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Effect of Hyperthermia on Poly(Adenosine Diphosphate-Ribose) Glycohydrolase[1]

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ABSTRACT

The effects of supranormal temperature on the activity of poly(ADP-ribose) glycohydrolase were studied by assaying the enzyme in cell extracts derived from cells subjected to hyperthermia and comparing with extracts that were heated *in vitro*. The enzyme activity was reduced by both hyperthermic treatment of cells and by heating of cell extracts; however greater reductions were observed when intact cells were subjected to hyperthermia. The additional reduction observed when intact cells were heated was reversed when cells were allowed to recover at 37°C following hyperthermia. We postulate that hyperthermia alters poly(ADP-ribose) glycohydrolase activity by two mechanisms, an irreversible thermal denaturation of the enzyme and a reversible metabolic alteration. Changes in poly(ADP-ribose) glycohydrolase activity can account in full for the observed alterations of poly(ADP-ribose) metabolism that occur following hyperthermia.

INTRODUCTION

Poly(ADP-ribose) glycohydrolase is a nuclear enzyme which catalyses the degradation of poly(ADP-ribose) by splitting the ribose-ribose bonds, liberating ADP-ribose residues (1). Following DNA damage, there is a rapid synthesis and turnover of poly(ADP-ribose) in intact cells that is required for processes in the cell nucleus necessary for cellular recovery from DNA damage (2, 3). We have shown previously in C3H10T1/2 and SVT2 cells that poly(ADP-ribose) metabolism is altered by hyperthermia (4–6). Following hyperthermic treatment, we observed a large increase in the accumulation of poly(ADP-ribose) yet the NAD pool, the substrate for poly(ADP-ribose) synthesis, was unaffected (6). Hyperthermia also increased the half-life of poly(ADP-ribose) following DNA damage, which suggested an effect of hyperthermia on poly(ADP-ribose) glycohydrolase activity. Furthermore, a recovery period at 37°C following the hyperthermic treatment resulted in the restoration of near normal polymer turnover rates demonstrating the potential for cellular recovery (6).

Here we report direct measurements of poly(ADP-ribose) glycohydrolase activity in C3H10T1/2 cells following heat shock which show that the glycohydrolase is inactivated by hyperthermia and recovers partially during a 24-h period. The decrease and recovery of glycohydrolase activity accounts for the changes observed in poly(ADP-ribose) metabolism following heat shock.

MATERIALS AND METHODS

Cell Cultures and Experiments with Intact Cells. These experiments were conducted with confluent cultures of C3H10T1/2 cells (clone 8). For studies of poly(ADP-ribose) glycohydrolase activity, cells were grown in 25-cm^2 tissue culture flasks. Each flask contained about 1.5 × 10^6 cells. For measurement of poly(ADP-ribose) and nucleotide pools, cells were grown in 5.5-cm^2 Nunclon tubes (Nunc/Intermed, Denmark) containing approximately 300,000 cells. The cells were maintained as described previously (6). Hyperthermic treatment at various temperatures was performed by adding preheated medium to the cultures and immediately immersing them in a circulating water bath at the appropriate temperature. The precision of temperature control was ±0.1°C. In some experiments the hyperthermic treatment medium was removed and replaced with medium at 37°C and the cells were incubated at 37°C for various recovery periods. At the end of the incubation period the culture medium was removed and the cells were rinsed twice with ice-cold phosphate-buffered saline (0.01 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl).

Permeabilization and Homogenization. After rinsing the flasks with phosphate-buffered saline, 2 ml ice-cold permeabilization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM NaF, 300 mM sucrose, 1 mM 2-mercaptoethanol, and 0.1% NP-40) was added and the cells were left on ice for 10 min. The cells were scraped from the flasks and the flasks were rinsed with 0.5 ml permeabilization buffer. The cells were homogenized with 10 strokes (tight piston) in a Dounce homogenizer and the homogenizer was washed with 0.5 ml permeabilization buffer. The cell extract was then stored at −70°C.

Heating of Cell Extracts. The effect of temperature on the stability of poly(ADP-ribose) glycohydrolase in cell extracts was studied. One hundred μl aliquots of cell extract in Eppendorf tubes were heated at various temperatures for different time periods. After 10 min of equilibration at 37°C the extract was tested for enzyme activity as described below.

