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HEAT ENHANCEMENT OF CYTOTOXICITY INDUCED BY OXIDATION PRODUCTS OF SPERMINE IN CHINESE HAMSTER OVARY CELLS

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Abstract—This study investigates the potential of using polyamines as thermosensitizers, in the presence of bovine serum amine oxidase (BSAO), as a new anticancer strategy. The effect of hyperthermia on cytotoxicity of spermine oxidized by purified bovine serum amine oxidase was investigated in Chinese hamster ovary cells. Several different spermine concentrations were employed in the presence of BSAO at 37° and 42°. Cytotoxicity was considerably enhanced at 42°. Heat also increased the individual cytotoxicity of both exogenous H_2O_2 and the exogenous aldehyde acrolein. Thus, both of these species could contribute to the thermal enhancement of cytotoxicity caused by BSAO and spermine. The effect of temperature was especially marked in the presence of exogenous catalase. This cytotoxicity cannot be accounted for by H_2O_2 and was attributed to aldehyde(s). The involvement of aldehyde(s) in cytotoxicity at 42° was also confirmed by the complete inhibition of cytotoxicity with both exogenous aldehyde dehydrogenase and exogenous catalase. A particularly interesting finding, in the presence of exogenous catalase, was that conditions of BSAO and spermine ($\leq 50\ \mu M$) which were non-toxic at 37° became cytotoxic at 42°. This suggests that spermine-derived aldehyde(s), that were non-toxic at 37°, contributed to cytotoxicity at 42° and resemble thermosensitizers. The thermosensitizing activity of aldehyde(s) produced in the BSAO-catalysed oxidation of spermine has potential value for improving the therapeutic effects of hyperthermia and could be considered for future application in cancer therapy. Polyamines are present at elevated levels in tumour cells and have been considered as heat sensitizers. By delivering BSAO into tumour cells, toxic oxidation products of polyamines could be produced *in situ* for selective killing of tumour cells.

Key words: aldehydes; thermosensitizers; polyamines; hyperthermia; amine oxidase; cytotoxicity

Hyperthermia has been used in cancer treatment for many years. It has been shown to be particularly beneficial against tumours when used together with radiation. The combination of hyperthermia with systemic chemotherapy is also likely to be useful because localized heating may provide intensification of drug cytotoxicity within a defined target region. Despite the developments in the methods of heat delivery, there is still a number of technical difficulties with applying hyperthermia in an effective way. New biological techniques need to be found in order to improve the efficacy of hyperthermia treatment [1]. One such way is to use heat together with compounds that become much more toxic at elevated temperatures. Of primary interest here are thermosensitizers. These compounds are characterized by an almost complete lack of cytotoxicity at 37°; however, at elevated temperatures they become potent inducers of cytotoxicity [2].

Naturally occurring polyamines were considered to be heat sensitizers [3–5], although their toxic action is essentially due to their oxidation products [6, 7]. We previously described the cytotoxic effect of exogenous spermine in the presence of purified BSAO§ in CHO cells [8, 9]. BSAO is a copper enzyme (EC 1.4.3.6) involved in the catabolism of polyamines [10], which are oxidized to yield the corresponding aldehydes, hydrogen peroxide and ammonia [11]. Aldehydes and H_2O_2 are known for their cytotoxicity [12] and their ability to inhibit DNA replication in cells [13, 14]. Rapidly dividing cancerous cells contain high concentrations of polyamines [15]. To take advantage of the high polyamine content of tumours for cancer treatment, toxic oxidation products of polyamines could be generated *in situ* by amine oxidases for the selective killing of tumour cells. There is evidence that hyperthermia could be beneficial in this application [9, 16]. It has also been shown that tumour cells are selectively killed by hyperthermia alone [17].

