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Mechanisms of spermine toxicity in baby-hamster kidney (BHK) cells

The role of amine oxidases and oxidative stress

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Spermine was toxic to BHK-21/C13 cells in the absence of any extracellular metabolism of the amine. Inhibition of copper-containing amine oxidases with aminoguanidine partially prevented the response, whereas inhibition of polyamine oxidase with MDL-72,527 exacerbated the effect. Oxidation by an intracellular copper-containing amine oxidase may be involved in the toxicity of spermine, whereas the polyamine-interconversion pathway appears to play a cytoprotective role. There was no evidence for spermine imposing a state of oxidative stress within the cells. Inhibition of catalase and glutathione reductase did not alter the cytotoxicity of spermine, and there was no excretion of oxidized glutathione into the extracellular medium. The results suggest that spermine itself can exert a toxic effect directly on the cells.

INTRODUCTION

The cytotoxicity *in vitro* of the naturally occurring polyamines, spermidine [$NH_2(CH_2)_3NH(CH_2)_4NH_2$] and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$], has been recognized for many years [1–3]. Early work in this area demonstrated that the inhibitory effect on the growth of a number of different cell lines was dependent on the presence of ruminant serum. When this was replaced with non-ruminant serum, such as horse serum, the inhibitory action of spermine was abolished [1]. These workers proposed that the inhibition of cell growth was dependent on the presence of a serum amine oxidase (EC 1.4.3.6) [4], which is found in bovine serum, but not in horse serum [5]. Furthermore, inhibition of bovine serum amine oxidase by aminoguanidine, an inhibitor of copper-dependent amine oxidases such as bovine serum amine oxidase, abolished the cytotoxicity of the polyamines [2,6].

Bovine serum amine oxidase catalyses the oxidative deamination of spermine and spermidine to produce respectively an aminomonoaldehyde [*N'*-(4-aminobutyl)-aminopropionaldehyde] or a dialdehyde [*NN'*-bis-(3-propionaldehyde)-2,4-diaminobutane], with ammonia and H_2O_2 [7]. It is believed that these aminoaldehydes are responsible for the toxicity of the polyamines [8–10]. It has been proposed that acrolein, formed as a by-product to polyamine oxidation, is responsible for the cytotoxicity observed in bovine serum [11–13]. However, there has been significant opposition to this proposal, since the cytotoxic effects of acrolein cannot be substituted for those of oxidized polyamines [14,15].

However, there have been few reports of polyamine-induced toxicity in the presence of horse serum [16–18], and it has been suggested that the amine itself may be inhibitory [16], or that oxidation of the amine by a cellular amine oxidase may be involved [17].

Intracellular oxidation of polyamines can occur by two possible routes. Polyamine oxidase (PAO) is an FAD-dependent enzyme which was first isolated and characterized from rat liver [19]. This enzyme is involved in the retroconversion pathway for polyamines, which converts spermine and spermidine back into their precursor, putrescine, via a series of acetylation/oxidation reactions. PAO catalyses the oxidation of spermidine and spermine and their acetylated derivatives at the secondary amino groups to cleave the appropriate amine and 3-aminopropanal or 3-acetaminopropanal, depending on the substrate, with the concomitant formation of H_2O_2. The second possible route of intracellular oxidation is by a series of intracellular copper-dependent amine oxidases which are known to be involved in the terminal catabolism of polyamines [20].

Although polyamines are known to be essential for normal cell growth and differentiation, their oxidative products may act as negative regulators of cell growth. Recent work by Pierce and co-workers [21–23] has implicated polyamine oxidation and subsequent cytotoxicity in the mechanism of programmed cell death. This aspect of polyamine function is independent of the presence of bovine serum amine oxidase, and before this theory can be established it is necessary to determine the mechanisms involved in the cytotoxicity of polyamines in the absence of this enzyme.