Measurement of Poly(ADP-Ribose) Glycohydrolase Activity. Activity of the glycohydrolase was assayed by a method described previously (7). Briefly, 10 μl of reaction buffer (300 mM potassium phosphate buffer, pH 7.5, 60 mM NaF and 60 mM 2-mercaptoethanol) and 10 μl of ^{32}P-labeled poly(ADP-ribose) (60 μM, 20–25 mCi/mmol, prepared as described in Reference 7) was prewarmed to 37°C. The mixture was then incubated with 40 μl of the cell extract for 40 min. A typical assay contained extract derived from 1.5 to 2.0 × 10^3 cells. The reaction was stopped by boiling for 3 min. Twenty-μl aliquots were applied to a thin-layer plate (0.1 mm PEI-F-cellulose; Merck Chemical Division, St. Louis, MO) together with unlabeled carrier of ADP-ribose (Sigma Chemical Co., St. Louis, MO). The plates were developed, first in 100% methanol and then in 0.9 M acetic acid, 0.3 M LiCl.

Measurement of Poly(ADP-Ribose) Accumulation and NAD Pools. The accumulation of poly(ADP-ribose) and the corresponding NAD pools were measured by a radiolabeling technique described previously (6, 8). MNNG[4] (Sigma) at 64 μM was used to induce poly(ADP-ribose) synthesis. MNNG was dissolved in acetone so that the final concentration in the medium was less than 0.5%. MNNG was administered immediately after the hyperthermic treatment and treatment was for 30 min.

RESULTS

Activity of poly(ADP-ribose) glycohydrolase was measured by the release of monomers of ADP-ribose from ^{32}P-labeled poly(ADP-ribose) which was detected by thin-layer chromatography (7). While the glycohydrolase is presumably a nuclear enzyme, cell disruption results in the recovery of a significant fraction (up to one-half) of the activity in the postnuclear

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[4] The abbreviation used is: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

supernatant fraction (data not shown). We have obtained similar values for total activity by assaying unfractionated extracts or separately assaying nuclear and postnuclear fractions. Thus, we assayed unfractionated extracts in the present study. Under the assay conditions utilized, release of product was linear with time until 30% of the substrate was hydrolyzed (data not shown). In the experiments shown here hydrolysis never exceeded 30% during the 40-min assay period. Further, the amount of activity was directly proportional to the amount of cell extract added for all experiments shown.

The effect on poly(ADP-ribose) glycohydrolase activity of heating cell extracts at 45°C was compared to the effects of hyperthermic treatment of intact cells (Table 1). Incubation of extracts from control cells at 37°C for 30 min did not result in a change of activity but incubation at 45°C for 20 and 30 min result in a decrease to 53 and 41% of controls, respectively. Hyperthermic treatment of intact cells at 45°C for 20 and 30 min prior to preparation of extracts resulted in greater decreases in enzyme activity than the heating of cell extracts for the corresponding times. However, when the cells were allowed to recover at 37°C before preparation of cell extracts, enzyme activity partially recovered. It was interesting that, following recovery, the activities were very similar to those of heated extracts.

Fig. 1 shows activity of poly(ADP-ribose) glycohydrolase as a function of temperature. In each case, the cell cultures or extracts were held 30 min at each temperature. Again, values are shown for heated extracts and for cells subjected to hyperthermia in culture and either harvested immediately for assay or allowed to recover for 24 h prior to assay. Over the entire temperature range studied activity was more temperature resistant in heated extracts. Further, again the activities in heated extracts were very similar to those obtained from extracts of cultures made following 24 h of recovery from hyperthermia. The enzyme activity decreased with increasing temperature in all cases in a biphasic pattern with a breakpoint around 42°C. This pattern was similar to that observed previously in studies on biological effects of hyperthermia such as cell survival and development of thermotolerance (9). At temperatures above 42°C the decrease in enzyme activity with increasing temperature suggested thermal inactivation of the enzyme. Utilizing the Arrhenius equation we calculated an inactivation energy of approximately 110 kcal/mol, which is well within the range of

Table 1

Activity of poly(ADP-ribose) glycohydrolase relative to 37°C controls following 20- and 30-min heating times at 45°C. Data are shown for heated cell extracts of control cells and for extracts prepared following hyperthermic treatment of intact cells. For cell extracts heated *in vitro*, the activity was measured immediately after heat treatment. For intact cells, extracts were made immediately (no recovery) and following a recovery period of 24 h. Values are the mean of three independent determinations with standard deviations.

