Stress induction of the spermidine/spermine *N*¹-acetyltransferase by a post-transcriptional mechanism in mammalian cells

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Heat shock and diethyldithiocarbamate stimulate polyamine catabolism in animal cells by a mechanism involving the induction of spermidine/spermine *N*¹-acetyltransferase (*N*¹-SSAT) activity. Steady-state levels of RNA encoding this enzyme remain essentially unchanged during periods after these stresses when *N*¹-SSAT activity is increased by 3.5–10-fold or more in three different cell lines of hamster and human origin. Depletion of intracellular spermidine pools by α -difluoromethylornithine (DFMO) inhibits stress induction of *N*¹-SSAT activity. Exogenous spermidine can restore stress inducibility of *N*¹-SSAT to

DFMO-treated cells, and induce this enzyme activity in non-heat-shocked but polyamine-depleted cells. Acetylation at *N*¹ suppresses the ability of spermidine to induce *N*¹-SSAT activity, relative to this same modification at *N*⁸. Fluorinated spermidine analogues, which decrease the *pK_a* values of the amine groups at positions 4 and 8, neither induce nor inhibit *N*¹-SSAT activity in DFMO-treated cells. These data demonstrate that certain stresses induce *N*¹-SSAT by a spermidine-dependent post-transcriptional mechanism. The mode of induction is affected by both the propyl and butyl moieties of spermidine.

INTRODUCTION

Polyamines are ubiquitous polycations present in near-millimolar concentrations in most living cells [1,2]. Suppression of polyamine contents by either genetic or pharmacological methods results in a sub-optimal growth in both bacteria [3] and animal cells [4,5]. Recent gene knock-out experiments demonstrate that polyamines are essential for growth in yeast [6].

Polyamine metabolism is highly regulated. The activities of enzymes involved in polyamine synthesis are controlled by transcriptional [7], translational [8] and post-translational [9,10] mechanisms in various systems. Two of these synthetic enzymes, ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase, are negatively regulated by spermidine [9–11].

The activity of the first enzyme in polyamine catabolism, spermidine/spermine *N*¹-acetyltransferase (*N*¹-SSAT), is positively regulated by spermidine [12]. Both *N*¹-SSAT activity and polyamine catabolism are induced in response to a variety of stresses in both rodent and human cells in culture [12–16]. Polyamines are necessary for the induction of *N*¹-SSAT by heat shock and diethyldithiocarbamate (DDC) [12,13]. High levels of endogenous spermidine are sufficient to induce *N*¹-SSAT in both cells [12] and tissues [17].

Heat shock stimulates the transcription of a set of genes in both pro- and eu-karyotic cells [18]. Thus one aim of this study was to determine whether stresses caused an increase in steady-state levels of *N*¹-SSAT RNA. A second aim was to define further the features of spermidine involved in *N*¹-SSAT activity induction.

EXPERIMENTAL

Cell culture techniques

Chinese hamster (line CHO) and human tumour (lung-cancer-derived A549 and melanoma-derived C8146C) cells were

maintained in monolayer cultures in McCoy's 5A medium supplemented with 2% fetal-bovine serum and 8% newborn-calf serum, as reported elsewhere [12,16]. Cultures were heat shocked by submerging sealed plastic flasks in a temperature-controlled (± 0.05 °C) water bath as previously reported [12–14]. Fluorinated spermidine analogues were kindly given by Dr. Pierre Mamont, Marion Merrell Dow Research Institute, Strasbourg, France. α -Difluoromethylornithine (DFMO) and the specific polyamine oxidase inhibitor MDL 72.521 were also graciously provided by Marion Merrell Dow.