This study examines the thermal enhancement of cytotoxicity induced in CHO cells by spermine oxidized by purified BSAO. We used exogenous catalase to eliminate H_2O_2 from the incubation

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§ Abbreviations: ALDH, aldehyde dehydrogenase; BSAO, bovine serum amine oxidase; CHO, Chinese hamster ovary; FBS, fetal bovine serum; MEM, minimum essential medium; DFMO, α-difluoromethylornithine.

mixture in order to study the effect of other oxidation products of spermine. We confirmed the role of aldehydes in cytotoxicity at 42° by means of exogenous NAD-dependent ALDH (EC 1.2.1.5). Cytotoxicity of each of the oxidation products, H_2O_2 and aldehydes, was enhanced at 42°. The effect of temperature, however, was especially marked in the presence of exogenous catalase and this was attributed to aldehyde(s). With exogenous catalase present, conditions of BSAO and spermine which were non-toxic at 37° became cytotoxic at 42°. The findings here showed that spermine-derived aldehyde(s) behaved as thermosensitizer(s) and could have potential use in cancer treatment together with clinical hyperthermia.

MATERIALS AND METHODS

BSAO purification. BSAO was purified by the method of Mondovi *et al.* [18], with two additional purification steps on Q-Sepharose columns, pH 8.0 and pH 6.0 [19]. The BSAO purification factor was about 1400-fold and a single band was obtained on SDS gel electrophoresis. The enzymatic activity was assayed spectrophotometrically at 25° by monitoring the formation of benzaldehyde at 250 nm (E = 12,500/M/cm) [20]. The specific activity of BSAO used was 0.35 IU/mg, with IU defined as μmol of benzylamine oxidized per min. The protein concentration was determined from the absorbance at 280 nm, assuming an absorption coefficient of 1.74 L/g/cm [21].

ALDH assay. The enzymatic activity of ALDH (EC 1.2.1.5) was assayed spectrophotometrically at 25° by monitoring the formation of NADH at 340 nm [22]. To determine the stability of ALDH, activity of the enzyme was assayed before and after incubation at 42°, in tubes containing 1 mL of PBS–1% BSA–10 mM glucose. There was no loss of enzyme activity after 1 hr of incubation.

Tissue culture. CHO cells (AuxB1) [23] were grown in monolayer in MEM-Alpha (Gibco, Burlington, Ontario, Canada) supplemented with 10% FBS (Gibco) and 1% penicillin (50 U/mL)–streptomycin (50 μg/mL) in an atmosphere of 5% CO_2 in a water-jacketed incubator at 37° as previously described [24]. The cells were grown to near confluence and then incubated for 24 hr with fresh culture medium. Confluent cells were harvested with citrated PBS (0.14 M NaCl, 0.01 M Na_2HPO_4/NaH_2PO_4 and 0.015 M sodium citrate) and then washed by centrifugation (1000 *g*, 2 min) and resuspended in PBS containing 1% BSA and 10 mM glucose for use in cell survival experiments.

Cell survival experiments. Cell survival experiments were carried out in 1 mL of PBS–1% BSA–10 mM glucose. CHO cells (10^5/mL) were incubated in the presence or absence of the following reagents: BSAO (5.7×10^{-3} U/mL), spermine (340 μM), ALDH (0.4 U/mL, from yeast), NAD^+ (1.8 μg/mL) (Boehringer Mannheim, Mannheim, F.R.G.), catalase (300 U/mL, from bovine liver), acrolein (Sigma Chemical Co., Dorset, England) and H_2O_2 (Fisher Scientific Co., Fairlawn, NJ, U.S.A.). Spermine (Fluka Chemie, Buchs, Switzerland) was freshly prepared before each experiment and, if present, was added last. The cells were incubated in tubes in a waterbath at 37° or 42°, washed, diluted in culture medium and plated in tissue culture-coated Petri dishes. After 8 days at 37°, the macroscopic colonies were fixed with 95% ethanol and stained with methylene blue. Percentage cell survival was determined as the mean number of colonies obtained relative to the mean number of colonies obtained in the control [24].

RESULTS

BSAO (EC 1.4.3.6) oxidatively deaminates polyamines which contain primary amine groups. The reaction involves amine, dioxygen and water as substrates. The products are H_2O_2, the corresponding aldehyde and ammonia. We examined the effect of hyperthermia on the cytotoxicity of the toxic species produced during the enzymatic oxidation of spermine, H_2O_2 and aldehyde. There is still controversy concerning the chemical nature of the aldehyde(s) formed in this reaction. It was shown that both monoaldehyde and dialdehyde are produced [11]. However, the dialdehyde is unstable and may undergo spontaneous β-elimination to form acrolein [25]. We are currently attempting to clarify which aldehydes are produced in our experimental conditions by HPLC analysis.

