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Differentiation of PC12 cells induced by N^8-acetylspermidine and by N^8-acetylspermidine deacetylase inhibition

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Abstract

Spermidine is one of the simple polyamines found in cells of virtually all living organisms. It undergoes a metabolic conversion to N^8-acetylspermidine catalyzed by an enzyme in cell nuclei and is converted back to spermidine by a deacetylase in the cytoplasm. In this study, two different mechanisms were used to produce an elevation in the level of N^8-acetylspermidine in PC12 cells: inhibition of N^8-acetylspermidine deacetylase and direct addition of N^8-acetylspermidine to the cell culture. The increasing intracellular concentration of N^8-acetylspermidine was accompanied by signs of PC12 cell differentiation including increased content of dopamine and morphological changes (neurite outgrowths), suggesting a strong and perhaps causal relationship among these effects. This effect on differentiation appears to be specific for N^8-acetylspermidine as the addition of other polyamines including spermidine and N^1-acetylspermidine did not elicit these changes. Nerve growth factor (NGF) and dexamethasone, commonly used inducers of differentiation in PC12 cells, produced differentiation without measurable changes in N^8-acetylspermidine levels, suggesting that different (or multiple) mechanisms may be involved in these differentiation processes.

Keywords: Polyamine; N^8-Acetylspermidine; N^8-Acetylspermidine deacetylase; APAH; Cell growth; Differentiation; Dopamine; PC12 cells

1. Introduction

The polyamines, found in virtually all living organisms, are a ubiquitous group of compounds that appear to play a vital role in many cellular processes involving nucleic acids including cell growth and differentiation [1]. The three primary polyamines, putrescine, spermidine, and spermine, can each undergo *N*-acetylation to form monoacetylated derivatives through two functionally different pathways. Putrescine and spermine are both symmetrical, and each can give rise to only one, N^1-monoacetylated, product. Spermidine, which is asymmetrical with three nitrogens joined by a 3-carbon and a 4-carbon aliphatic chain, has been shown to undergo two different enzyme-catalyzed *N*-acetylation reactions in mammalian tissues [2]. Acetylation on the nitrogen adjacent to the 3-carbon chain yields N^1-acetylspermidine, which is a key intermediate in the conversion of spermidine back to putrescine via oxidative deamination by polyamine oxidase [2]. Acetylation on the terminal nitrogen adjacent to the 4-carbon chain produces N^8-acetylspermidine. This reaction is catalyzed by spermidine N^8-acetyltransferase [3] and does not result in the conversion of spermidine to putrescine but, instead, the product undergoes deacetylation [2]. This acetyltransferase appears to be associated with chromatin in the cell nucleus and has been reported to be the same as (or related to) the enzyme(s) responsible for histone acetylation [3,4]. N^8-Acetylspermidine does not accumulate in tissues but rather appears to be rapidly deacetylated back to spermidine by a relatively specific cytosolic deacetylase, N^8-acetylspermidine deacetylase [1,5].

The function of this N^8-acetylation/deacetylation pathway in cellular processes is not understood clearly, but several observations have suggested a role in cell growth and differentiation. Addition of N^8-acetylspermidine, but not N^1-acetylspermidine, to the culture medium induces differentiation of HL60 human leukemia cells [6]. Increases in the intracellular concentrations of N^8-acetylspermidine stimulate the growth of L1210 mouse leukemia cells [7] but not of HTC rat hepatoma cells [8]. Acetylation of spermidine, either at the N^1- or N^8-position, decreases its ability to stabilize DNA [9] and nucleosome core particles

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Abbreviations: APAH, 7-[*N*-(3-aminopropyl)-amino]-heptan-2-one; DAH, 1,7-diaminoheptane; NGF, nerve growth factor.

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[10]. It has been proposed that the *N*-acetylation of spermidine may alter the interaction of spermidine with DNA, thereby affecting processes such as DNA replication or transcription and cell proliferation [11]. Moreover, the unique nuclear localization of N^8-acetyltransferase would suggest that N^8-acetylation might have a greater role than N^1-acetylation in influencing DNA stability and transcription and cell proliferation.