Analysis of *N*¹-SSAT activity

After harvesting and washing cultures in cold PBS (140 mM NaCl, 2 mM KCl, 8.1 mM Na₂HPO₄, 0.9 mM K₂HPO₄), cell pellets were resuspended in lysis buffer (50 mM Tris/HCl, 2.5 mM dithiothreitol, 0.1 mM EDTA, pH 7.5) at a concentration of 10⁷ cells/ml and stored frozen at –80 °C until enzyme analysis. The radiochemical assay of *N*¹-SSAT activity was performed by estimation of labelled *N*¹-acetylspermidine (*N*¹AcSpd) synthesized from [¹⁴C]acetyl-CoA and unlabelled spermidine, as described in detail elsewhere [12,17]. One unit of enzyme activity is defined here as the amount which catalysed the formation of 1 pmol of *N*¹AcSpd/min at 30 °C.

Activity measurements were verified as deriving from *N*¹-SSAT by immunoprecipitation experiments using a specific anti-*N*¹-SSAT antiserum, obtained as a gift from Dr. Anthony E. Pegg, University of Pennsylvania, Hershey, PA, U.S.A. This antiserum has been described in detail elsewhere [17]. Briefly, cell lysates were incubated with various amounts of anti-*N*¹-SSAT antiserum overnight at 4 °C in a final volume of 100 μ l. The enzyme was cleared from the solution by adding 12 μ l of 10% (w/v) suspension of formalin-fixed bacterial Protein A adsorbent and centrifuging at 7500 *g* for 2 min to remove immune

Abbreviations used: DDC, diethyldithiocarbamate; *N*¹-SSAT, spermidine/spermine *N*¹-acetyltransferase; DFMO, α -difluoromethylornithine; *N*¹AcSpd, *N*¹-acetylspermidine; *N*⁸AcSpd, *N*⁸-acetylspermidine; 6²DFS, 6,6-difluorospermidine; 7²DFS, 7,7-difluorospermidine.

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complexes. N^1 -SSAT enzyme activity was then assessed in the antiserum-cleared lysates.

RNA isolation and analysis

Total RNA was obtained from cells by extraction with guanidinium thiocyanate and phenol/chloroform as described by others [19]. RNA was separated on 1% agarose gels and transferred to nylon membranes (Hybond-N, Amersham). Efficiency of transfer and location of rRNAs were monitored by analysis of ethidium bromide-stained material. Membranes were prehybridized at 42 °C for 4 h in 50 mM Tris/HCl containing 1 M NaCl, 1% SDS, 10% dextran sulphate, 50% formamide and 150 µg/ml salmon sperm DNA. Hybridization was performed under the same conditions with an N^1 -SSAT-specific cDNA probe, as described elsewhere [20], which was labelled by random priming [21] by using a kit from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Membranes were washed at 65 °C and then at room temperature in $0.1 \times$ SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.5% SDS before autoradiography.

Polyamine-content determinations

These were done by reverse-phase ion-paired h.p.l.c. techniques as reported previously [9,12–16]. Briefly, washed cell pellets were disrupted in 0.1 M HCl, and acid-soluble and -insoluble fractions were obtained after adjusting the lysates to 0.2 M HClO₄. Acid-soluble fractions were analysed directly by h.p.l.c. with post-column derivative formation of amines with *o*-phthaldialdehyde. Amine contents were normalized to cell number used to prepare the lysate. Cell counts were determined with an automatic particle counter (Coulter Electronics, Hialeah, FL, U.S.A.).

RESULTS

Unstressed exponential-phase hamster and human tumour cells expressed an acetyltransferase activity, which was increased after heat shock (Table 1). The level of acetyltransferase activity in unstressed cells varied somewhat between cell lines, as did the degree of induction after heat shock. To determine what fraction of these activities was due to N^1 -SSAT, extracts were treated with specific antiserum to precipitate this enzyme (Figure 1). Essentially all the heat shock-inducible acetyltransferase activity was inactivated by the antiserum, indicating the inducible activity was due to N^1 -SSAT enzyme protein. Approx. 25% of the CHO-cell and 15% of the A549-cell acetyltransferase activity was unaffected by the antiserum, which corresponded to the levels of enzyme activity in unstressed cells. The enzyme activity in unstressed cells was unaffected by the antiserum (results not shown). Together, these results showed that the acetyltransferase activity other than N^1 -SSAT and that essentially all the inducible activity was due to N^1 -SSAT. Thus the degree of heat-shock induction of N^1 -SSAT activity was greater than 3.5–10-fold, as suggested by the activity measurements in Table 1.

