Polyamine regulation of heat-shock-induced spermidine N^1 -acetyltransferase activity

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The enzyme spermidine/spermine N^1 -acetyltransferase (N^1 -SAT) is rapidly induced by heat shock in CHO and A549 cells, with activity declining by 24 h. Depletion of intracellular polyamines by α -difluoromethylornithine, an inhibitor of ornithine decarboxylase, blocks this induction. Re-addition of putrescine to these cultures restores the response to heat shock, with a concomitant increase in intracellular N^1 -acetylspermidine. Diaminopropane is more than twice as effective as the naturally occurring diamine putrescine, suggesting that the propylamine moiety of spermidine is involved in the regulation of N^1 -SAT induction. Inhibitor studies indicate transcriptional activation and that the enzyme has an apparent half-life of 30–60 min. A second heat shock rapidly inhibits induced N^1 -SAT activity, which decays with a half-life of 2–3 min. Despite its induction by heat, N^1 -SAT is not a stable enzyme, suggesting that the activity observed is not due to a modification of an existing peptide, but is due to a transcriptional event, which may justify the inclusion of this enzyme in the family of heat-shock proteins.

INTRODUCTION

The metabolism of putrescine, spermidine and spermine has been the subject of considerable scrutiny in the literature. Although widely accepted to be involved in processes as diverse as cellular proliferation and differentiation (for reviews, see Pegg, 1986; Pegg & McCann, 1988), oncogene expression (Celano *et al.*, 1988) and oestrogen-receptor stabilization (Thomas & Kiang, 1987), the precise role(s) played by these molecules remains unclear. The synthesis of the irreversible enzyme-activated inhibitor α -difluoromethylornithine (DFMO) has provided a means for effective polyamine depletion with low toxicity in many systems (Metcalf *et al.*, 1978). The target of this ornithine analogue is the rate-limiting enzyme of the polyamine-biosynthetic pathway, ornithine decarboxylase (ODC; EC 4.1.1.17). Inhibition of ODC results in the rapid depletion of its product, putrescine, and subsequently the higher polyamine spermidine, which is synthesized from putrescine by the addition of a propylamine moiety derived from the decarboxylation of *S*-adenosylmethionine by *S*-adenosylmethionine decarboxylase (EC 4.1.1.50). Depletion of polyamines by DFMO results in cytostasis in many cell lines, which has generated interest in the compound as a potential anti-neoplastic drug. Although trials with DFMO in human cancer patients have been inconclusive, the drug has been found to be effective against protozoan diseases in man, such as trypanosomiasis, malaria and *Pneumocystis carinii* (for reviews see Pegg, 1988; Pegg & McCann, 1988; Sjoerdsma *et al.*, 1984).

Catabolism of the polyamines occurs intracellularly by two steps. Spermidine is initially acetylated in the N^1 position by spermidine/spermine N^1 -acetyltransferase (N^1 -SAT). The acetylated polyamine is then oxidized by a flavin-dependent enzyme, polyamine oxidase (PAO), with the formation of the parent polyamine (putrescine or spermidine) and the oxidation products H_2O_2 and acetamidopropanal (Pegg, 1986). N^1 -SAT is

a short-lived protein that is rapidly induced by a wide variety of stresses and trophic agents (Matsui *et al.*, 1981; Danzin *et al.*, 1982; Della Ragione & Pegg, 1983; Persson & Pegg, 1984; Stefanelli *et al.*, 1986; Erwin & Pegg, 1986; Halline *et al.*, 1989). This burst of oxidative polyamine catabolism may be part of a general cellular response to stressors, although there may be species-specific differences in the regulation and expression of PAO (Hirvonen *et al.*, 1989).

Interest has centred in recent years on the potential of hyperthermia as a therapeutic adjunct to radiation and chemotherapy in the treatment of cancer (for review, see Oleson *et al.*, 1988). Our laboratory has reported in the past that depletion of intracellular polyamines by DFMO greatly sensitizes monolayer cultures to the cytotoxic effects of heat shock (Fuller & Gerner, 1987). In the present paper we show that N^1 -SAT is rapidly induced by the exposure of Chinese-hamster and human tumour cells to elevated temperatures and that the inhibition of polyamine biosynthesis by DFMO blocks this induction. In addition, we have investigated the differences in the regulation of N^1 -SAT between the human and rodent cell lines.