To test this hypothesis, we have developed a means of raising the levels of N^8-acetylspermidine in tissues or cultured cells using an inhibitor of N^8-acetylspermidine deacetylase. Bioisosteric replacement of the 8-nitrogen of N^8-acetylspermidine with a carbon produced APAH, which we have shown in *in vitro* studies to be a selective and potent N^8-acetylspermidine deacetylase inhibitor with a K_i of 0.18 μM [12]. When mice are administered 200 mg/kg of APAH intraperitoneally, N^8-acetylspermidine is raised to detectable levels in liver and spleen [13]. N^8-Acetylspermidine is also increased in HeLa cells after APAH treatment [13]. The availability of this inhibitor has enabled us to examine in the present study the effects of inhibition of N^8-acetylspermidine deacetylation and of the resultant elevated levels of intracellular N^8-acetylspermidine on growth, differentiation, and polyamine content of PC12 cells in culture. PC12 cells, a clonal cell line derived from a rat pheochromocytoma, undergo neuronal differentiation when stimulated with NGF and have been used extensively as a model of neuronal differentiation [14–16]. The present study focuses on the N^8-acetylation process and the effects of accumulation of intracellular N^8-acetylspermidine on the differentiation of PC12 cells.

2. Materials and methods

2.1. Materials

Putrescine, N^1-acetylspermidine, N^8-acetylspermidine, spermidine, spermine, DAH, aminoguanidine bicarbonate, rat tail collagen, NGF (2.5S), dexamethasone, dopamine hydrochloride, and epinephrine hydrochloride were purchased from the Sigma Chemical Co. RPMI 1640 medium, penicillin/streptomycin, horse serum, and fetal bovine serum were obtained from GIBCO. 7-[*N*-(3-Aminopropyl)-amino]heptan-2-one·2HCl (APAH) was synthesized as described previously [12].

2.2. Cell culture conditions and procedures

PC12 cells were obtained from the American Type Culture Collection (Cat. ATCC-CRL1721). Cells were grown as a monolayer on rat tail collagen-coated 25 and 75 cm^2 plastic tissue culture flasks in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum containing 50 μg/mL of streptomycin and 50 IU/mL of penicillin. The flasks were coated with 1 mL of a 0.1% solution of rat tail collagen (sterilized by filtration) and left overnight or until the coating dried under UV light. Cultures were maintained at 37° in a CO_2 incubator (VWR model 1710) under humidified air containing 5% CO_2. Aminoguanidine (1 mM) was added to the medium in all experiments including controls to inhibit the amine oxidases present in the serum, and the medium was changed every third day.

For drug treatment studies, cells were removed from maintenance flasks when they reached confluence by scraping with a rubber policeman, and were suspended and distributed equally into 25 cm^2 flasks (3×10^6 cells/flask) or wells (10^6 cells/well) of 6-well plates. The flasks were used for the morphology studies and polyamine assays, and the 6-well plates for the dopamine assays. Stock drug solutions were prepared in distilled water and then filtered through sterile Nalgene disposable filters (0.25 μm) for sterilization. Solutions were diluted with RPMI medium and kept at 4°. In all experiments, cells were incubated under standard conditions for at least 24 hr before drug treatments were begun, and the time at which drug solutions were added to the culture medium is designated as 0 time.

2.3. Evaluation of PC12 cell morphology in treated and control cultures

Morphological differentiation of PC12 cells was characterized by the presence of neurite outgrowths, i.e. neurological-like processes observed on the cultured cells. Differentiated cells were defined as cells with neurites at least one body diameter long with a well-defined growth cone [14,15]. The number of cells exhibiting such processes was determined using a phase-contrast microscope for each treatment and time. The number of differentiated cells were counted in six microscopic fields for each flask (treatment time) as well as the total number of cells in these fields. The percentage of differentiated cells was calculated as the number of cells with well-defined neurite outgrowths divided by the total number of cells (differentiated and undifferentiated) multiplied by 100.

2.4. Preparation of PC12 cell samples for dopamine, polyamine, and protein assays

At the appropriate treatment times, cells were harvested by scraping from the well or flask with a rubber policeman and pelleting from the medium by centrifugation at 1800 *g* for 5 min at 4°. Pellets were washed twice with 1 mL of ice-cold PBS. PBS supernatants were removed carefully with cotton swabs. The remaining cell pellet was extracted at 4° with 0.5 mL of 0.6 N perchloric acid for 30 min and then centrifuged for 5 min at 12,000 *g* at 4° in an Eppendorf Microcentrifuge (Brinkmann Instruments). For samples prepared for polyamine assays, the 0.6 N perchloric acid also contained 1 μM DAH as an internal standard. Following centrifugation, supernatants were removed and

stored at −80° for dopamine or polyamine assays, and pellets were saved for protein assays.

