]. PolyADP-ribosylation may be involved in various biological functions, including not only DNA repair but also differentiation and transcription [16,17]. Various biological functions may be regulated physiologically by PAR through gene regulation in the absence of substantial DNA damage [18]. Inhibitors of PARPs lead to centrosome amplification in CHO-K1 cells in the absence of apparent DNA damage [19].

The physiological level of PAR in the absence of DNA damage is not well characterized because of the low levels and rapid turnover of PAR in intact cells. PAR metabolism is regulated not only through the activity of PARPs but also through the activity of poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase 3 [20]. In our previous study, we showed that PARG knockout *Drosophila melanogaster* accumulate high levels of PAR in brain cells, cannot fly because of neuronal cell death, and die within 2 weeks [21]. Similarly, PARG knockout mice show embryonic lethality [22]. PAR binds apoptosis-inducing factors in mitochondria and is involved in neuronal cell death by a process termed parthanatos [23]. Thus, further investigation of the physiological functions of polyADP-ribosylation is necessary and requires accurate measurement of PAR levels.

The purpose of the current study was to measure the physiological levels of PAR, which are quite low, in cultured cells. Various methods have been reported for this purpose, including radioimmunoassay [24], radioisotope labeling methods [25,26], the use of nonisotopic compounds [27,28], enzyme-linked immunosorbent assay (ELISA) [29,30], and stable isotope dilution mass spectrometry [31]. However, some current assays have low sensitivity for measuring PAR in intact cells, are not amenable to high-throughput measurements, or need expensive instrumentation. In addition, the level of PAR is difficult to determine under similar experimental conditions because of the rapid turnover of PAR by PARPs and PAR-degrading enzymes. In particular, artifactual synthesis and/or degradation of PAR frequently occur during lysis of cells in culture. Therefore, establishing more sensitive and suitable methods for measuring the physiological level of rapidly turned-over PAR is necessary. In this study, we developed a sensitive ELISA to measure the physiological levels of PAR in cultured cells.

Materials and methods

Materials

Radioimmunoprecipitation assay (RIPA) buffer, DNase I, and RNase A were purchased from Nacalai Tesque. Nuclease P1 was obtained from Yamasa (Japan). Trichloroacetic acid (TCA), skim milk, *o*-phenylenediamine, and hydrogen peroxide were obtained from Wako Pure Chemical Industries. Proteinase K was purchased from Merck Millipore. Sheared salmon sperm DNA was obtained from BioDynamics Laboratory. RNA from baker's yeast was purchased from Worthington Biochemical.

PAR antibody (10H, IgG3 kappa) secreted from hybridoma cells was purified using a Protein A Sepharose Fast Flow column (GE Healthcare) [32]. Anti-PAR polyclonal antibody was produced in a rabbit by injecting PAR mixed with methylated bovine serum albumin and was purified with a Protein A Sepharose Fast Flow column [33]. Goat anti-rabbit IgG (H&L) conjugated to horseradish peroxidase (HRP) was obtained from Rockland Immunochemicals. Mouse monoclonal anti-tubulin antibody was purchased from Sigma-Aldrich. Mouse monoclonal anti-human PARP1 antibody (F2) and HRP-conjugated goat anti-mouse immunoglobulin antibody and goat anti-rabbit immunoglobulin antibody were obtained from Santa Cruz Biotechnology.

PAR was prepared and purified as described previously [34], but commercially available PAR (Trevigen) showed similar immunoreactivity and was also used in these experiments.

Cell culture

HeLa cells and HEK 293T cells, human embryonic kidney cells having SV40 T antigen, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). HepG2 cells, a human hepatocellular carcinoma cell line, were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). The above cells (5×10^5) were cultured in 100-mm Petri dishes for 48 h without replacing the culture medium at 37 °C in a humidified cell culture incubator containing 5% CO₂.

Sample preparation for ELISA

HeLa cells were washed with ice-cold phosphate-buffered saline (PBS), and then the cells were fixed immediately by the addition of ice-cold 20% TCA, followed by incubation on ice for 20 min. The fixed cells were collected by scraping with a rubber policeman. The cell suspension in 20% TCA was centrifuged at 800 *g* for 10 min at 4 °C, and the cell pellet was washed two times with ethyl ether and then centrifuged at 800 *g* for 10 min at 4 °C. The cell pellets were resuspended in 0.1 N NaOH.