MATERIALS AND METHODS

Chemicals

MDL 71.728A (DFMO) was given by Merrell–Dow Research Institute, Cincinnati, OH, U.S.A. Actinomycin D, aminoguanidine hemisulphonate, 1,5-diaminopentane, 1,8-diaminooctane, N^1 -acetylspermidine and spermidine were obtained from Sigma Chemical Corp., St. Louis, MO, U.S.A., as were h.p.l.c.-grade *o*-phthalaldehyde, sodium acetate, octanesulphonic acid, mercaptoethanol and Brij-35. Other h.p.l.c. solvents were acetonitrile from Baxter Healthcare Corp., Muskegon, MI, U.S.A., and acetic acid from Mallinckrodt, Paris, KY, U.S.A. Cycloheximide was from Calbiochem, La Jolla, CA, U.S.A. New

Abbreviations used: N^1 -SAT, spermidine N^1 -acetyltransferase; ODC, ornithine decarboxylase; DFMO, α -difluoromethylornithine; CHO, Chinese-hamster ovary; PAO, polyamine oxidase.

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England Nuclear (Boston, MA, U.S.A.) supplied [14 C]acetyl-CoA. 1,3-Diaminopropane was purchased from Pfaltz and Bauer, Stamford, CT, U.S.A., 1,4-diaminobutane (putrescine) from Alfa Products, Danvers, MA, U.S.A., 1,6-diaminohexane from ICN Pharmaceuticals, Plainsview, NY, U.S.A., and 1,7-diaminoheptane from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Cell culture

Chinese-hamster ovary (CHO) and human lung carcinoma (A549) cells were maintained as monolayer cultures in McCoy's modified 5A medium supplemented with 10% (v/v) fetal-bovine serum plus 1% penicillin/streptomycin solution (10000 units of penicillin G/ml and 10000 μ g of streptomycin sulphate/ml, from Grand Island Biological Co., Grand Island, NY, U.S.A.). Cultures were incubated at 37 °C in CO₂/air (1:19). In some experiments, exogenous polyamines were added to the culture medium. We have previously shown that treatment of CHO cells with putrescine concentrations up to 1 mM for 8 h (Gerner *et al.*, 1980), or a series of diamines for 24 h (Fuller & Gerner, 1987), is not toxic when the cultures are incubated at 37 °C. This lack of toxicity of exogenous diamines at concentrations up to 1 mM was confirmed for A549 cells as part of the present study (results not shown).

Hyperthermia treatments

Cells were exposed to elevated temperatures by sealing 100 mm-diam. dishes or T-75 flasks with Parafilm and immersing them in water at 43 or 45 °C for times indicated in the text. Temperature was controlled to ± 0.1 °C by a recirculating water bath.

Polyamine analysis

Cells to be analysed for polyamine content were released by trypsin from the monolayers and counted before sonication into 0.1 M-HCl (3×10^6 cells/ml). This sonicate preparation was made 0.2 M with HClO₄, and the clarified supernatant was assayed by reverse-phase h.p.l.c. (Seiler & Knodgen, 1980), with 1,7-diaminoheptane as an internal standard. Protein was determined by the method of Bradford (1976).

N¹-SAT assay

Cells were trypsin-treated, washed three times in ice-cold phosphate-buffered saline and counted before being frozen in lysis buffer (2×10^7 cells/ml) at -80 °C. On thawing, 50 μ l samples of supernatant were taken for assay. Activity was estimated by the transfer of 14 C from [14 C]acetyl-CoA to N¹-[14 C]acetyl-spermidine at 30 °C as previously described (Persson & Pegg, 1984); 1 unit of enzyme activity is defined as 1 pmol of N¹-acetylspermidine formed/min per 10^6 cells.