2.5. Determination of dopamine levels in PC12 cells by HPLC

Dopamine was assayed by HPLC as described by Emy *et al.* [17]. The supernatants obtained from the 0.6 N perchloric acid extract of the PC12 cells were taken through a clean-up step in preparation for injection into the HPLC column. The pH of the supernatant was raised above 8.5 by the addition of NaOH, and 50 mg of alumina was added to adsorb the dopamine. Alumina was pelleted by centrifugation (600 *g* for 10 min at room temperature) and washed twice with distilled water. Dopamine was removed from the alumina with 250 μL of 0.2 N perchloric acid containing 10 nmol epinephrine hydrochloride as an internal standard. The sample was filtered through a 0.45 μm filter (HPLC certified, Gelman Sciences) prior to injection into the HPLC column. Percent recovery through the alumina extraction was determined, using known amounts of dopamine, as greater than 90% (91.5 to 97.1% in three determinations).

The HPLC assay system (Spectra-Physics) used a 4.6 × 250 mm C18 Ultrasphere-ODS column (Alltech Scientific) for separation and a model CC-5 electrochemical detector (Bioanalytical Systems). The mobile phase was 100 mM phosphoric acid containing 200 μM sodium octyl sulfate, 100 μM EDTA, and 9% methanol (pH 2.6) pumped at a flow rate of 1 mL/min. The output from the detector was recorded and integrated with a model 3390A integrator (Hewlett-Packard). Quantitative analyses were based on a standard curve established for dopamine, and individual analyses were compared using epinephrine as an internal standard. Even though epinephrine is a metabolite of dopamine, it can be used as the internal standard for analyses of PC12 cells because they lack the enzyme responsible for its synthesis (phenylethanolamine-*N*-methyltransferase) and because epinephrine synthesis is not induced in PC12 cells by glucocorticoids or other stimuli [14].

2.6. Determination of polyamine contents in PC12 cells by HPLC

Polyamines were assayed by a reversed-phase HPLC system with a μBondapak C18 column as described previously from this laboratory [7]. Supernatants obtained from the 0.6 N perchloric acid extracts of the PC12 cells were filtered through 0.45 μm filters prior to analysis by HPLC.

Post-column derivatization (in-line) of polyamines with *o*-phthaladehyde reagent delivered by a mini-pump allowed the rapid detection of these compounds in a flow cell equipped fluorimeter (Perkin-Elmer 204A) [7]. The excitation wavelength was set at 345 nm, and emission was measured at 455 nm. Output from the fluorimeter was recorded and integrated with a model 3390A integrator (Hewlett-Packard). Quantitation was achieved using polyamine standards with DAH as an internal standard to control variations among individual samples. The minimum level of detection achieved with the sensitivity of the detector for standard polyamine solutions was 5 pmol.

2.7. Protein determinations

Protein assays were performed on the cell residue (pellets) recovered after extraction of the dopamine and polyamines. The pellets were dissolved in 0.1 M NaOH, neutralized with an equal volume of 0.1 M HCl, and assayed for protein using the method of Bradford [18] with bovine serum albumin as the standard protein.

2.8. Statistical analysis

Student's *t*-test (for single test group) [19] and Dunnett's *t*-test (for multiple comparisons) [20] were used for statistical analyses, as applicable.

3. Results

3.1. Induction of morphological changes

PC12 cells undergo characteristic morphological changes in culture when exposed to NGF, and this serves as a model for studies of differentiation [14]. The primary morphological change was the growth of neurites, which began to appear within 24 hr after the addition of NGF to the medium and involving more than 90% of cells within 7 days. Over this 7-day period, less than 3% of the cells underwent morphological differentiation (neurite outgrowths) in the absence of NGF. The number of cells affected over time by NGF (50 ng/mL) is illustrated in Fig. 1. In parallel experiments, the effects of N^8-acetylspermidine and APAH were tested on PC12 cell morphology. As shown in Fig. 1, the results indicate that both these compounds induced morphological changes (neurite outgrowth) but to a lesser degree than seen with NGF. At the concentrations studied, APAH (10^{-5} M) produced neurite outgrowths in a greater percentage of cells than did N^8-acetylspermidine (10^{-6} M), reaching a maximum at day 5 of 30.4% compared with 15.1% on day 7, respectively. These two concentrations were chosen because in other experiments they produced the maximum effects over a range (10^{-7} to 10^{-4} M) of concentrations tested. Thus, APAH appears to be the more effective of these two agents in inducing morphological differentiation. To determine if additive or synergistic effects can be produced by these two compounds, another set of flasks were treated with both N^8-acetylspermidine (10^{-6} M) and APAH (10^{-5} M), and morphological changes were recorded. As can be seen in