To prepare RIPA buffer samples, HeLa cells were washed with PBS, harvested by scraping with a rubber policeman, suspended in ice-cold RIPA buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, protease inhibitor cocktail, and 0.1% sodium dodecyl sulfate [SDS]), and incubated on ice for 20 min. Then, 0.5 N NaOH was added to bring the final concentration to 0.1 N.

The samples treated with TCA or RIPA buffer were sonicated for 30 min and then incubated for 1 h at 37 °C in a water bath to completely solubilize the cell pellets and hydrolyze the ester bonds between PAR and acceptor proteins in alkaline conditions [35,36]. The reaction was stopped by adding equal amounts of a mixture of 0.5 N HCl and 1 M Tris-HCl (pH 7.2). For extensive digestion of DNA and RNA in the samples, DNase I (20 µg/ml), RNase A (20 µg/ml), and nuclease P1 (1 U/ml) were added to the samples, which were incubated overnight at 37 °C in the presence of 5 mM MgCl₂ [34]. The samples were incubated with proteinase K (200 µg/ml) overnight at 50 °C to digest all of the proteins in cell extract, including DNase I, RNase A, and nuclease P1, and then were boiled for 5 min at 100 °C to inactivate proteinase K. Then, 50 µl of the samples was transferred to the ELISA plate.

ELISA

Sandwich ELISA was performed as follows. Microtiter plates with 96 wells (Thermo Fisher Scientific, 442404) were coated (16 h at 4 °C) with a mouse monoclonal antibody against PAR (10H) as the PAR-capturing antibody (10 µg/ml) in 50 µl of coating buffer (15 mM Na₂CO₃ and 37 mM NaHCO₃, pH 9.6) [32]. The plates were blocked with 200 µl of 5% skim milk in 0.1% Tween 20 in PBS (0.1% T-PBS) for 1 h at 25 °C. The blocking solution was removed, and the plates were washed three times with 0.05% Tween 20 in phosphate buffer (0.05% T-PBS). Samples or PAR standards were diluted with buffer (100 mM mannitol, 100 mM Tris-HCl, and 0.1 M NaCl, pH 9.0), and a 50-µl sample or standard (purified PAR) solution containing 0, 5, 10, 25, 50, and 75 pg of PAR was added to each well and incubated for 2 h at 25 °C. The plates were washed three times with 0.05% T-PBS. A 50-µl sample of rabbit anti-PAR polyclonal antibody (the detection antibody), diluted to 10 µg/ml with 2% skim milk in 0.1% T-PBS, was added to each well [33]. The plates were incubated for 2 h at 25 °C and washed three times with 0.05% T-PBS. A 50-µl sample of goat anti-rabbit IgG polyclonal antibody conjugated to HRP, diluted to 0.08 µg/ml, was added to each well, and the plates were incubated for 1.5 h at 25 °C. The plates were then washed three times with 200 µl of 0.05% T-PBS.

The antigen-antibody complex was visualized by the addition of 100 µl *o*-phenylenediamine solution (0.5 mg/ml *o*-phenyl-enediamine in 0.05% hydrogen peroxide) and incubated for 30 min at 25 °C. The reaction was terminated with 25 µl of 4 N H₂SO₄. The absorbance was measured at 492 nm with the reference wavelength at 620 nm. All experiments were performed in triplicate.

Western blotting

HeLa cells were washed with PBS with or without 7.5 mM 3-aminobenzamide (3-AB) in dimethyl sulfoxide (DMSO), a PARP inhibitor, and then the cells were fixed immediately by adding ice-cold 20% TCA with or without 7.5 mM 3-AB, followed by incubation for 20 min at 4 °C. Then, the cells were collected by scraping with a rubber policeman. The cell suspension in 20% TCA was centrifuged at 16,000 *g* for 10 min at 4 °C. The precipitates were washed two times with ice-cold ethyl ether and were collected by centrifugation at 16,000 *g* for 5 min at 4 °C. Next, 2% SDS in 20 mM Tris-HCl (pH 8.0) was added to the cell pellets, which were sonicated and boiled for 10 min at 100 °C. Then, the cell lysates were sonicated and precleared by centrifugation at 16,000 *g* for 10 min at 4 °C.

To prepare samples with RIPA buffer, HeLa cells were washed with PBS with or without 7.5 mM 3-AB. The cells were then harvested by scraping with a rubber policeman and suspended in ice-cold RIPA buffer containing 3-AB or its solvent DMSO, followed by incubation on ice for 20 min. Cell lysates were sonicated and pre-cleared by centrifugation at 16,000 *g* for 10 min at 4 °C.