In order to understand the mechanism of induction of N^1 -SSAT activity, we measured steady-state levels of RNA encoding this enzyme in controls and cells treated with two different stresses, heat shock or DDC (Figure 2). N^1 -SSAT message was apparent in RNA isolated from all three unstressed cell lines. Neither heat shock nor DDC caused a significant change in the steady-state levels of this message. The results shown in Figure 2 were obtained from cells incubated at 37 °C for 6 h after heat

shock or for 6 h in DDC. This time corresponded to maximal induction of enzyme activity [12,13]. In other experiments, RNAs were analysed from cells incubated at 37 °C for up to 24 h after heat shock, or for up to 24 h of continuous DDC treatment. Steady-state levels of N^1 -SSAT RNA remained essentially unchanged under both conditions for all of these times (results not shown).

Depletion of intracellular putrescine and spermidine pools with DFMO inhibited the induction of N^1 -SSAT activity by heat shock (Table 2). Treatment of polyamine-depleted (DFMO-treated) cells with exogenous spermidine restored inducibility of this enzyme activity to levels twice that of non-DFMO treated but heat-shocked cells. Enzyme activity was also induced by exogenous spermidine in non-heat-shocked but DFMO-treated cells, although to a lesser degree than in heat-shocked cells.

Acetylation of spermidine at N^1 or N^8 decreased enzyme inducibility to 18 or 53%, respectively, of the spermidine control value in non-heat-shocked cells. Two fluorinated analogues of spermidine, 6,6-difluorospermidine (6²DFS) and 7,7-difluorospermidine (7²DFS), did not restore enzyme inducibility in non-heat-shocked DFMO-treated cells. In fact, these analogues further decreased the levels of non- N^1 -SSAT activity in DFMO-treated cells (from ~15% to 5% or less of the N^1 -SSAT immunoreactive activity).

It was possible that the lower levels of N^1 -SSAT inducibility caused by spermidine analogues (Table 2) might be a result of

Table 1 Induction of N^1 -SSAT activity by heat shock

Heat shock was 45 °C for 20 min. All cells, which were in exponential-phase growth at the time of treatment, were incubated for an additional 6 h at 37 °C and then assayed for N^1 -SSAT activity. The values shown are means $\pm$ S.E.M. of triplicate enzyme-activity determinations from a single representative experiment which was replicated three times.

Cell line	N^1 -SSAT activity (units/ 10^6 cells)	
	37 °C Control	Heat shock
CHO (rodent)	2.5 $\pm$ 0.3	9.1 $\pm$ 0.2
A549 (human)	2.6 $\pm$ 0.4	18.3 $\pm$ 0.3
C8146C (human)	4.5 $\pm$ 0.2	44.6 $\pm$ 0.1

Figure 1 Immunoinactivation of heat-shock-induced N^1 -SSAT activity in rodent (CHO; ○) and human (A549; ●) cells

Extracts containing approx. 3 units of enzyme activity were incubated with various amounts of antiserum against N^1 -SSAT protein. After incubation with bacterial Protein A adsorbent, extracts were centrifuged and supernatants were assayed for N^1 -SSAT activity as described in the Experimental section. Values shown are the means of triplicate enzyme determinations from a single representative experiment in a series of three experiments.