The effect of 42° hyperthermia on cytotoxicity induced by BSAO during 60 min as a function of spermine concentration is shown in Fig. 1A. Figure 1B represents the corresponding data obtained in the presence of exogenous catalase. In order to evaluate more clearly the differences in cytotoxicity between 37° and 42°, the survival curves at 42° were normalized to the level of cell killing caused by heat alone (Fig. 1A, B, dashed line). Hyperthermia enhanced cytotoxicity under both these conditions. Without exogenous catalase, cytotoxicity occurred at concentrations of spermine above 5 μM at both 37° and 42° (Fig. 1A). In the presence of exogenous catalase where H_2O_2, one of the toxic products, was eliminated, cytotoxicity occurred at concentrations of spermine above 50 μM at 37° (Fig. 1B). The initial shoulder on the survival curve at 42° was smaller than that at 37°. Consequently, 3 log of cell killing occurred at 42° for spermine concentrations which were non-toxic at 37°. Heat alone, in the absence of BSAO and spermine, caused a decrease in percentage cell survival (Fig. 1A, vertical axis). This contribution of heat alone to cytotoxicity did not account for the magnitude of enhancement of cytotoxicity caused by heat in the presence of spermine and BSAO. The slopes of the survival curves, both with and without catalase, were much steeper at 42° than at 37° (Fig. 1A, B). Therefore thermal enhancement increased with spermine concentration.

Heat enhancement of cytotoxicity induced by BSAO, as a function of time, was demonstrated for two different spermine concentrations (Fig. 2). Without exogenous catalase (Fig. 2A), both spermine concentrations caused similar cytotoxicity at 42°, whereas at 37° a 60 μM spermine concentration caused less cytotoxicity than 340 μM spermine. Cytotoxicity that was attributed to all of the oxidation products of spermine, H_2O_2 and aldehyde(s), was

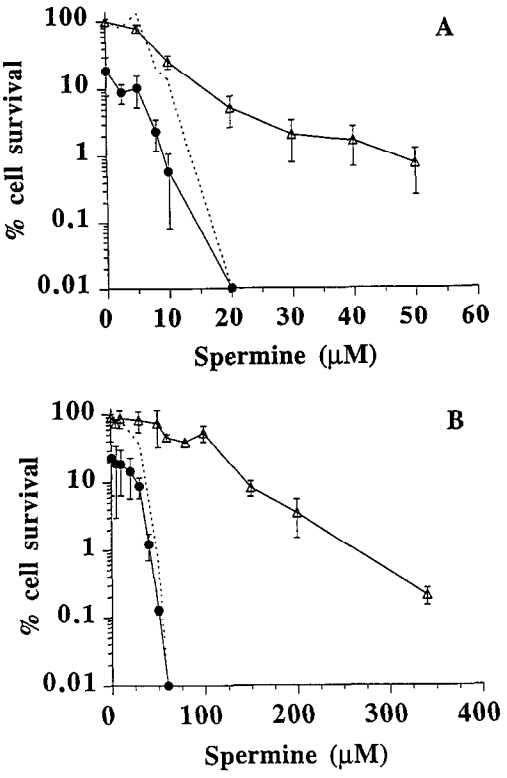

Fig. 1. Temperature dependence for cytotoxicity of BSAO as a function of spermine concentration. CHO cells (10^5/mL) were incubated with BSAO (5.7×10^{-3} U/mL) and spermine for 60 min at 37° (△) and 42° (●) in 1 mL of PBS–1% BSA–10 mM glucose. (A) No addition of catalase; (B) addition of catalase (300 U/mL). The differences in spermine concentration scale between A and B should be noted. Means and SD are shown for 2–6 determinations from two experiments. The survival curves at 42° were normalized to the killing caused by heat alone (dashed line).