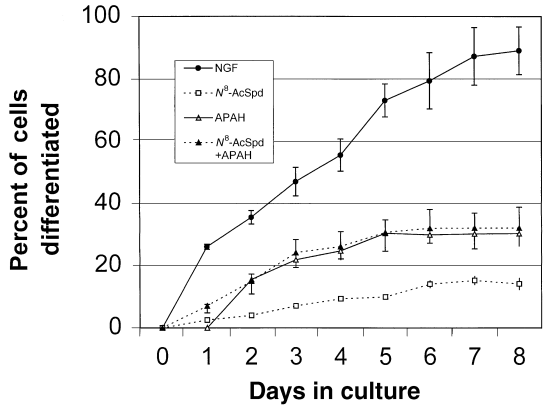

Fig. 1. Effects of nerve growth factor (NGF), 7-[*N*-(3-aminopropyl)-amino]-heptan-2-one (APAH), and N^8-acetylspermidine (N^8-AcSpd) on the morphological differentiation of PC12 cells. Cells were incubated from day 0 in the presence of 50 ng/mL of NGF, 10^{-6} M N^8-AcSpd, 10^{-5} M APAH, or 10^{-6} M N^8-AcSpd + 10^{-5} M APAH. Cell cultures were scored for the percentage of cells exhibiting features characteristic of differentiation at 24-hr intervals, as described in the text. Each point represents the mean ± SD of counts from 6 fields for each of three cell culture flasks.

Fig. 1, the combination produced no significant increase over that observed with APAH alone, suggesting that the mechanism of these two agents involves a common or rate-limiting step. Since both APAH and the added N^8-acetylspermidine would be expected to produce an increase in the intracellular concentration of N^8-acetylspermidine, this would be expected to be the common step, limiting or leading to a limiting step in the differentiation response. The fact that APAH and N^8-acetylspermidine (at any of the concentrations studied) produced differentiation in a much smaller proportion of cells than did NGF suggests a difference in the mechanisms of action of these agents.

A number of other polyamines were tested under identical conditions to determine their ability to produce morphological changes in PC12 cells. N^1-Acetylspermidine, putrescine, spermidine, and spermine were each tested at 10^{-5} M, 10^{-6} M, and 10^{-7} M. For each compound and concentration tested, less than 4% of the cells showed signs of morphological differentiation after 7 days of exposure. These results suggest that it is the N^8-acetylated form of spermidine and not other polyamines or even the closely related N^1-acetylated form that is involved in this differentiation process. This is consistent with the observations of Snyder *et al.* [6], that N^8-acetylspermidine, but not N^1-acetylspermidine, can induce differentiation in HL60 human leukemia cells. We have also observed specific growth stimulation of L1210 cells with N^8-acetylspermidine, but not N^1-acetylspermidine or other polyamines [7].

3.2. Effects of NGF, dexamethasone, and polyamines on dopamine levels

The effects of N^8-acetylspermidine on dopamine levels in PC12 cells over a range of concentrations and times are shown in Table 1. Significant increases in dopamine concentration compared with the control were seen as early as 48 hr after the addition of N^8-acetylspermidine (10, 100, and 1000 μM) to the culture medium. Increases were seen after 72 hr of exposure over the full range of concentrations tested with an apparent maximum at 100 μM N^8-acetylspermidine for both 48 and 72 hr.

Table 1
Concentration effects of N^8-acetylspermidine on dopamine levels in PC12 cells

N^8-Acetylspermidine (μM)	Dopamine content (nmol/mg protein)		
	24 hr	48 hr	72 hr
0 (control)	6.4 ± 1.1	8.1 ± 1.4	11.0 ± 1.8
0.1	5.8 ± 0.8	7.1 ± 0.9	12.4 ± 0.5*
1	6.9 ± 1.8	9.8 ± 0.9	14.7 ± 1.6*
10	9.6 ± 1.3	14.5 ± 2.1**	20.4 ± 2.8*,**
100	11.4 ± 1.7	21.6 ± 2.6*,**	30.5 ± 34*,**
1000	10.9 ± 0.9	18.7 ± 3.5**	26.7 ± 3.7*,**

Each value represents the mean (± SD) of values obtained by sampling each of three different flasks and analyzing each sample separately.
* $P < 0.05$, significantly different from the same treatment at 24 hr.
** $P < 0.5$, significantly different from control at the same time interval.