RESULTS

Effect of heat shock on N¹-SAT activity in CHO cells

Exposure of exponentially growing CHO cells to heat shock results in rapid induction of N¹-SAT activity (Fig. 1), which increases to a maximum between 8 and 24 h after return of the cultures to 37 °C. Activity is declining, but still elevated, at 24 h. Exposure to the polyamine-biosynthesis inhibitor DFMO inhibits this response. The low activities seen in control and DFMO-treated cultures are not inactivated by a polyclonal antibody against rat N¹-SAT (Persson & Pegg, 1984), whereas heat-shock-induced activities are (results not shown).

Fig. 1. Effect of polyamine depletion on the induction of N¹-SAT by heat shock

CHO cells were plated out into control medium or into medium supplemented with 5 mM-DFMO; 36 h later, dishes were sealed with Parafilm and heat-shocked for 90 min at 43 °C. Cells were harvested for N¹-SAT assay at times indicated. ●, Control cultures; ○, DFMO-treated cultures.

Fig. 2. Restoration of N¹-SAT induction in DFMO-treated cells by diamine homologues

CHO and A549 cells were depleted by growth for 36 h in medium supplemented by 5 mM-DFMO. Then 16 h before heat shock, cultures were made 1 mM with diamine homologues (■, control; ■, 1,3-diamine; ■, 1,4-diamine; ■, 1,5-diamine; □, 1,6-diamine; ■, 1,8-diamine); 1 mM-aminoguanidine was also added at this time to inhibit copper-dependent amine oxidases in the fetal-bovine serum. Heat shock was 43 °C for 90 min for the CHO cells as described in Fig. 1, and was 45 °C for 15 min for the A549 cells, which are isoeffect doses in terms of clonogenic survival. Cultures were harvested for N¹-SAT assay 6 h after these heat treatments.

Effect of exogenous diamines on the restoration of N¹-SAT inducibility in CHO and A549 cells

Fig. 2 shows that treatment of polyamine-depleted cultures with members of a homologous series of diamines 16 h before

Fig. 3. Effect of the concentration of exogenous putrescine on N^1 -SAT activity and intracellular polyamine content

A549 cells were subcultured in the absence and presence of 5 mM-DFMO for 36 h. At 16 h before heat shock, putrescine (1–1000 μ M) was added to the culture medium in the presence of 1 mM-aminoguanidine as described above. The heat shock in these experiments was 45 °C for 15 min, as in Fig. 2 for these cells. At 6 h after heat shock, the cells were trypsin-treated and harvested for N^1 -SAT assay, h.p.l.c. analysis and protein determination. ●, 37 °C; ○, heat shock; C, controls.

heat shock has differing effects on N^1 -SAT activity. The results demonstrate that in both lines putrescine (1,4-diaminobutane) will restore the inducibility of N^1 -SAT to control (100%) values. Higher homologues from cadaverine (1,5-diaminopentane) to 1,8-diamino-octane are not effective. However, 1,3-diaminopropane elicits a response that is 2–2.5 times that of the naturally occurring diamine putrescine.

Effect of exogenous putrescine concentration on N^1 -SAT activity and intracellular polyamine levels in A549 cells

In order to determine whether the suppressive effect of DFMO on N^1 -SAT activity was due to the depletion of a specific polyamine (putrescine or spermidine), exogenous putrescine was added to DFMO-treated A549 cells, and both endogenous polyamine levels and N^1 -SAT activities were determined. Putrescine was rapidly taken up to supranormal levels in these cells (Fig. 3c), with no detectable difference between cells that were heat-shocked or those maintained continuously at 37 °C. N^1 -SAT activity (Fig. 3a) and N^1 -acetylspermidine levels (Fig. 3b) were restored to maximal values by 1 mM exogenous putrescine, as was the endogenous spermidine pool (Fig. 3d). Endogenous

putrescine levels continued to increase with exogenous concentrations up to 1 mM-putrescine. Thus the restoration of N^1 -SAT inducibility correlated with the restoration of the endogenous spermidine pool, but not the putrescine pool. The accumulation of N^1 -acetylspermidine in heat-shocked cells is due to the very low level of expression of PAO in A549 cells (S. W. Carper, D. J. M. Fuller, L. Clay, J. R. Chen, P. M. Harari & E. W. Gerner, unpublished work). Fig. 3(e) shows that spermine content (normally somewhat elevated in DFMO-treated cells, owing to increased *S*-adenosylmethionine decarboxylase activity; results not shown) gradually declines as a function of exogenous putrescine concentration in both control and heat-shocked cultures.