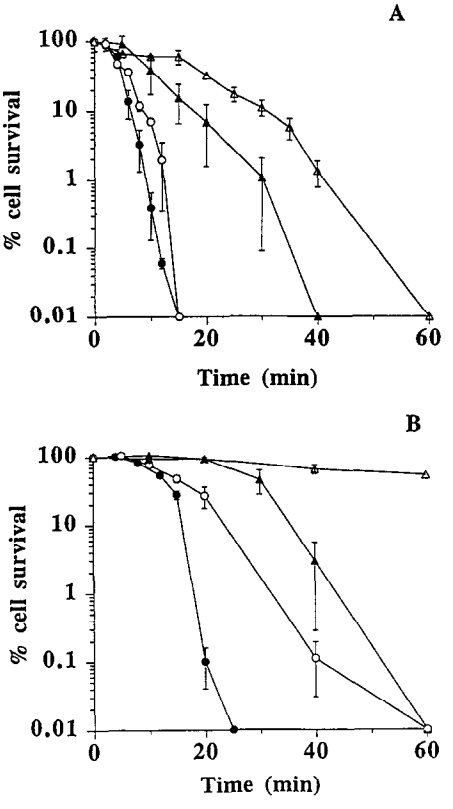

Fig. 2. Time course for temperature dependence of cytotoxicity of BSAO and spermine. CHO cells (10^5/mL) in 1 mL of PBS–1% BSA–10 mM glucose were incubated with BSAO (5.7×10^{-3} U/mL) and either 60 μM spermine at 37° (△) and 42° (○) or 340 μM spermine at 37° (▲) and 42° (●). (A) No addition of catalase; (B) addition of catalase (300 U/mL). Means and SD are shown for 2–19 determinations from six experiments.

therefore enhanced at 42°. However, the overall difference between cell survival at 37° and 42° was more pronounced at the lower spermine concentration.

In the presence of exogenous catalase, heat also caused enhancement of cytotoxicity at both spermine concentrations (Fig. 2B). Cytotoxicity, which was attributed to oxidation products other than H_2O_2, was therefore also markedly enhanced at 42°. During 60 min incubation in the presence of exogenous catalase, 60 μM spermine and BSAO were not toxic to cells at 37°, but caused considerable cell killing at 42°. With 340 μM spermine and BSAO, a similar difference in percentage cell survival between the two temperatures was observed after only 20 min.

To demonstrate the effect of 42° hyperthermia on the cytotoxicity of the toxic products individually, we examined the cytotoxic effect of exogenous H_2O_2 (Fig. 3A) and exogenous acrolein (Fig. 3B) at 37° and 42°. Acrolein served as an example of aldehyde under our experimental conditions. Cytotoxicity occurred for concentrations of H_2O_2 above 10 μM and for concentrations of acrolein above 40 μM. In order to illustrate more clearly the differences in cytotoxicity of H_2O_2 and acrolein at 37° and 42°, the cell survival curves at 42° were normalized to the level of cell killing caused by heat alone (Fig. 3A, B, dashed line). Hyperthermia enhanced cytotoxicity caused by each compound and this effect became more pronounced with concentration.

To clarify further the nature of the oxidation products responsible for the enhancement by heat, we examined the effect of exogenous NAD-dependent ALDH on cytotoxicity induced by 340 μM spermine and BSAO at 42° (Fig. 4). ALDH oxidizes aldehydes including acrolein to the corresponding acids. It can eliminate the toxic aldehyde intermediates formed during early stages of spermine oxidation and prevent possible formation of acrolein. Under conditions where catalase protected against cytotoxicity (first 15 min of the reaction), ALDH alone did not protect cells against cytotoxicity but did give some protection at later stages of the reaction. When used together with catalase, it also provided considerable protection at later stages of the reaction, but there was still a gradual decrease in percentage cell survival. This could not be