Figure 2 Measurement of steady-state *N*¹-SSAT RNA levels in hamster (CHO) and human (A549 and C8146C) cells stressed with either heat shock or DDC

RNA was isolated and analysed by Northern blotting as described in the Experimental section. The major band corresponds to the expected 1.3 kb size of *N*¹-SSAT mRNA [20]; the position of the 18 S rRNA marker is also shown. Lanes: C, untreated control cells growing in exponential phase at 37 °C; HS, heat-shocked (45 °C for 20 min) cells, which were then incubated at 37 °C for 6 h before harvest; DDC, cells in exponential phase treated with 10 μ M DDC for 6 h, which induces *N*¹-SSAT activity [13], before harvest. Films were exposed for periods up to 1 week to ensure linearity of response. The results shown here were 4-day exposures.

differences in uptake or metabolism of these spermidine derivatives. To test this hypothesis, we first added *N*¹AcSpd to cultures which were also incubated with the specific polyamine oxidase inhibitor MDL 72.521 [22]. Essentially no difference was observed in *N*¹-SSAT activity induction by *N*¹AcSpd (Table 2).

Endogenous polyamine contents were then determined in parallel cultures to those used for enzyme-activity analysis as shown in Table 2. This series of experiments was carried out in non-heat-shocked cells, as previous work from our group showed that heat shock caused cells to dump endogenous polyamines into the culture medium [23]. As shown in Table 3, *N*¹AcSpd was taken up by DFMO-treated A549 cells to a similar degree as *N*⁸-acetylspermidine (*N*⁸AcSpd). However, the level of *N*¹-SSAT activity induction was twice as high in the cells treated with *N*⁸AcSpd compared with those treated with *N*¹AcSpd. Inhibition of *N*¹AcSpd catabolism with MDL 72.521 affected specific polyamine pools as expected, but did not affect *N*¹-SSAT activity induction.

Table 2 Effects of spermidine (Spd) and Spd derivatives on *N*¹-SSAT activity induction in non-heat-stressed (NHS) and heat-shocked A549 cells

Cultures were divided into two groups, one treated with DFMO and the other not treated with this inhibitor (control group). Amines were added at final media concentrations of 100 μ M 36 h after cultures were seeded in the presence of 5 mM DFMO; 12 h later, some cultures were heat-shocked (45 °C for 20 min). All cultures were then incubated for an additional 6 h at 37 °C before enzyme assay. The values shown are means $\pm$ S.E.M. of triplicate enzyme-activity determinations from a representative experiment which was replicated twice. Abbreviation: NM, not measured. Values in parentheses are percentages of those with DFMO/Spd.

Group	Amine additions	<i>N</i> ¹ -SSAT activity (units/10 ⁶ cells)	
		NHS	Heat shock
Control	None	2.4 $\pm$ 0.4	13.8 $\pm$ 1.6
DFMO	None	1.8 $\pm$ 0.5	1.9 $\pm$ 0.3
	Spd	17.5 $\pm$ 2.5 (100)	43.8 $\pm$ 5.4
	<i>N</i> ¹ AcSpd (–MDL 72.521)	3.2 $\pm$ 0.2 (18)	NM
	<i>N</i> ¹ AcSpd (+MDL 72.521)	3.6 $\pm$ 0.4 (21)	NM
	<i>N</i> ⁸ AcSpd	9.3 $\pm$ 0.7 (53)	NM
	6 ² DFS	0.8 $\pm$ 0.1 (5)	NM
	7 ² DFS	0.6 $\pm$ 0.1 (3)	NM

The fluorinated spermidine derivatives (6²DFS and 7²DFS) accumulated to similar or even higher levels than the acetyl derivatives of spermidine (Table 3). However, neither of these amines induced *N*¹-SSAT activity in DFMO-treated A549 cells.

Spermidine was taken up to a greater degree than were either the acetyl- or fluoro-derivatives when each was added at an exogenous concentration of 100 μ M. Thus the difference between spermidine and *N*⁸AcSpd could be due to differences in amine uptake. However, the differences between *N*⁸AcSpd and the other analogues cannot be due to differential accumulation of these amines.