In the present study we have addressed this and looked at two different aspects of spermine toxicity. Firstly, we have used aminoguanidine and MDL-72,527 [*NN'*-bis-(2,3-butadienyl)-1,4-butanediamine], a specific inhibitor of PAO [24], to define the role of spermine oxidation in the absence of bovine serum. Secondly, we have investigated oxidative stress as a mechanism responsible for the cytotoxicity of polyamines. Excessive production of H_2O_2 leading to oxidative stress has previously been proposed to contribute to polyamine-induced cytotoxicity [25]. Cellular protection against the effects of H_2O_2 is provided by the activities of catalase and the glutathione reductase/peroxidase

Abbreviations used: PAO, polyamine oxidase; MDL-72,527, *NN'*-bis-(2,3-butadienyl)-1,4-butanediamine; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; DMEM, Dulbecco's modification of Eagle's medium; DH_{10}, DMEM supplemented with 10% (v/v) horse serum; FDNB, 2,4-dinitrofluorobenzene; AT, 3-amino-1,2,4-triazole.

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system. The activity of catalase can be inhibited by aminotriazole (AT) and that of glutathione reductase by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) [26]. Both BCNU and AT have been shown to enhance the toxicity of the quinone, menadione, the mechanism of action of which is known to generate reactive oxygen species [27]. During periods of oxidative stress the availability of NADPH limits the function of the glutathione redox cycle. This results in the accumulation of GSSG, which is excreted from the cell [28], and raised extracellular GSSG levels are an indication of toxicity caused by oxidative stress. We have investigated the role of oxidative stress in polyamine-induced toxicity, using BCNU and AT as enzyme inhibitors and by monitoring extracellular GSSG levels.

A preliminary report of these studies has appeared in abstract form [29].

MATERIALS AND METHODS

Materials

Spermine tetrahydrochloride, N^1-acetylspermine trihydrochloride, aminoguanidine, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid), pargyline, horseradish peroxidase, 2,4-dinitrofluorobenzene (FDNB), iodoacetic acid and AT were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [^{14}C]Spermine (0.25 Ci/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). BCNU was kindly given by Bristol-Myers Co. (Syracuse, NY, U.S.A.) and MDL-72,527 was generously given by Merrell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.). Bond-Elut aminopropyl columns were obtained from Analytichem International (Cambridge, U.K.).

Cell culture

BHK-21/C13 cells were routinely grown in monolayer culture in Dulbecco's medium (DMEM) supplemented with 10% (v/v) horse serum (DH_{10}), in a humidified atmosphere of air/CO_2 (19:1) at 37 °C. For experiments, the cells were seeded at a density of 1.6×10^4/cm^2 on 9 cm-diameter plastic plates and grown for 16 h before exposure to spermine for up to 24 h. At the required times the cells were harvested mechanically with a 'rubber policeman'. The polyamines and glutathione were extracted into 0.2 M-$HClO_4$ at 4 °C for 10 min and stored at −20 °C before analysis; they were extracted from samples of medium in the same way.

Measurement of polyamines and protein

Polyamines were measured by a modification of the h.p.l.c. method of Seiler & Knodgen [30] as described previously [31]. The acid-insoluble cell pellet was resuspended in 0.3 M-NaOH, and samples were used for the determination of protein by the Lowry method [32].

Measurement of enzyme activities

PAO activity. This was determined by a modification of the method of Suzuki *et al.* [33]. The H_2O_2 formed in the oxidase reaction, with N^1-acetylspermine as a substrate, was measured fluorimetrically by converting homovanillic acid into a highly fluorescent compound in the presence of horseradish peroxidase. Pargyline and aminoguanidine were present to inhibit monoamine oxidase and copper-containing amine oxidases respectively. The assay mixture contained 20 mM-sodium borate buffer, pH 9.0, 0.92 mM-homovanillic acid, 1 mM-aminoguanidine, 0.1 mM-pargyline, 0.2 mM-N^1-acetylspermine (all final concentrations), horseradish peroxidase (8.8 units) and 100 μl of homogenate (100–200 μg of protein) in a total volume of 0.6 ml. The homogenates were prepared by suspending the cell pellet in 0.5 ml of 10 mM-Tris/HCl, pH 7.5, and leaving on ice for 15 min. The cell suspension was homogenized by hand with 30 strokes of a PTFE Eppendorf homogenizer. Before addition of the homovanillic acid and substrate, the mixture was preincubated at 37 °C for 20 min. After incubation of the complete reaction mixture at 37 °C for 30 min, the reaction was stopped by addition of 2 ml of 0.2 M-NaOH. The fluorescence was measured at 323 nm excitation and 426 nm emission on a Perkin–Elmer LS-5B Luminescence Spectrometer. A blank reading was obtained by incubating the assay mixture in the same way but replacing the substrate with an equal volume of homogenizing buffer. The reaction was standardized and corrected for quenching by adding 1–2 nmol of H_2O_2 in place of the substrate. The reaction was linear with respect to protein content up to 250 μg of protein per assay.