Table 2 shows the effects of APAH on dopamine levels in PC12 cells over a range of concentrations and times. Dopamine content was increased within 24 hr of exposure to 100 μM APAH and continued to increase at 48 and 72 hr. At 72 hr, 10–1000 μM concentrations of APAH caused significant increases in dopamine content compared with control levels, with the maximum content observed at 100 μM APAH.

In Table 3, the effects of N^8-acetylspermidine and APAH are compared with those produced by NGF, dexamethasone, putrescine, spermidine, and spermine. Both NGF and glucocorticoids (dexamethasone) have been shown to induce differentiation in PC12 cells as evidenced by increased dopamine (and to a much lesser extent norepinephrine) synthesis [14,21]. The results in Table 3 confirm this observation in our system with increases in dopamine content compared with controls observed at 24 hr after the

Table 2
Concentration effects of APAH on dopamine levels in PC12 cells

APAH (μM)	Dopamine content (nmol/mg protein)		
	24 hr	48 hr	72 hr
0 (control)	7.3 ± 0.8	8.6 ± 1.1	12.3 ± 1.8*
0.1	7.2 ± 1.5	8.9 ± 1.8	11.8 ± 2.5
1	8.5 ± 2.2	9.4 ± 1.4	13.4 ± 2.4*
10	10.8 ± 1.3	15.9 ± 2.3**	22.8 ± 1.8**
100	14.8 ± 2.1**	19.9 ± 2.2**	28.1 ± 2.6*,**
1000	13.4 ± 3.8	18.6 ± 1.6**	26.2 ± 2.5*,**

Each value represents the mean (± SD) of values obtained by sampling each of three different flasks and analyzing each sample separately.
* $P < 0.05$, significantly different from the same treatment at 24 hr.
** $P < 0.05$ significantly different from the control at the same time interval.

Table 3
Effects of N^8-acetylspermidine (N^8-AcSpd) APAH, other polyamines, dexamethasone, or NGF on dopamine levels in PC12 cells

Treatment	Dopamine content (nmol/mg protein)		
	24 hr	48 hr	72 hr
Control	5.2 ± 1.2	8.1 ± 0.9	12.1 ± 1.4*
Putrescine (10 μM)	5.0 ± 0.5	6.8 ± 0.9	11.2 ± 0.6*
Spermidine (10 μM)	6.2 ± 0.8	7.0 ± 1.9	12.9 ± 1.3*
Spermine (10 μM)	6.1 ± 1.3	9.6 ± 0.4	14.8 ± 1.5*
N^8-AcSpd (10 μM)	8.2 ± 0.6	14.1 ± 0.8*,**	20.4 ± 1.7*,**
APAH (10 μM)	7.5 ± 0.9	13.1 ± 0.4*,**	19.2 ± 1.4*,**
Dexamethasone (10 μM)	10.4 ± 1.3**	16.0 ± 1.7*,**	30.7 ± 3.9*,**
NGF (25 ng/mL)	12.3 ± 1.4**	16.4 ± 3.4**	33.3 ± 4.2*,**

Each value represents the mean (± SD) of values obtained by sampling each of three different flasks and analyzing each sample separately.
* $P < 0.05$, significantly different from the same treatment at 24 hr.
** $P < 0.05$, significantly different from the control at the same time interval.

addition of dexamethasone or NGF to the medium. Further increases were observed with these agents up to 72-hr treatment times. Both N^8-acetylspermidine and APAH also produced increases in dopamine content compared with control levels at 48- and 72-hr treatment times. No increases in dopamine levels compared with controls were seen in cells exposed to 10 μM putrescine, spermidine, or spermine. At 100 μM, putrescine, spermidine, and spermine significantly increased dopamine levels only after 72 hr, and these increases were significantly less than those produced by 10 μM N^8-acetylspermidine or APAH (data not shown). Over the 72-hr incubation period, dopamine content increased in all treatment groups, as well as in controls, compared with cells incubated for only 24 hr. To compare various treatment effects, dopamine content should be compared with data from untreated (control) cells at the comparable incubation time. At the 10 μM concentrations of N^8-acetylspermidine and APAH studied, the increases in dopamine content were not as large as those seen with dexamethasone or NGF, but in contrast with the three nonacetylated polyamines, only N^8-acetylspermidine and APAH produced significant increases at 48 and 72 hr compared with the controls.