Effect of cycloheximide and second heat shock on N^1 -SAT activity

The addition of 20 μ g of cycloheximide/ml to CHO-cell cultures, a concentration sufficient to suppress protein synthesis to less than 5% of control values within 5 min (results not shown), at times after heat shock results in decay of N^1 -SAT activity, as shown in Fig. 4. The apparent half-time of decay of

Fig. 4. Apparent half-life of N^1 -SAT activity after inhibition of protein synthesis and after a second heat shock

Exponential-phase CHO cultures were heat-shocked at 45 °C for 15 min to induce N^1 -SAT initially. At times (○, 4 h; □, 8 h; △, 24 h) after N^1 -SAT induction, some cultures (white symbols) were made 20 µg/ml with cycloheximide. These cells were rapidly harvested for N^1 -SAT determination at 30 min intervals after protein-synthesis inhibition. To test the stability of the enzyme in response to elevated temperature *in vivo*, other cultures (black symbols) were subjected to a second 45 °C heat shock beginning 6 h after N^1 -SAT induction, and the cells (●, CHO; ■, A549) were harvested for assay after treatment times up to 20 min as shown.

N^1 -SAT activity is between 30 and 60 min. However, in both CHO and A549 cells that have had an inducing heat shock 6 h before the start of a second one, enzyme activity decreases very rapidly, with an apparent half-time of 2–3 min, demonstrating the acute sensitivity of the protein, once induced, to the effects of elevated temperature.

Inhibition of N^1 -SAT induction by actinomycin D

Fig. 5 shows that heat-shock-induced N^1 -SAT activity was inhibited by actinomycin D, an inhibitor of RNA synthesis, in both CHO and A549 cells. In these experiments, 4 µg of actinomycin D/ml was added to exponential-phase cultures 30 min before heat shock, since previous work has shown that incorporation of radioactive precursors into RNA is inhibited under these conditions (results not shown). N^1 -SAT activity was determined 6 h after heat shock (45 °C for 15 min). For both human and rodent cells, this antiserum-sensitive heat-inducible enzyme activity was suppressed by actinomycin D levels above 1 µg/ml.

Fig. 5. Effect of actinomycin D on the induction of N^1 -SAT by heat shock in CHO and A549 cells

Actinomycin D was added to cultures 30 min before heat shock to final concentrations of 0.1–100 µg/ml. At 6 h after hyperthermia (15 min at 45 °C), cells were harvested for N^1 -SAT assay and polyamine analysis by h.p.l.c. (●, CHO; △, A549).

DISCUSSION

The enzyme N^1 -SAT has been shown to be inducible in a variety of systems. Specific polyamines are themselves potent inducers of N^1 -SAT in polyamine-depleted cells (Erwin & Pegg, 1986). These authors have suggested that polyamines are localized into subcellular compartments and that the disruption of these compartments by stressors, such as carbon tetrachloride, ethanol or, as reported here, heat, is the initiator for the induction of N^1 -SAT. The rapid influx of exogenous polyamines into the cytoplasm of depleted cells, presumably as a prelude to compartmentalization, may accomplish the same 'triggering' event. The data presented support the contention not only that the polyamines are involved in the induction of N^1 -SAT but that polyamines, specifically spermidine, regulate induction by other agents such as heat and the copper chelator diethyldithiocarbamate (Harari *et al.*, 1989).