Treatment	Activity of poly(ADP-ribose) glycohydrolase	
	nmol/10^6 cells/min	%
Cell extracts		
37°C	0.24 ± 0.02	100
45°C, 20 min	0.13 ± 0.00	53
45°C, 30 min	0.10 ± 0.03	41
Intact cells		
45°C, 20 min		
No recovery	0.06 ± 0.01	23
24-h recovery	0.13 ± 0.01	53
Intact cells		
45°C, 30 min		
No recovery	0.02 ± 0.01	8
24-h recovery	0.10 ± 0.02	41

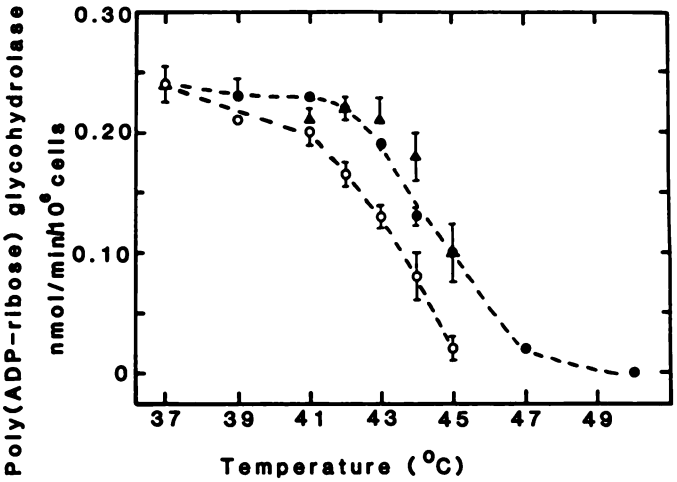

Fig. 1. Activity of poly(ADP-ribose) glycohydrolase as a function of temperature. C3H10T1/2 cells or cell extracts were heated at each temperature for 30 min. ○, intact cells, no recovery; ▲, intact cells, 24-h recovery at 37°C; ●, heating of cell homogenates. Data are the mean of two separate experiments; *error bars*, where the duplicates differed by a % standard deviation of more than 5%.

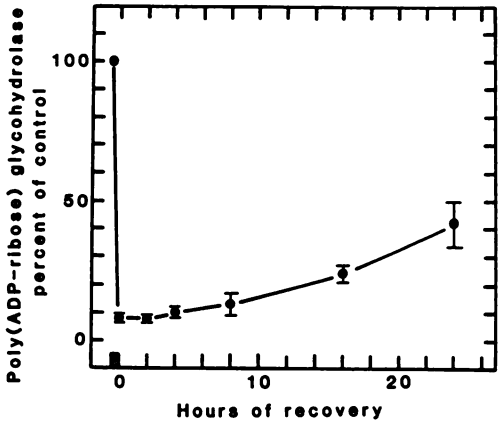

Fig. 2. Activity of poly(ADP-ribose) glycohydrolase in C3H10T1/2 cells as a function of time of recovery at 37°C following 30 min at 45°C. Values are the mean of two separate experiments; *error bars*, SD.

energies known to cause denaturation of several other enzymes and proteins (10).

Fig. 2 shows the glycohydrolase activity as a function of recovery time at 37°C following hyperthermia. The enzyme activity dropped immediately following hyperthermia and remained at less than 10% of control for about 4 h after which the activity gradually returned to approximately 40% of control by 24 h. Similar results were obtained from cell extracts from cultures in which 100 μg/ml cyclohexamide was present during the recovery period at 37°C (results not shown), indicating that the recovery of activity was not due to *de novo* synthesis of the enzyme.