Protein concentrations were determined using a BCA (bicinchoninic acid) assay kit (Thermo Scientific). Each sample was subjected to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis using the mouse monoclonal anti-PAR antibody (10H antibody), the mouse monoclonal anti-PARP-1 antibody, and the mouse monoclonal anti-tubulin antibody. Blots were then incubated with HRP-conjugated secondary antibodies, and polyADP-ribosylated proteins were visualized with enhanced chemiluminescence (GE Healthcare) and analyzed using LAS-1000 Plus (GE Healthcare).

Results

Developing an ELISA to detect PAR

Using the protocol described in the “ELISA” section above, a typical standard curve was generated with purified PAR (Fig. 1). Levels of purified PAR from 5 pg (9 fmol as ADP-ribose residues) to 75 pg could be detected in a dose-dependent manner. PAR saturation occurred at a mean of 100 pg.

Requirement for DNase I, nuclease P1, RNase A, and proteinase K treatment

We investigated potential interference from cellular components in the ELISA. First, we investigated the influence of purified DNA (Fig. 2A). A single HeLa cell has approximately 15 pg of total DNA [37]. The amount of DNA in the cell extract used to measure PAR was approximately 1.1 µg/50 µl. Wells of microtiter plates were coated with 10H antibody. Salmon sperm DNA and 25 pg of PAR in 50 µl of MTN buffer (0.1 M mannitol, 0.1 M Tris–HCl, and 0.1 M NaCl, pH 9.0) were then incubated in the wells and reacted with rabbit polyclonal antibody against PAR. Detection of PAR was inhibited by salmon sperm DNA in a dose-dependent manner (Fig. 2A). To prepare DNA samples treated with DNase I + nuclease P1, 200 µg/ml salmon sperm DNA was incubated with DNase I (20 µg/ml) and nuclease P1 (1 U/ml) in MTN buffer overnight at 37 °C and then boiled for 5 min at 100 °C. When purified PAR and DNase I + nuclease P1-treated DNA were incubated in the wells, detection of PAR was not affected by 0.05–0.5 µg of DNA but was affected by 5 µg of DNA (Fig. 2A). However, when DNA was incubated with DNase I and nuclease P1 in MTN buffer in the presence of 5 mM MgCl₂, which is required for optimal digestion by DNase I in the wells, detection of PAR was not affected (Fig. 2A).

Second, we investigated interference from purified RNA in the ELISA (Fig. 2B). A single HeLa cell has approximately 30 pg of total RNA [37]. The amount of RNA in the cell extract used to measure PAR was approximately 2.2 µg/50 µl. Detection of PAR was not affected by yeast RNA up to 5 µg in the presence or absence of RNase A (Fig. 2B). To prepare RNA samples treated with RNase A + nuclease P1, 2 mg/ml yeast RNA in MTN

buffer was incubated with RNase A (20 µg/ml) and nuclease P1 (1 U/ml) overnight at 37 °C and then boiled for 5 min at 100 °C.

Third, we investigated interference from FBS proteins, which are representative of commonly encountered proteins, in the ELISA (Fig. 2C). FBS is widely used for in vitro culture of cells. A single HeLa cell has approximately 300 pg of total protein [37]. The amount of protein in the cell extract used to measure PAR was approximately 22 µg/50 µl. Purified PAR and various amounts of FBS protein were then incubated in the wells with rabbit polyclonal antibody against PAR. Detection of PAR was inhibited by protein in a concentration-dependent manner (Fig. 2C). To prepare protein samples treated with proteinase K, FBS protein at 1 mg/ml in MTN buffer was incubated with proteinase K (200 µg/ml) overnight at 50 °C and then boiled for 5 min at 100 °C. Proteinase K-treated FBS protein in the well up to 25 µg did not interfere with PAR detection (Fig. 2C).

Comparison of cell lysis procedures with TCA or RIPA buffer for extraction of PAR

We tested various conditions for decreasing artifactual production or reduction of PAR during PAR extraction. We analyzed the effects of cell lysis procedures with TCA and RIPA buffer on PAR levels (Fig. 3A). Each sample was subjected to SDS-PAGE followed by Western blot analysis using the indicated antibodies (Fig. 3A). With RIPA buffer treatment, detection of polyADP-ribosylated proteins was dramatically increased compared with TCA-fixed samples. The increase in polyADP-ribosylated proteins observed with RIPA buffer treatment was substantially blocked with 3-AB treatment (Fig. 3A). No clear change was seen with TCA extraction. It was also confirmed that human PARP-1 in the cell lysates prepared with RIPA buffer treatment was clearly auto-polyADP-ribosylated and showed a characteristic shift on Western analysis (Fig. 3A, middle panel).