Catalase activity. This was measured by the method of Aebi [34], which measures the decrease in A_{240} corresponding to the loss of H_2O_2.

Glutathione reductase activity. This was determined by the method of Carlberg & Mannervik [35], which monitors the oxidation of NADPH during the reduction of GSSG.

Measurement of glutathione

Intracellular GSH and glutathione in the medium were measured by the h.p.l.c. method of Reed *et al.* [36]. This method allows for the separation of thiols as their *N*-dinitrophenyl derivatives, after derivative formation with FDNB and iodoacetic acid. Before analysis, the *N*-dinitrophenyl derivatives were concentrated 20-fold by passage through aminopropyl Bond-Elut columns. They were eluted from these columns in 0.5 M-sodium acetate in 60% (v/v) methanol, which is the mobile phase for the h.p.l.c. separation.

RESULTS

Inhibition of intracellular amine oxidases

The ID_{50} (dose causing 50% inhibition of cell growth) for spermine in BHK cells after a 24 h exposure is about 1.0 mM [37], and this concentration was used in the present inhibitor studies. Pretreatment with aminoguanidine partially reversed the inhibition of cell growth by spermine (Fig. 1). After a 12 h exposure, spermine caused a significant decrease in cell growth, which was completely prevented by pretreatment with aminoguanidine. After a 24 h exposure to spermine, aminoguanidine did not reverse completely the inhibitory effects of spermine; spermine alone produced a 54% inhibition of cell growth, whereas spermine in the presence of aminoguanidine produced a 24% inhibition. Aminoguanidine alone had no effect on cell growth.

The pretreatment protocol for aminoguanidine was taken from the data on inhibition of bovine serum amine oxidase by this agent: 1 mM-aminoguanidine completely inhibited spermine oxidation in foetal-calf-serum-supplemented medium after a 30 min incubation (results not shown).

In BHK cells treated with 15 μM-MDL-72,527 no PAO activity was measurable after 16 h in culture (Table 1). This inhibition of enzyme activity was maintained for a further 24 h in culture, which covers the time of exposure of BHK cells to spermine in this study. Pretreatment of BHK cells with 15 μM-MDL-72,527 for 16 h significantly enhanced the toxicity of spermine after a 24 h exposure to the amine (Fig. 2): spermine caused a 57% inhibition of cell growth, which was increased to 78% by MDL-72,527 pretreatment. MDL-72,527 alone had no effect on cell growth. Spermine alone had no effect on PAO activity in BHK cells (results not shown). Although N^1-acetylspermine was used as the substrate for PAO in these experiments, spermine was also metabolized by the enzyme, but at a much

Table 1. Effect of MDL-72,527 on PAO activity in BHK-21/C13 cells

BHK cells were grown in the absence or presence of 15 μM-MDL-72,527 and PAO activity was measured over a 40 h culture period. One unit of enzyme activity is defined as 1 nmol of H_2O_2 produced/min. Values are means ± S.D. ($n = 3$); n.d., not detected.

Time in culture (h)	Treatment	PAO activity (units/mg of protein)	Inhibition (%)
4	None	3.11 ± 0.05	0
	MDL	2.24 ± 0.01	28.0
16	None	2.08 ± 0.02	0
	MDL	n.d.	100
40	None	1.75 ± 0.06	0
	MDL	n.d.	100