The results of Fig. 1 indicated that poly(ADP-ribose) glycohydrolase was very heat sensitive above 42°C. Above this temperature, the intracellular content of poly(ADP-ribose) increased although no effect on NAD content was observed (6). In order to examine more sensitively the effects of hyperthermia on glycohydrolase activity, pulses of MNNG (64 μM for 30 min) were used as a probe to increase the available substrate for the enzyme. Fig. 3, *top*, shows the cellular content of poly(ADP-ribose) induced by an MNNG pulse following various times of hyperthermic treatment at three different temperatures. The lower panel shows the corresponding amount of NAD consumed during each MNNG pulse. After treating the

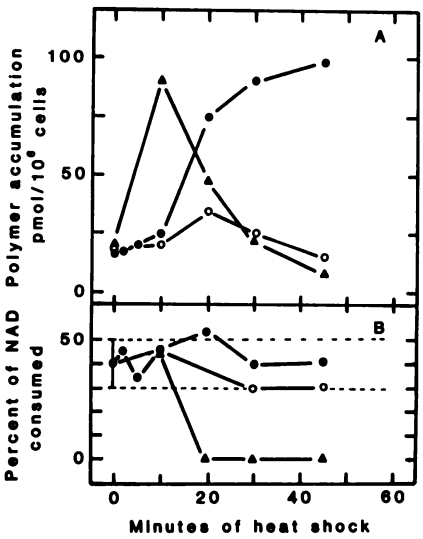

Fig. 3. Effect of hyperthermic treatment on cellular poly(ADP-ribose) content and NAD consumption. C3H10T1/2 cells were subjected to hyperthermia for various times at 42.5°C (○), 45°C (●), and 47°C (▲). Cells were returned at 37°C and treated for 30 min with a pulse of 64 μM MNNG. *Top*, polymer accumulation as a function of time of hyperthermic treatment; *bottom*, NAD consumption *versus* time of hyperthermic treatment. *Dashed lines*, range of NAD consumption following MNNG treatment at 37°C.

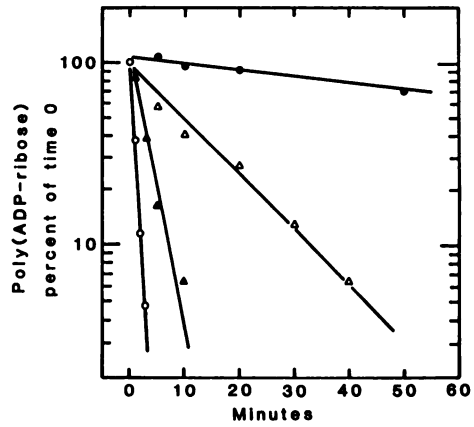

Fig. 4. Analysis of poly(ADP-ribose) turnover as a function of temperature. The cells were given 30 min of hyperthermia and returned to 37°C after which 64 μM MNNG was added. After 30 min (*time 0* in the figure) benzamide was added to the culture medium to a final concentration of 5 mM to inhibit new synthesis. A stable component of approximately 10% of total polymer was subtracted from the data prior to calculation. *Solid lines*, least squares fit of exponential decay curves for the experimental data 37°C (○), 42.5°C (▲), 45°C (△), and 47°C (●).

cells at 42.5°C a small increase in accumulation of polymer was observed compared to MNNG treatment alone. The amount of NAD consumed was unaffected as compared to controls where about 40% of the NAD pool was consumed during the MNNG pulse. Following 45°C treatment a detectable increase in the accumulation of poly(ADP-ribose) was caused by a 10-min treatment. This increase continued up to 45 min where polymer accumulation was four to five times higher than unheated controls. However, no effect on NAD consumption occurred, demonstrating that the primary effect of hyperthermia on poly(ADP-ribose) metabolism was a decreased rate of polymer degradation. Following incubation at 47°C there was a prompt response in polymer accumulation followed by a rapid loss. Initially, there was no detectable change in NAD consumption; however, when the polymer accumulation began to decrease, NAD consumption also dropped to zero, indicating loss of poly(ADP-ribose) polymerase activity. In order to estimate poly(ADP-ribose) glycohydrolase activity in intact cells as a function of temperature, the relative levels of poly(ADP-ribose) were measured as a function of time following inhibition of poly(ADP-ribose) polymerase by addition of 5 mM benzamide to the culture medium (Fig. 4). The calculated half-lives were 0.8 min at 37°C, 2 min at 42.5°C, 10 min at 45°C, and 90 min at 47°C. Using these values we have calculated the relative activity in intact cells as a function of temperature. When these data are normalized relative to the activity in unheated cell extracts, the values obtained were in close agreement with those determined by the direct measurement of enzyme activity as shown in Fig. 1.