Next, we measured PAR levels with an ELISA using these conditions. With TCA treatment, the amount of PAR was $(2.11 \pm 0.18) \times 10^{-5}$ pg/cell or (0.039 ± 0.003) amol as ADP-ribose residue/cell ($n = 3$) (Fig. 3B). In contrast, with RIPA buffer treatment, the amount of PAR was $(950 \pm 7.73) \times 10^{-5}$ pg/cell ($n = 3$), which was a 450-fold increase in PAR levels in HeLa cells with RIPA buffer relative to TCA treatment (Fig. 3B).

Next, we investigated whether the artifactual increase in PAR was due to activation of PARPs in response to DNA damage that occurred during cell lysis using RIPA buffer. The cells were treated with TCA or RIPA buffer containing 3-AB, a PARP inhibitor. With TCA extraction, the amount of PAR was $(2.24 \pm 0.15) \times 10^{-5}$ pg/cell ($n = 3$). In contrast, with RIPA extraction, the amount of PAR was $(115 \pm 7.73) \times 10^{-5}$ pg/cell ($n = 3$) (Fig. 3B). Although we observed no significant change with TCA treatment, the increase in PAR observed with RIPA buffer treatment was significantly blocked with 3-AB treatment but not to the level measured following TCA treatment.

For confirmation of the usefulness of the current ELISA system to show the increase of PAR after DNA damage, we treated cells with 10 µM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 30 min to induce DNA damage and observed an increase in PAR from $(2.08 \pm 0.13) \times 10^{-5}$ pg/cell ($n = 3$) to $(346 \pm 25.1) \times 10^{-5}$ pg/cell ($n = 3$). The physiological levels of PAR differ

in cell types. For examples, the PAR level was $(2.05 \pm 0.09) \times 10^{-5}$ pg/cell ($n = 3$) in HEK 293T cells, whereas it was $(0.10 \pm 0.06) \times 10^{-5}$ pg/cell ($n = 4$) in HepG2 cells (Fig. 4).

Discussion

To analyze the function of polyADP-ribosylation using cell culture under conditions without DNA damage, it is necessary to detect physiological levels of PAR. Among several methods for quantifying polyADP-ribosylated proteins, ELISA is sensitive and can be used to measure many samples at one time [38]. However, because the level of PAR under physiological conditions without DNA damage is low and changes quite rapidly, the sensitivity and specificity of ELISA should be improved and the metabolism of PAR should be immediately stopped by inactivation of PAR-synthesizing and -degrading enzymes during sample preparation.

First, we developed a sensitive and specific ELISA and successfully measured small amounts of PAR. The sensitivity is quite high as compared with previous methods [28–30], and the limit of detection is 5 pg of PAR, which corresponds to 9 fmol of ADP-ribose residues in PAR. Further improvement of the sensitivity awaits further study. As for the specificity, the detection of PAR was affected by excess amounts of high-molecular-weight DNA and protein. These high-molecular-weight compounds interfered with the assay system, presumably by nonspecific binding to the PAR-capturing antibody or to the ELISA plate, thereby modifying nonspecific binding by the first and/or secondary antibodies. DNase I, nuclease P1, RNase A, and proteinase K treatment was used to prepare samples for ELISA. Although the yeast RNA used in the current work did not interfere with the ELISA system, combined treatment with RNase A and nuclease P1 treatments is recommended to avoid possible interference from unknown RNA that can be present in various cell types.

Second, PAR levels detected with Western blotting of proteins from HeLa cells treated with RIPA buffer were higher than those from cells treated with TCA (Fig. 3A). Surprisingly, in the current ELISA system, the amount of PAR following treatment with RIPA buffer was 450-fold higher than that following treatment with TCA (Fig. 3B). We believe that PARPs might not have been completely inactivated in RIPA buffer during extraction on ice and that this increase in PAR could be due to polyADP-ribosylation following DNA damage that occurred during the cell lysis step with RIPA buffer.

Western blot analysis with anti-PAR antibodies is frequently used to indicate an increase in PAR after DNA damage with the characteristic appearance of high-molecular-weight molecules. We showed that Western blot analysis is not sensitive enough to measure low levels of PAR because this technique did not show clear changes in PAR levels between cells treated with RIPA buffer in the presence of a PARP inhibitor and cells treated with TCA (Fig. 3A). However, with our ELISA, we observed a clear difference between the PAR level in cells treated with RIPA buffer in the presence of the PARP inhibitor and that in cells treated with TCA in the absence or presence of the PARP inhibitor (Fig. 3B), demonstrating the sensitivity of this ELISA for detecting such differences.