Depletion of putrescine and spermidine by DFMO blocks the induction of N^1 -SAT by heat and diethyldithiocarbamate. The hypothesis that the spermidine molecule is regulatory in the process is supported by the observation that 1,3-diaminopropane is 2–3 times more effective than putrescine at restoring the inducibility of N^1 -SAT. Some restoration of induction is observed with 1,5-diaminopentane (cadaverine), but none with the longer-chain homologues. This suggests that there is a binding site that is sensitive to the internal imino group of spermidine. Previously reported data (Fuller & Gerner, 1987) have demonstrated by clonogenic assay that these compounds display minimal toxicity at this concentration. Since 1,3-diaminopropane and cadaverine are not metabolized to detectable levels of spermidine analogues in these cells, the data suggest that it is the propylamine moiety of spermidine that recognizes the putative site(s) involved in the induction process. That spermine is uninvolved is inferred from the observation that N^1 -SAT is not inducible in cells that have been depleted of spermidine and putrescine, but exhibit control or higher spermine levels despite DFMO treatment. Even though two propylamine moieties are present on the molecule, suggesting that it should induce N^1 -SAT, it is apparently not available for this purpose, perhaps because of sequestration by compartmentalization or ionic binding. Our contention, therefore, is that

putrescine must be 'activated' by the action of spermidine synthase to become regulating. We have shown in the past that polyamine depletion by DFMO, although not cytotoxic in CHO cells, renders them two orders of magnitude more sensitive to the effects of elevated temperature (Fuller & Gerner, 1987). Here we report that DFMO inhibits the induction of *N*¹-SAT, an enzyme that appears to be part of a generalized cellular response to stress. Although these phenomena are not necessarily causally related, polyamines may be intrinsic to an ordered reaction of cells to potentially damaging stimuli. There are clearly cell-line-related differences in polyamine catabolism. Hirvonen *et al.* (1989) analysed a number of mouse and human cell lines and determined that the human cells did not express PAO. It was found (S. W. Carper, D. J. M. Fuller, L. Clay, J. R. Chen, P. M. Harari & E. W. Gerner, unpublished work) that PAO was constitutively expressed in rodent cells, but was expressed in a variety of human normal and tumour cells at very low levels (4 % of activities expressed in CHO cells grown under identical conditions). In contrast with PAO, *N*¹-SAT appears to be commonly regulated in human and rodent cells. In both, (a) *N*¹-SAT is stress-inducible, (b) the induction is polyamine-dependent, (c) *N*¹-SAT is heat-sensitive, and (d) the induction is dependent on RNA transcription. *N*¹-SAT, the first enzyme involved in the stress-induced catabolism of the polyamines, is probably rate-limiting for this process in rodent cells. Although *N*¹-SAT is regulated in a similar manner in human cells, the regulation of polyamine catabolism is complicated by the very low level of PAO expression. The significance of this metabolic difference between rodent and human cells is not yet understood.

The effect of actinomycin D on the induction of *N*¹-SAT suggests that transcriptional activation is involved. Inhibition of protein synthesis by cycloheximide shows that *N*¹-SAT is a rapidly turned-over molecule, with a half-life similar to that of ODC (30–60 min), a value in the range of that observed by Persson & Pegg (1984). In addition, a second heat treatment 6 h after the first, at a time when *N*¹-SAT levels are increasing and protein synthesis is recovering, rapidly inactivates the induced enzyme activity, indicating that the enzyme is highly sensitive to the effects of heat, with activity decreasing with a half-time of 2–3 min. This correlates well with the results of Persson & Pegg (1984), showing that purified *N*¹-SAT was a heat-labile enzyme. Since the enzyme activity is suppressed by transcription inhibitors, these findings suggest that heat shock induces *N*¹-SAT activity by a polyamine-dependent transcriptional mechanism, rather than by a polyamine-dependent post-translational modification of the enzyme peptide. Thus *N*¹-SAT may be a

member of a class of proteins known collectively as heat-shock proteins, which share the properties of being transcriptionally activated by heat and a number of other stressors (Carper *et al.*, 1987). Further work needs to be done to determine whether the *N*¹-SAT gene sequence contains a heat-inducible regulatory element, as has been found to be the case for other heat-inducible genes (Pelham, 1982).

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