DISCUSSION

Poly(ADP-ribose) glycohydrolase appears to be a ubiquitous enzyme (11) and a variety of evidence has led to the conclusion that it is the primary degrading enzyme for poly(ADP-ribose) *in vivo* (12, 13). In this study we have shown that hyperthermia alters the rate of poly(ADP-ribose) degradation in intact cells (Figs. 3 and 4) and the activity of poly(ADP-ribose) glycohydrolase when intact cells are subjected to hyperthermia or when cell extracts are heated (Table 1 and Fig. 1). Further, we postulate that two mechanisms are involved in the decrease of poly(ADP-ribose) glycohydrolase activity following hyperthermia, an irreversible partial thermal denaturation of the enzyme and a reversible metabolic modulation of enzyme activity.

The data of Table 1 and Fig. 1 show that poly(ADP-ribose) glycohydrolase activity is partially thermally denatured by the hyperthermic conditions used in this study. However, thermal denaturation can account for only a portion of the decreased activity observed when intact cells are subjected to hyperthermia and assayed immediately (Table 1 and Fig. 1). In contrast, when cells are allowed to recover prior to preparation of cell extracts, thermal denaturation can account for the failure to recover total enzyme activity. Since the recovery of normal polymer turnover rates occurs in the presence of cyclohexamide (6) and the recovery of activity was observed in cell extracts from cultures in which cyclohexamide was present during recovery, the recovery does not appear to be due to resynthesis of the enzyme. The simplest explanation for these data is that part of the decrease in activity observed in intact cells following hyperthermia and the recovery following return to 37°C is due to a reversible metabolic alteration of enzyme activity in response to hyperthermia. However, we cannot rule out more complex mechanisms. For example, it has been previously shown that hyperthermia can result in sequestion of enzymes in the nucleus (14, 15) and it is possible that a portion of the decrease in the activity in crude extracts may be due to a failure to release the enzyme for assay in extracts from cells subjected to hyperthermia.

The data presented here also indicate that the alteration by hyperthermia of poly(ADP-ribose) metabolism can be accounted for by this alteration of glycohydrolase activity. When the cells were allowed to recover at 37°C following hyperthermic treatment, both polymer accumulation and polymer half-life returned to near normal (6). At this time, the glycohydrolase activity had recovered to about 40% of control level. This argues that there is an excess of poly(ADP-ribose) glycohydrolase in these cells. This conclusion can also be drawn from simple kinetic considerations. The NAD content of these cells is about 1600 pmol/10^6 cells (6) and about 50% of the NAD pool was consumed following a 30-min MNNG treatment (Fig. 4). Assuming negligible resynthesis of NAD, we can

estimate the activity of poly(ADP-ribose) polymerase under these conditions to be about 30 pmol/min/10^6 cells, whereas maximal poly(ADP-ribose) glycohydrolase activity under our assay conditions was approximately 240 pmol/min/10^6 cells. Thus glycohydrolase activity would appear to be in excess. However, a 90% decrease in enzyme activity following hyperthermic treatment brings the activity to a level where it would be below the activity poly(ADP-ribose) polymerase which can explain the observed increase in polymer levels and half-life. After a 24-h recovery, when the glycohydrolase activity had recovered to a level comparable to the polymerase activity or higher, polymer accumulation and half-life returned to normal.

In conclusion, the data in this paper and the preceding paper (6) show that changes in the activity of poly(ADP-ribose) glycohydrolase can fully account for the changes seen in poly(ADP-ribose) metabolism following heat shock and that thermal denaturation of the enzyme is not the only mechanism of regulation. It is not known what the other mechanism(s) might be, but it is possible that these mechanisms could differ with cell type making some cells more sensitive to hyperthermia than others. With regard to the clinical applications of hyperthermia, it should be of interest to investigate the effects of hyperthermia on poly(ADP-ribose) metabolism in various human cell types.

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