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Effect of mild temperature shift on poly(ADP-ribose) and γ H2AX levels in cultured cells

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Abstract

Poly (ADP-ribose) (PAR) is rapidly synthesized by PAR polymerases (PARPs) upon activation by DNA single- and double-strand breaks. In this study, we examined the quantitative amount of PAR in HeLa cells cultured within the physiological temperatures below 41 °C for verification of the effect of shifting-up or -down the temperature from 37.0 °C on the DNA breaks, whether the temperature-shift caused breaks that could be monitored by the level of PAR. While PAR level did not change significantly when HeLa cells were cultured at 33.5 °C or 37.0 °C, it was significantly increased 2- and 3-fold when cells were cultured for 12 h and 24 h, respectively, at 40.5 °C as compared to 37.0 °C. Similar to the results with HeLa cells, PAR level was increased 2-fold in CHO-K1 cells cultured at 40.5 °C for 24 h as compared to 37.0 °C. As the cellular levels of PAR polymerase1 (PARP1) and PAR glycohydrolase (PARG), a major degradation enzyme for PAR, did not seem to change significantly, this increase could be caused by activation of PARP1 by DNA strand breaks. In fact, γ H2AX, claimed to be a marker of DNA double-strand breaks, was found in cell extracts of HeLa cells and CHO-K1 cells at elevated temperature vs. 37.0 °C, and these γ H2AX signals were intensified in the presence of 3-aminobenzamide, a PARP inhibitor. The γ H2AX immunohistochemistry results in HeLa cells were consistent with Western blot analyses. In HeLa cells, proliferation was significantly suppressed at 40.5 °C in 72 h-continuous cultures and decreased viabilities were also observed after 24–72 h at 40.5 °C. Flow cytometric analyses showed that the HeLa cells were arrested at G2/M after temperature shift-up to 40.5 °C. These physiological changes were potentiated in the presence of 3-aminobenzamide. Decrease in growth rates, increased cytotoxicity and G2/M arrest, were associated with the temperature-shift to 40.5 °C and are indirect evidence of DNA breaks. In addition to γ H2AX, PAR could be a sensitive

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Disclosures

No conflicts of interest are declared by the author(s).

marker for DNA single- and double-strand breaks. These two molecular markers provide evidence of physiological changes occurring within cells.

Keywords

Thermal environment; DNA double-strand break; DNA single-strand break; γ H2AX; Poly(ADP-ribose); DNA repair

1. Introduction

Temperatures higher than physiological condition, known as hyperthermia, cause abnormalities in various cellular functions [1,2] and thus it is preclinically and clinically used as a sensitizer to enhance the effect of cytotoxicity, which is induced by ionizing radiation or anticancer drugs [3]. Strict temperature regulation is crucial for cell survival within the body [4].

Precise molecular mechanism(s) of the effects of heat on cellular components and cell viability, and the synergistic effect of ionizing radiation on cell killing is not yet clear [3]. Since 1988, γ H2AX, a form of histone H2A phosphorylated at serine 139, has been used as a sensitive marker for DNA double-strand breaks [5]. Heating cells, such as human lung carcinoma cells and other mammalian cell lines at 45.5 °C for 20 min [6,7] or human primary fibroblast, human squamous carcinoma cells and bovine endothelial cells at 45 °C for 30 min [8], was reported to increase γ H2AX-positive foci formation, arguing that heat exceeding 41 °C can induce DNA double-strand breaks. To the contrary, other researchers argue that heat-induced chromatin conformation change triggered to induce γ H2AX foci and heat-induced γ H2AX response did not play a role in hyperthermic cell killing [9,10]. Thus, it is not yet certain whether heat can induce DNA strand breaks or heat-induced sensitization is due to DNA strand breaks. We are interested to see cellular events, especially DNA strand breaks, induced by physiologically experienced temperatures, i.e. below 41 °C.

Poly(ADP-ribose) (PAR) is rapidly and abundantly synthesized by PAR polymerases (PARPs) after activation by DNA single- or double-strand breaks. Several reports state that PAR synthesis by PARPs is tightly linked to DNA excision repair mechanisms [11–14]. Therefore, precise measurement of PAR levels may lead to better understanding of whether DNA strand breaks could occur at physiological temperatures below 41 °C, which we may experience under disease conditions. If DNA strand breaks occur under mild temperature shift and not repaired, it could be an important factor in mutagenesis, carcinogenesis and aging.

2. Materials and methods

2.1. Cells and reagents

HeLa cells and Chinese hamster CHO-K1 cells provided by Riken (Tsukuba, Japan) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μ g/ml) in a humidified incubator with 5% CO₂.