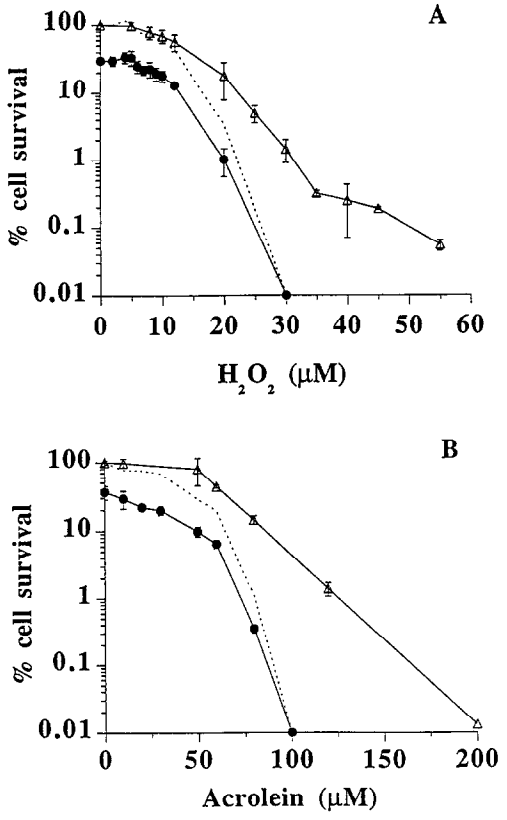

Fig. 3. Temperature dependence for cytotoxicity of (A) H_2O_2, and (B) acrolein. CHO cells (10^5/mL) were incubated with H_2O_2 or acrolein for 1 hr in 1 mL of PBS–1% BSA–10 mM glucose at 37° (△) and 42° (●). Means and SD are shown for 2–10 determinations from four experiments. The survival curves at 42° were normalized to the killing caused by heat alone (dashed line).

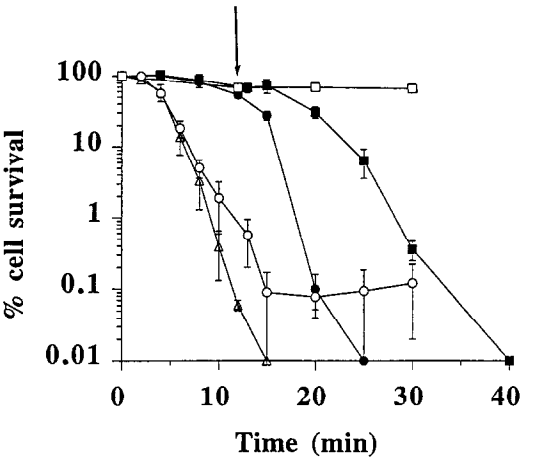

Fig. 4. The effect of ALDH and catalase on cytotoxicity of BSAO and spermine at 42°. CHO cells (10^5/mL) in 1 mL of PBS–1% BSA–10 mM glucose were incubated with BSAO (5.7×10^{-3} U/mL) and spermine (340 μM) (△), or with addition of catalase (300 U/mL) (●), ALDH (0.4 U/mL) (○), ALDH and catalase (■), or ALDH and catalase with further addition of 0.4 U/mL of ALDH at 12 min (indicated by arrow) (□). Means and SD are shown for 2–6 determinations from two experiments.

accounted for by heat inactivation of ALDH at 42°. The addition of another 300 U/mL of catalase did not prevent this cytotoxicity (data not shown). However, the further addition of 0.4 U/mL of ALDH entirely prevented cytotoxicity. Therefore, the additional amount of enzyme was necessary to remove high amounts of aldehydes that had accumulated during oxidation of spermine after longer times. These results show that aldehydes produced during enzymatic oxidation of spermine at 42° were responsible for cytotoxicity that cannot be accounted for by H_2O_2. Control points using heat-inactivated catalase or with inactivation of both enzymes (60 min at 100°) showed no inhibition of cytotoxicity in the presence of BSAO and spermine at 20 min. This indicates that the enzymatic properties of catalase and ALDH are responsible for their protective effects.