It was possible that the lack of induction of *N*¹-SSAT activity by the fluoro-derivatives of spermidine was due to an inhibition of enzyme activity. In order to test this hypothesis, these analogues were evaluated for both substrate and inhibitory activities in cell-free enzyme assays (Table 4). For saturating concentrations, both fluoro-derivatives were poor (20% or less of spermidine controls) substrates in this reaction. For con-

Table 3 Effects of exogenous spermidine (Spd) and Spd analogues on endogenous polyamine contents in DFMO-treated A549 cells

Cultures were treated with DFMO (5 mM) for 36 h, at which time various amines were added as indicated at a final concentration of 100 μ M in the medium. Then 18 h later, corresponding to the same cell harvest time as in Table 2, cultures were harvested for polyamine analysis. MDL 72.521 was added to cultures 30 min before *N*¹AcSpd was added. Polyamine contents are given in nmol/10⁶ cells; the values shown are single determinations from a representative experiment, which was replicated twice. Abbreviations: Put, putrescine; Spm, spermine; nd, none detected (limit of detection $\sim$ 0.005 nmol/10⁶ cells).

Amine added	MDL 72.521	Polyamine contents						
		Put	Spd	Spm	<i>N</i> ¹ AcSpd	<i>N</i> ⁸ AcSpd	6 ² DFS	7 ² DFS
None	–	nd	0.10	0.85	nd	nd	nd	nd
	+	nd	0.07	0.92	nd	nd	nd	nd
Spd	–	0.10	1.41	0.57	1.61	nd	nd	nd
	+	nd	0.62	0.95	0.28	nd	nd	nd
<i>N</i> ¹ AcSpd	–	nd	0.28	0.89	0.36	nd	nd	nd
	+	nd	0.70	1.07	nd	0.20	nd	nd
<i>N</i> ⁸ AcSpd	–	nd	0.12	1.18	nd	nd	0.87	nd
6 ² DFS	–	nd	nd	0.36	nd	nd	nd	0.40
7 ² DFS	–	nd	nd					

Table 4 Evaluation of substrate or inhibitory activities of difluoro-spermidine analogues in the *N*¹-SSAT-activity reaction

Approx. 50 units of *N*¹-SSAT activity from A549-cell extracts was assayed in reactions containing various concentrations (mM) of spermidine (Spd) and Spd analogues as indicated. Other components of the reaction mixtures were as described in the Experimental section. The values shown are means $\pm$ S.E.M. of triplicate enzyme-activity determinations from a single representative experiment which was replicated once.

Spd concn.	6 ² DFS concn.	7 ² DFS concn.	<i>N</i> ¹ -SSAT activity (%)
3	0	0	100.0 $\pm$ 12.9
0	3	0	16.6 $\pm$ 1.6
0	0	3	19.7 $\pm$ 2.5
3	0.01	0	102.2 $\pm$ 14.5
3	0.1	0	95.3 $\pm$ 4.9
3	1	0	90.8 $\pm$ 2.0
3	10	0	89.5 $\pm$ 2.3

centrations up to 10 mM, 6²DFS was a poor ($\leq 10\%$) inhibitor of *N*¹-SSAT activity; 7²DFS was not tested as an inhibitor.

DISCUSSION

Heat shock and DDC both induce *N*¹-SSAT activity and subsequent polyamine catabolism [12–16]. In one hamster and two human tumour cell lines investigated here, induction of this enzyme activity by factors exceeding 3.5–10-fold occurs in the absence of any significant increase in *N*¹-SSAT mRNA. These data strongly suggest that the mechanism of *N*¹-SSAT induction by these stresses is post-transcriptional in nature. Thus the gene encoding *N*¹-SSAT protein appears not to be a member of the so-called heat-shock genes, whose mRNA levels increase dramatically (~ 15 -fold; see [24], for example) in response to heat shock.