3-Aminobenzamide (3AB) was obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Hoechst 33342 was purchased from Dojindo (Kumamoto, Japan).

2.2. Cell proliferation and cell viability assays

For quantifying cell proliferation or viability, we used trypan blue exclusion assay. HeLa cells (5.8×10^4) were plated into 35-mm cell culture dishes. After growth, cells were trypsinized, resuspended in medium, and mixed with 0.4% trypan blue solution. Viable cells were those not stained with trypan blue.

2.3. Antibodies

The following antibodies were used: mouse anti-PARP antibody (F2), goat anti- β -actin antibody (I-19), mouse anti-p53 antibody (DO-1), goat HRP-conjugated anti-mouse antibody (sc-2005), goat HRP-conjugated anti-rabbit antibody (sc-2004) and donkey HRP-conjugated anti-goat antibody (sc-2056) were purchased from Santa Cruz Biotechnologies. Mouse anti-PARG antibody (ab1606) was purchased from Abcam. Mouse anti-phospho-Histone H2A.X antibody (Ser139) (05-636) was purchased from Millipore. Goat Alexa Fluor 488-conjugated anti-mouse antibody was purchased from Invitrogen. Mouse anti-PAR (10H) antibody was produced and purified [15]. Rabbit anti-PAR antibody was prepared and purified [16].

2.4. Flow cytometric analysis

Flow cytometric analysis was performed to evaluate the cell-cycle distribution. Trypsinized cells were centrifuged ($100 \times g$) for 3 min and resuspended in phosphate-buffered saline (PBS). The suspension was fixed with ice-cold 100% ethanol to a final concentration of 70%, followed by staining with propidium iodide (Sigma-Aldrich). Flow cytometry was performed using BD AccuriTM C6 Flow Cytometer (BD Biosciences) following manufacturer's Instrument Manual (BD AccuriTM C6 Flow Cytometer, BD Biosciences).

2.5. Cell lysis and immunoblotting

Cells were washed with PBS, and then were fixed immediately by addition of ice-cold 20% TCA, followed by incubation on ice for 20 min. Then, the fixed cells were collected by scraping with a rubber policeman. The cell suspension was precipitated with 20% TCA, centrifuged at $600 \times g$ for 5 min and the precipitates were washed twice with ethyl ether. The cell pellets were dissolved by addition of 2% SDS in 20 mM Tris-HCl (pH8.0) and sonicated. After adjustment of protein concentration using BCA kit (Thermo Scientific), cell lysates were subjected to SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% (v/v) non-fat dry milk (Wako) in Tris-buffered saline (pH7.5) for 1 h at room temperature and then incubated with primary antibody. The target proteins were visualized with enhanced chemiluminescence by using ImageQuant Las-4000 (GE Healthcare Life Sciences).

2.6. Indirect immunofluorescence

HeLa cells grown on coverslips were fixed with 3.7% formalin for 10 min at room temperature followed by 100% methanol for 10 min at -20°C , washed with PBS, and

permeabilized with 1% TritonX-100 in PBS for 5 min. Cells were incubated with blocking solution (5% FBS in PBS) for 30 min and immunostained. For immunostaining for γ H2AX, cells were probed with mouse anti- γ H2AX antibody for 12 h at 4 °C. Antibody-antigen complexes were detected by incubation for 2 h with Alexa 488-conjugated goat anti-mouse IgG at room temperature. Samples were counterstained with Hoechst 33342.

ELISA—Methods for sample preparation for PAR and ELISA system were as described [17,18], except for using goat HRP-conjugated anti-rabbit antibody (sc-2004, Santa Cruz Biotechnologies) as a secondary antibody.

3. Results

3.1. Mild temperature shift decreased cell proliferation rate and viability, an effect enhanced by a PARP inhibitor

Proliferation of HeLa cells cultured under different temperatures is shown in Fig. 1A, showing an optimal temperature of 37.0 °C. Mild temperature shift at 40.5 °C delayed cell proliferation and reduced viability as compared to 33.5 °C or 37.0 °C (Fig. 1A, B). Addition of 3AB further reduced cell proliferation at 33.5 °C and 37.0 °C, but it did not affect cell viability and cell cycle pattern (Fig. 1A, B). But at 40.5 °C, accumulation of cells at G2/M increased and viability was decreased in the presence of 3AB (Fig. 1B, D, E). The effect of 3AB on cell proliferation phenotypes at 37 °C was in accordance with our previous findings with CHO-K1 cells [19]. HeLa cells cultured at 40.5 °C in the presence of 3AB showed the slender shape (Fig. 1C).