3.3. Effects of NGF, dexamethasone, and polyamines on polyamine levels

Fig. 2 illustrates the effects of N^8-acetylspermidine, APAH, or a combination of the two on intracellular N^8-acetylspermidine levels in PC12 cells. In the top two panels, it can be seen that intracellular N^8-acetylspermidine levels increased with increasing concentrations of N^8-acetylspermidine or APAH added to the medium and with incubation times up to 7 days. Under these conditions, both N^8-acetylspermidine and APAH appeared to be of comparable potency with increases in N^8-acetylspermidine observed at 10 μM, but not at 1 μM. However, N^8-acetylspermidine raised intracellular levels of N^8-acetylspermidine to a greater extent (approximately 2-fold greater) than did equimolar concentrations of APAH. It should be emphasized that our assay technique can only measure total cellular N^8-acetylspermidine with there being no way to determine compartmentalization in the nucleus versus the cytoplasm. These two methods may produce a difference in distribution of increased N^8-acetylspermidine within cells and may account for some differences in effects. The combination of the two agents produced an apparently greater increase in N^8-acetylspermidine levels, but this was markedly greater than that produced by N^8-acetylspermidine alone only at the 100 μM concentration for 3 days.

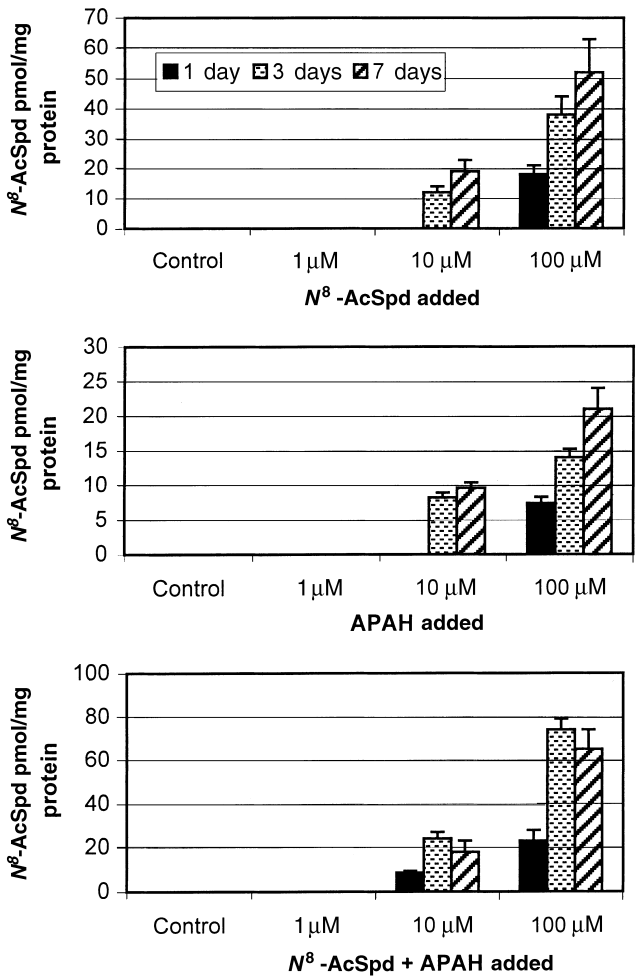

Fig. 2. Concentrations of N^8-acetylspermidine in PC12 cells cultured for 1, 3, and 7 days in the presence of increasing concentrations of 7-[*N*-(3-aminopropyl)-amino]-heptan-2-one (APAH) and N^8-acetylspermidine (N^8-AcSpd). Cells were incubated from day 0 in the presence of N^8-AcSpd, APAH, or N^8-AcSpd + APAH at the concentrations indicated. Cells were harvested after 1, 3, and 7 days of exposure, and N^8-acetylspermidine levels were measured by HPLC. Each bar represents the mean ± SD of values obtained by sampling each of three different flasks and analyzing each sample separately. The absence of bars for the control and the 1 μM concentration indicates that N^8-acetylspermidine levels were below detection limits.