In a previous paper, we reported that the increase in heat-induced *N*¹-SSAT activity in CHO and A549 cells was inhibited by the transcription inhibitor actinomycin D [12], whereas here we show that steady-state levels of *N*¹-SSAT mRNA do not change after this stress. It appears from these two studies that actinomycin D suppresses enzyme activity in CHO and A549 cells by a mechanism not affecting *N*¹-SSAT mRNA synthesis. Our results, suggesting that *N*¹-SSAT activity is regulated by a post-transcriptional mechanism, are similar to those reported by Obayashi et al. [25], for sodium arsenite, and our previous studies [20,26] for polyamine-analogue treatments. In fact, Obayashi et al. [25] found that actinomycin D inhibited the sodium arsenite-induced increase in *N*¹-SSAT activity without affecting steady-state mRNA levels, just as we report here for heat shock. Those authors suggested that other factors transcriptionally induced by stress may stimulate *N*¹-SSAT activity by a post-transcriptional mechanism.

Our finding that not all spermidine-acetylating activity in animal cells is inhibited by *N*¹-SSAT-specific antiserum corroborates the original report by Persson and Pegg [17]. Similarly to results shown in Figure 1, these workers found that essentially all spermidine-acetylating activity induced by several chemical stresses or spermidine was precipitated by anti-*N*¹-SSAT antiserum. In rat liver, uninduced levels were not immunoreactive with this antiserum. These authors also concluded that the level of enzyme induction was underestimated by activity measurements.

Heat shock and DDC induction of *N*¹-SSAT activity is inhibited when putrescine and spermidine contents are depleted by DFMO [12,13], whereas polyamines and polyamine analogues

act to induce this enzyme activity [12,17,20,26]. Some polyamine analogues do cause an increase in *N*¹-SSAT mRNA levels [20,26]. The increase, however, is generally insufficient to account for analogue-induced increases in enzyme activity [20,26]. Together with the results shown here, these findings suggest that the post-transcriptional mechanism of *N*¹-SSAT regulation is polyamine-dependent. Pegg and co-workers [27] have shown that *N*¹*N*¹²-bis(ethyl)spermine, which does stimulate *N*¹-SSAT activity in intact cells [20], does not affect *N*¹-SSAT mRNA translation in the reticulocyte-lysate system. Their results imply that enzyme-activity induction is not simply explained by a translational mechanism involving an interaction between *N*¹*N*¹²-bis(ethyl)spermine and *N*¹-SSAT mRNA.

In a previous study comparing a series of diamine homologues, we found that the propylamine moiety of spermidine was important for the restoration of *N*¹-SSAT inducibility in DFMO-treated cells [12]. Here we have further characterized structural features of spermidine that affect this induction. We found that differences in *N*¹-SSAT induction by acetyl derivatives may be due to differences in uptake of these amines. However, for equivalent amine uptake, suppression of *N*¹-SSAT induction in non-heat-shocked cells was greater when spermidine was acetylated at N-1 than when it was acetylated at N-8. The differential effect of acetylation at N-1 versus N-8 in non-heat-shocked cells independently corroborates the conclusion that the propylamine group of spermidine is important for the induction of *N*¹-SSAT activity [12].

Modification of butylamine groups of spermidine, with fluorine atoms that differentially affect the p*K*_a values of the *N*⁴ and *N*⁸ derivatives [28], also suppressed *N*¹-SSAT inducibility. The effects of the fluorinated spermidine analogues were due to their inability to replace structural features of spermidine in this enzyme-induction mechanism, as these analogues were found to be poor substrates for *N*¹-SSAT and 6²DFS did not inhibit enzyme activity.

In summary, we have shown that heat shock and DDC induce *N*¹-SSAT activity by a spermidine-dependent post-transcriptional mechanism in both human tumour and hamster cell lines. Both the propyl- and butyl-amine moieties of spermidine affect the process(es) regulating the induction of this enzyme. *N*¹-SSAT induction is known to be associated with a stabilization of enzyme protein in at least some cases [17]. It will be of interest to determine if spermidine or polyamine analogues are acting to regulate *N*¹-SSAT activity by suppressing *N*¹-SSAT protein degradation. Spermidine is already known to regulate negatively ornithine decarboxylase, the first enzyme in polyamine synthesis, at the level of protein degradation [2,3,9,10]. Thus spermidine could be co-ordinately regulating polyamine synthesis and catabolism by mechanisms involving protein degradation.

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