3.2. Mild temperature shift induced phosphorylation of H2AX in HeLa cells and CHOK1 cells and 3AB potentiated this effect

It has been reported that hyperthermia (41.5°C–45.5 °C) induced expression of γ H2AX proteins and foci [6–10]. We investigated whether mild temperature shift also induced γ H2AX signals by immunoblotting and immunofluorescence. The γ H2AX signal was found at 17 kDa by Western blot of a HeLa cell extract, following HeLa cell culture at 40.5 °C for 24 h, and was increased in the presence of 3AB, a PARP inhibitor (Fig. 2A). No changes of PARP1, PARP2 and p53 proteins in HeLa cells were observed in cells cultured at either temperature for 24 h (Fig. 2A). Similar to the results in HeLa cells, the γ H2AX signal was found in CHO-K1 cells cultured at 40.5 °C for 24 h as compared to 37.0 °C, and was increased in the presence of 3AB (Fig. 2B). With immunocytochemistry, γ H2AX foci were also found in HeLa cells cultured at 40.5 °C for 24 h (Fig. 2C), and they were significantly increased in the presence of 7 mM 3 AB (Fig. 2D). Interestingly, the significant increase of γ H2AX foci and its potentiation by 3AB were already observed when HeLa cells were cultured for 12 h at 40.5 °C (Fig. 2E). Thus, mild temperature shift induced expression of γ H2AX proteins and foci and an inhibitor of PARPs, 3AB, significantly potentiated this effect.

3.3. Mild temperature shift induced PAR in HeLa cells and CHO-K1 cells

The level of PAR was determined with recently developed sensitive ELISA system (Fig. 3A). The level of PAR in HeLa cells was $(1.33 \pm 0.15) \times 10^{-5}$ pg/cell at 37.0 °C and $(1.25$

$\pm 0.50) \times 10^{-5}$ pg/cell at 33.5 °C, and did not change significantly in cells cultured for 24 h. However, the level of PAR in cells cultured for 24 h at 40.5 °C was $(4.54 \pm 0.82) \times 10^{-5}$ pg/cell and was significantly higher than that at 37.0 °C ($n = 3$, $P < 0.01$). At all temperatures, addition of 3AB lowered PAR levels (Fig. 3B). In a different set of experiments, the level of PAR in HeLa cells cultured at 37.0 °C for 12 h was $(3.93 \pm 0.13) \times 10^{-6}$ pg/cell, while it was significantly increased to $(8.29 \pm 0.50) \times 10^{-6}$ pg/cell ($n = 2$, $P < 0.01$) at 40.5 °C (Fig. 3D). The level of PAR in CHO-K1 cells cultured at 37.0 °C for 24 h was $(3.37 \pm 0.70) \times 10^{-6}$ pg/cell, while it was significantly increased to $(6.82 \pm 0.70) \times 10^{-6}$ pg/cell at 40.5 °C ($n = 2$, $P < 0.05$) (Fig. 3C). Addition of 3AB significantly reduced PAR levels at either temperature in CHO-K1 cells cultured for 24 h (Fig. 3C).

4. Discussion

We found for the first time that the amount of PAR in HeLa and CHO-K1 cells significantly increased at 40.5 °C for 12 h- and 24 h-cultivation as compared with the normal temperature of 37.0 °C. Since PAR is synthesized by PARPs and degraded by PARG, increased PAR level could be explained by the increase of PARP protein and/or decrease of PARG protein. However as there appeared to be no significant change of the major PAR-metabolizing enzymes, PARP1 and PARG (Fig. 2A), the increase of PAR might well be explained by DNA strand breaks [20]. Furthermore, 3AB, a general inhibitor of PARPs, significantly inhibited the increased PAR level at 40.5 °C (Fig. 3), thereby potentiating heat effects on γ H2AX signals, cell proliferation and viability (Figs. 1 and 2). γ H2AX can also lead to G2 arrest through p53/p21 pathway [21]. These effects of 3AB could be explained by the inhibition of DNA excision repair activity of PARPs, leaving the DNA breaks unrepaired. This is the first indication that PAR level and γ H2AX signals were apparently increased and γ H2AX signals were potentiated by a PARP inhibitor at 40.5 °C, below the temperature known as hyperthermia that is preclinically and clinically used in combination with ionizing radiation or DNA damaging anticancer drugs.

Previously, it was reported that the level of PAR in SV40-transformed Balb/c 3T3 cells did not increase when cultured up to 42 °C or below for 8 h, but it increased to 46 pmoles/ 10^8 cells at 43 °C for 4.5 h [22]. They did not detect DNA strand breaks with velocity sedimentation on alkaline sucrose gradient. But it might be due to the sensitivity of the method to detect DNA strand breaks, since γ H2AX was not found as a marker of DNA double-strand breaks until 1998 [5].