Table 4 provides results of studies on polyamine and acetylpolyamine levels in PC12 cells incubated for 72 hr in

Table 4
Effects of a 72-hr exposure to N^8-acetylspermidine (N^8-AcSpd), APAH, dexamethasone, or NGF on polyamine levels in PC12 cells

Treatment	Polyamine content (pmol/mg protein)				
	PUT	SPD	SPM	N^1-AcSpd	N^8-AcSpd
Control	71 ± 11	2622 ± 139	1428 ± 59	ND	ND
NGF (25 ng/mL)	74 ± 12	2994 ± 137	1448 ± 39	ND	ND
Dexamethasone (10 μM)	82 ± 08	2984 ± 168	1441 ± 26	ND	ND
N^8-AcSpd (10 μM)	94 ± 09	3162 ± 162	1544 ± 45	ND	12 ± 02
APAH (10 μM)	92 ± 17	2871 ± 174	1185 ± 51	ND	8.2 ± 0.7
N^8-AcSpd plus APAH (10 μM each)	108 ± 12	1741 ± 112	1942 ± 59	ND	24 ± 03

Each value represents the mean (± SD) of values obtained by sampling each of three different flasks and analyzing each sample separately. The polyamines are putrescine, spermidine, spermine, N^1-acetylspermidine, and N^8-acetylspermidine. ND: not detectable.

the presence of NGF, dexamethasone, N^8-acetylspermidine, or APAH. No significant changes were observed in the polyamine or acetylpolyamine content of PC12 cells exposed to dexamethasone or NGF. Similarly, N^8-acetylspermidine or APAH exposure produced no significant changes in polyamine (nonacetylated) or N^1-acetylspermidine content; however, these two agents produced an increase in N^8-acetylspermidine to detectable levels at 72 hr. In combination, N^8-acetylspermidine and APAH (10 μM each) produced an even greater increase in N^8-acetylspermidine without significant changes in the other polyamines but with an interesting shift in the ratio of spermidine/spermine concentration (control ratio = 1.84 and treated ratio = 0.9). This decrease in spermidine compared with spermine concentrations is consistent with results from a previous study [7] with L1210 cells in which N^8-acetylspermidine and APAH in various concentrations appeared to decrease spermidine concentrations. Whether the mechanism for this effect on spermidine levels involves increased turnover, decreased synthesis, or an effect on transport remains unclear.

Based on our experience with limits of detection of polyamines in this and in previous studies [7], the minimum concentration of N^8-acetylspermidine detectable in cultured cells using this HPLC assay is 3–5 pmol/mg protein. These data are consistent with the results presented in Fig. 2, indicating significant increases in N^8-acetylspermidine content with N^8-acetylspermidine or APAH treatments.

In a parallel set of experiments, addition of N^1-acetylspermidine to the medium (1, 10, and 100 μM) resulted in detectable levels of N^1-acetylspermidine, but not N^8-acetylspermidine, at all three concentrations studied (data not shown), but no morphological changes were observed in these N^1-acetylspermidine-treated cells.

4. Discussion

Our findings that increasing intracellular concentrations of N^8-acetylspermidine correlate with the increased content of dopamine and morphological changes in PC12 cells suggest a strong and perhaps causal relationship among these effects. Two different mechanisms were used to produce elevations in N^8-acetylspermidine levels: inhibition of N^8-acetylspermidine deacetylase and direct addition of N^8-acetylspermidine to the cell culture. Both of these mechanisms produced increases in dopamine levels (Tables 1 and 2) and morphological signs of differentiation (Fig. 1), and a direct relationship was seen between these effects and the increases in concentrations of N^8-acetylspermidine measured (Fig. 2). This effect on differentiation appears to be specific for N^8-acetylspermidine as the addition of the other polyamines was ineffective (Table 3), and no differentiation was observed with the addition of N^1-acetylspermidine (data not shown). The fact that NGF (Fig. 1 and Table 3) and dexamethasone (Table 3) can produce differentiation without measurable changes in N^8-acetylspermidine levels (Table 4) suggests that different (or multiple) mechanisms may be involved in these differentiation processes.

The polyamines have long been known to promote tumor growth and to be involved in processes related to transformation, growth, and differentiation [22]. Polyamine deficiency can affect many growth-related processes including those involving epidermal growth factor [23], c-Myc expression and the differentiation of F9 teratocarcinoma cells [24], and proliferation and hormone production of pancreatic β-cells [25]. Most of these and other studies of polyamine function have examined the effects of depletion of polyamine levels using inhibitors of ornithine decarboxylase, the rate-limiting enzyme in polyamine synthesis. Thus, the polyamines have been shown to be essential for the normal functioning of these processes, but the specific mechanisms or steps in polyamine metabolism influencing these processes remain unknown.