Artificial PAR synthesis clearly occurred during cell lysis with RIPA buffer and was not completely prevented with a PARP inhibitor. The usefulness of this ELISA system under conditions during which DNA damage occurs was also confirmed. Therefore, we emphasize that the sample preparation protocol is very important to accurately determine the physiological level of PAR in cultured cells. TCA was first used by Jacobson's group to precipitate the cultured cells for determination of PAR levels in carcinogen-treated cells [27].

We confirmed that cold 20% TCA was able to inactivate PARPs, PARG, and other enzymes in cultured cells quickly enough to measure PAR in extracts from lysed cells. To our knowledge, this is the first versatile ELISA system for measurement of the physiological levels of PAR in cultured cells.

Third, physiological levels of PAR differed depending on cell types. Among three cell lines used, the levels were between 0.10×10^{-5} and 2.11×10^{-5} pg/cell or between 0.002 and 0.039 amol as ADP-ribose residue/cell (Fig. 4). The level of PAR in HeLa cells was consistent with the data of Martello and coworkers [31].

Recently, the use of PARP inhibitors has become a promising therapeutic approach for diseases such as cancer and brain ischemia–reperfusion injury [6,39]. Thus, determining the efficiency of each inhibitor for decreasing the intracellular level of PAR is important. To do this, an accurate assay system for measuring the in vivo level of PAR at any given time is required.

In summary, the current ELISA method is useful for measuring a small amount of PAR under physiological conditions without substantial DNA damage to the cells and should be useful for analyzing the physiological functions of polyADP-ribosylation and application for clinical screening of effective inhibition of polyADP-ribosylation.

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Abbreviations used

PAR	poly(ADP-ribose)
PARP	poly(ADP-ribose) polymerase
ART2	ADP-ribosyltransferase 2
PARG	poly(ADP-ribose) glycohydrolase
ELISA	enzyme-linked immunosorbent assay
RIPA	radioimmunoprecipitation assay
TCA	trichloroacetic acid

HRP	horseradish peroxidase
FBS	fetal bovine serum
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate
T-PBS	Tween 20 in phosphate-buffered saline
3-AB	3-aminobenzamide
DMSO	dimethyl sulfoxide
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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Fig. 1. Sensitivity and linearity of the ELISA system for measurement of PAR. A typical dose–response curve for purified PAR was generated using the 10H antibody as the capturing antibody and rabbit polyclonal antibody against PAR as the detection antibody. Each data point is the mean of triplicate measurements.

Fig. 2.

Effects in the ELISA of DNase I, RNase A, and proteinase K treatment. A 25-pg sample of purified PAR was added to each well. (A) Purified PAR and the indicated amount of DNA were incubated with buffer, 1 μg of DNase I, or 1 μg of DNase I with 5 mM MgCl₂. (B) Purified PAR and the indicated amount of RNA were incubated with buffer or 1 μg RNase A. (C) Purified PAR and the indicated amount of protein were incubated with buffer or 10 μg of proteinase K. The results are the means ± standard deviations from three independent experiments. The data were analyzed for statistical significance using Dunnett's *t*-test. **P* < 0.01.

Fig. 3. Effect of cell lysis procedures on PAR levels. (A) The amount of PAR was estimated with Western blotting. HeLa cells were treated with TCA or RIPA buffer in the absence or presence of 7.5 mM 3-AB during cell lysis. The PAR signal was observed as a smeared band at greater than 150 kDa. Human PARP-1 (hPARP1) was observed at around 110 kDa. α -Tubulin was used as the loading control. (B) The amount of PAR was measured using ELISA. HeLa cells were treated with TCA or RIPA buffer in the absence or presence of 7.5 mM 3-AB during cell lysis. The results are the means $\pm$ standard deviations from three independent experiments. The data were analyzed for statistical significance using Dunnett's *t*-test. * $P < 0.01$.

Fig. 4. Physiological level of PAR in cultured cells. HeLa cells, HEK 293T cells, and HepG2 cells were treated with TCA during cell lysis. The results are the means $\pm$ standard deviations from three independent experiments. The data were analyzed for statistical significance using Dunnett's *t*-test. **P* < 0.01.