Our results support the notion that severe hyperthermia up to 45.5 °C could induce DNA double-strand breaks [6–8], although its significance on cytotoxicity by hyperthermia is a subject of future studies, since severe hyperthermia above 42 °C could induce denaturation of various proteins [1,9,10], whereas mild hyperthermia up to 41 °C could not.

Considering that 2000–10,000 DNA purine bases turn over every day in each human cell [23], base excision repair mechanism should be very important in preventing DNA single-strand breaks, and our results suggest that the function of DNA base excision repair system could be less efficient at 40.5 °C as compared to 37.0 °C. That the addition of 3AB, which

should inhibit excision repair [24], potentiated the effect of mild temperature shift on cells in increasing γ H2AX signals, support the above notion.

Low levels of DNA single-strand breaks induced by mild temperature shift, when occurring in adjacent locus in DNA, could cause DNA double-strand breaks and cell death, unless the broken ends of DNA are correctly ligated by the DNA repair machinery.

Synthetic lethality by PARP inhibitor to inhibit base excision repair in homologous recombination-deficient BRCA mutant cells was reported [25,26]. Thus, DNA single-strand breaks, even at low levels, occurring at 40.5 °C, which we may experience some time under disease conditions, could become one of the important factors in mutagenesis, carcinogenesis and aging.

We would like to propose that in addition to γ H2AX, PAR level in cell could be a sensitive marker for DNA single- and double-strand breaks. These two molecular markers may provide evidence of physiological changes occurring within cells.

Acknowledgments

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Abbreviations

PAR	poly(ADP-ribose)
PARPs	poly(ADP-ribose) polymerases
PARG	poly(ADP-ribose) glycohydrolase

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Fig. 1.

Mild temperature shift decreased cell proliferation and viability, which was enhanced by a PARP inhibitor. (A) Growth of HeLa cells was determined at indicated temperatures with or without 7 mM 3AB for 24 h, 48 h and 72 h. (B) Cell viability, expressed as a percentage, was calculated as the number of cells that did not stain with trypan blue, divided by the total number of cells. Cells that did not stain with trypan blue were counted on a hemocytometer. (C) Morphology of HeLa cells cultured for 48 h was shown. (D) Flow cytometric analysis of HeLa cells cultured for 24 h was performed. (E) The percentage of cells in each phase of the cell cycle was determined using DNA profile shown in (D). The results are the means $\pm$ standard deviations from two (A, B) and four (D, E) independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. 37 °C.

Fig. 2.

Mild temperature shift induced phosphorylation of H2AX in HeLa cells and CHO-K1 cells, and 3AB potentiated this effect. (A) HeLa cells were incubated at 37.0 °C or 40.5 °C with or without 7 mM 3AB for 24 h. Cells were subjected to Western blotting by using anti-phosphorylated H2AX, PARP1, PARG and p53 antibodies. β-Actin was used as a loading control. n.s.; nonspecific band. (B) CHO-K1 cells were incubated at 37.0 °C or 40.5 °C with or without 7 mM 3AB for 24 h. Cells were subjected to Western blotting by using anti-phosphorylated H2AX antibody. β-Actin was used as a loading control. (C) HeLa cells were grown on coverslips, incubated for 24 h at 37.0 °C or 40.5 °C with or without 7 mM 3AB, fixed, and were examined for γH2AX foci by immunofluorescence. (D) Quantification of γH2AX foci-positive HeLa cells cultured for 24 h was performed. For each point, 200 cells were analyzed. This experiment was repeated thrice and the mean number of foci was plotted. (E) Quantification of γH2AX foci-positive HeLa cells cultured for 12 h was performed. For each point, 200 cells were analyzed. This experiment was repeated thrice and the mean number of foci was plotted. (D, E) ** $p < 0.01$, n.s.; not significant.

Fig. 3.

Mild temperature shift induced PAR in HeLa cells and CHO-K1 cells. (A) A typical dose response curve for purified PAR was generated using 10H antibody [15] as the capturing antibody and rabbit anti-PAR polyclonal antibody [16] as the detection antibody. (B) The level of PAR in HeLa cells at indicated temperature with and without 7 mM 3AB for 24 h. (C) The level of PAR in CHO-K1 cells at indicated temperature with or without 7 mM 3AB cultured for 24 h. (D) The level of PAR in HeLa cells at indicated temperature with or without 7 mM 3AB cultured for 12 h. HeLa cells and CHO-K1 cells were treated with TCA during cell lysis. The results are the means $\pm$ standard deviations from three (B) and two (C, D) independent experiments. (B, C, D) * $P < 0.05$, ** $P < 0.01$. n.s.; not significant.