In the present study, we have focused on a specific metabolic step, N^8-acetylation of spermidine, to determine its role in growth and differentiation. A few studies have implicated the N^8-acetylation of spermidine as a specific mechanism influencing cell growth and differentiation. N^8-Acetylspermidine, but not N^1-acetylspermidine, has been shown to induce differentiation in HL60 human leukemia cells [6]. We have reported significant increases in L1210 leukemia cell growth as a result of increases in intracellular N^8-acetylspermidine, and not N^1-acetylspermidine,

concentrations [7]. However, not all studies have found a significant or specific role for N^8-acetylspermidine in cell growth or differentiation as demonstrated by the lack of an effect of N^8-acetylspermidine deacetylase inhibition on the growth of HTC hepatocytes [8]. Thus, we might assume that differences in mechanisms can exist in various tissues or cell types just as differences exist in differentiation mechanisms involving NGF, dexamethasone, and the polyamines.

Any discussion of the mechanisms by which N^8-acetylation of spermidine might influence cell growth and differentiation should include a comparison with the remarkably similar role of histone acetylation in these processes. Histone acetyltransferases *N*-acetylate internal lysines of core histones, and this activity has been found to correlate with transcriptional activation in eukaryotes [26]. Several transcriptional regulators have been shown to possess histone acetyltransferase or histone deacetylase activities, suggesting that this acetylation/deacetylation process plays an important role in regulating transcription and gene expression [26]. The similarities between spermidine N^8-acetylation and histone acetylation include: the location of acetylating enzyme activities in the nuclear chromatin, the occurrence of specific deacetylases, the identity of the 4-carbon amino terminus of lysine and spermidine as the targets for acetylation, and the apparent influence of these processes on growth and differentiation.

In fact, there has been speculation that the same enzymes, acetyltransferases and deacetylases, may be responsible for histone and spermidine acetylation/deacetylation [4,27]. While spermidine acetylation in the N^1-position definitely involves a separate enzyme than the histone acetyltransferases [27], differences have also been shown between histone and N^8-acetylspermidine acetyltransferases [4]. With the discovery of a multiplicity of histone acetyltransferase activities associated with many different transcriptional activators [26], it is quite possible that some histone and spermidine acetylation involves the same enzymes, but certainly in some cases differences appear to exist [28].

With respect to the deacetylase activities, we have evidence that at least in one system with HeLa cells the histone and N^8-acetylspermidine deacetylases are different enzymes. In this system, the inhibitor, APAH, blocks deacetylation of N^8-acetylspermidine without any apparent effects on deacetylation of histones H2B or H4 [13]. In the same study, it was observed that sodium butyrate, a commonly used histone deacetylase inhibitor, did not elevate N^8-acetylspermidine levels in HeLa cells as APAH did [13]. Thus, different enzymes seem to be involved in histone and N^8-acetylspermidine deacetylation. It is interesting that sodium butyrate has been used to inhibit cell growth and induce differentiation in a number of cancer cell lines [29], and a number of other inhibitors of histone deacetylase have anti-tumor activity [30].

Our findings suggest that the nuclear acetylation of the polyamines, most specifically spermidine, may be involved in the differentiation of rat pheochromocytoma (PC12) cells. If we consider these cells a model of normal differentiation or just of activation of dopamine synthesis, then perhaps this N^8-acetylation of spermidine is a step in transcriptional activation leading to protein synthesis as has been described for histones [26]. In recent reports, mitogen-activated protein kinase (MAP kinase) would appear to be a common mediator between deacetylation inhibition and PC12 differentiation. Obara *et al.* [16] reported that activation of the MAP kinase cascade leads to the differentiation of PC12 cells, and Espinos and Weber [31] have shown that inhibition of histone deacetylation by either of the two inhibitors can activate the MAP kinase cascade and synthesis of the enzyme choline acetyltransferase in transfected CHP126 neuroepithelioma cells. This raises questions to be answered in future studies regarding the importance of spermidine N^8-acetylation, histone acetylation, and MAP kinase activation in the processes leading to the differentiation of PC12 and other cells.

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