DISCUSSION

The diversity between normal and cancerous cells is an important consideration in the development of new treatments against tumours. One such difference is concerned with polyamine content and metabolism. Polyamine concentrations are high in growing tissues such as tumours. Compounds that inhibit the enzymes involved in the biosynthesis of polyamines can decrease their concentrations in cells, resulting in a cytostatic effect. One such compound, DFMO, has been extensively studied for its antiproliferative activity against tumours, either alone or in combination with other modalities [26–29]. We have been exploring another approach that takes advantage of the high polyamine content of tumour cells. This could be developed as a new strategy for cancer treatment. By delivering purified BSAO into tumour cells, toxic oxidation products of polyamines could be produced *in situ* for the selective killing of tumour cells [9, 30].

The present study focuses on the effect of heat on cytotoxicity induced in CHO cells by purified BSAO and exogenous spermine. We clarified the contribution of H_2O_2 and aldehyde(s) to cytotoxicity at both 37° and 42°, and demonstrated a marked effect of heat on cytotoxicity attributed to aldehyde(s).

The effect of elevated temperatures on the cytotoxicity of BSAO was more pronounced at low spermine concentrations. At concentrations of spermine below 60 μM, exogenous catalase completely protected the cells for 60 min at 37°. This shows that H_2O_2 was the sole contributor to cytotoxicity under these conditions. Aldehyde(s) present were probably not at sufficient levels to contribute to cytotoxicity. At higher spermine concentrations (340 μM), aldehyde(s) accumulated more rapidly and contributed to cytotoxicity after only 20 min at 37°. Exogenous acrolein also became toxic to cells at higher concentrations compared to exogenous H_2O_2. However, the magnitude of

thermal enhancement of cytotoxicity caused by exogenous H_2O_2 alone did not appear sufficient to explain the thermal enhancement of cytotoxicity caused by low concentrations of spermine and BSAO. This suggests that under these conditions there were other contributors to cytotoxicity apart from H_2O_2 at 42°. We propose that aldehyde(s), which were non-toxic at 37°, contributed to cytotoxicity at 42°. In this respect, they behaved similarly to other thermosensitizers. However, they appeared to be even more toxic at elevated temperatures than cysteamine [31] or the aminothiol WR-1065 [32]. In the presence of exogenous catalase, BSAO and 60 μM spermine were cytotoxic at the higher temperature only and caused 4 log of cell killing in 60 min. This effect could not be accounted for by H_2O_2 and was attributed to aldehyde(s). These findings indicate a marked enhancement by heat of cytotoxicity attributed to aldehydes generated by BSAO and spermine. The involvement of aldehyde(s) in cytotoxicity was also confirmed by total inhibition of cytotoxicity by ALDH and catalase.

The mechanisms underlying thermal enhancement of cytotoxicity induced by oxidation products of spermine are not yet understood. The explanation that the elevated temperature increases the concentration of spermine oxidation products, as a consequence of increased enzymatic activity of BSAO, is unlikely to account for the thermal enhancement. The activity of BSAO was scarcely affected by temperatures between 35° and 45°. A Q_{10} value of 1.17 was reported for BSAO (8, 9, 33), whereas enzymes usually have a higher Q_{10} of approx. 2. The present study shows that heat enhanced the cytotoxicity of both H_2O_2 and aldehyde(s). The mechanisms involved are probably complex since heat seems to affect the behaviour of the oxidation products. Several target sites may be involved, such as DNA or cell membranes. Heat caused a very marked enhancement of the cytotoxicity of aldehydes. This could be attributed to the interaction of the aldehydes with cell membranes. Polyamine-derived aldehydes have a structure of long polycations that bind to cell membranes [34]. Cell membranes are also important sites of cell damage by heat [35–37]. The interaction of benzaldehyde, a product of benzylamine oxidation by BSAO, with membrane proteins under hyperthermic conditions was also reported [38]. Benzaldehyde has antitumour activity in humans [39].

The idea of using BSAO in cancer treatment has received little attention but in view of these results merits further investigation. The enzyme could be used to further advantage in combination with hyperthermia. We have incorporated BSAO into liposomal vesicles [40] and have shown that BSAO could be bound and internalized by cultured cells [41]. Thus, endogenous polyamines present in tumour cells could be targeted and oxidized by the enzyme. These results suggest that BSAO may prove to be a powerful tool with biomedical applications in cancer treatment, especially together with clinical hyperthermia.

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