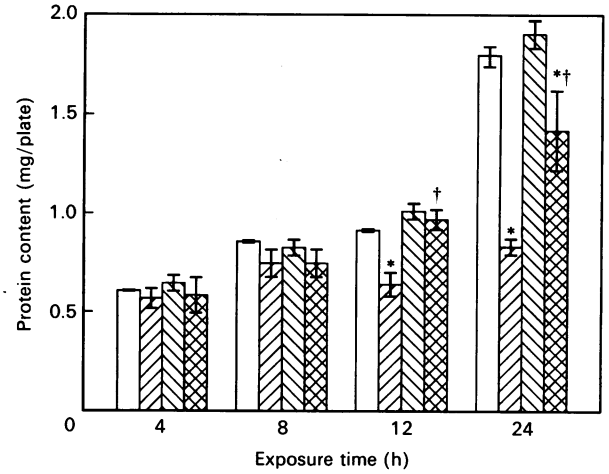


Fig. 1. Effect of aminoguanidine on spermine toxicity in BHK-21/C13 cells

BHK cells were exposed to 1 mM-spermine in the absence or presence of 1 mM-aminoguanidine. When aminoguanidine was present, it was added to the cells 30 min before exposure to spermine and was present throughout the experiment. Protein content was used as a measure of cell growth. Values are means ± S.D. ($n = 3$): *significantly different ($P < 0.01$) from control values, i.e. spermine against untreated and aminoguanidine + spermine combination treatment against aminoguanidine alone, and †significantly different ($P < 0.01$) from spermine treatment alone, by ANOVA with Dunnett's test. Key: □, untreated; ▨, spermine; ▧, aminoguanidine; ⊠, aminoguanidine + spermine.

lower rate (0.31 ± 0.02 versus 1.95 ± 0.08 nmol of H_2O_2 produced/min per mg of protein for spermine and N^1-acetylspermine respectively).

After addition of spermine to the extracellular medium, it is rapidly taken up into the cells, where it accumulates to very high levels (Table 2). In the presence of MDL-72,527 the intracellular spermine levels achieved were 1.5–2.5-fold greater than with spermine treatment alone. MDL-72,527 treatment alone had no effect on the spermine content of BHK cells.

Involvement of oxidative stress in spermine toxicity

We have previously reported that the intracellular GSH content of BHK cells was depleted after exposure to spermine [38]. As the excretion of GSSG from cells is often taken as an indicator of oxidative stress within the cell [39,40], we monitored the excretion of thiols from BHK cells. In control cells, low levels of GSH were detected in the medium, and after exposure of the

Table 2. Effect of MDL-72,527 on the intracellular spermine content of control and spermine-treated BHK-21/C13 cells

BHK cells were grown in the absence or presence of 15 μM-MDL-72,527 for 16 h before addition of 1 mM-spermine (SPM). The intracellular spermine content was measured during the 24 h exposure to the amine. Values are means ± S.D. ($n = 3$): *significantly different ($P < 0.01$) from the corresponding control values, i.e. spermine against untreated and the combination MDL + spermine treatment against MDL alone, and †significantly different ($P < 0.01$) from spermine treatment alone, by ANOVA with Dunnett's test.

Exposure time (h)	Treatment	Intracellular spermine content (nmol/mg of protein)
4	None	5.7 ± 0.5
	SPM	32.8 ± 3.7*
	MDL	4.5 ± 0.1
	MDL + SPM	35.8 ± 0.9*
8	None	4.3 ± 0.2
	SPM	46.7 ± 3.1*
	MDL	5.7 ± 0.5
	MDL + SPM	35.9 ± 5.6*
12	None	5.1 ± 0.3
	SPM	30.6 ± 1.7*
	MDL	4.7 ± 0.3
	MDL + SPM	71.0 ± 2.6*†
24	None	5.6 ± 0.6
	SPM	28.8 ± 1.9*
	MDL	5.8 ± 0.5
	MDL-SPM	50.8 ± 4.9*†

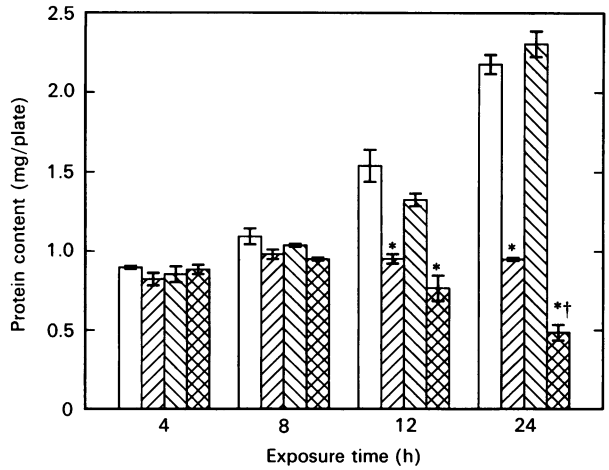


Fig. 2. Effect of MDL-72,527 on spermine toxicity in BHK-21/C13 cells

BHK cells were grown in the absence or presence of 15 μM-MDL-72,527 for 16 h before addition of 1 mM-spermine, when the MDL-72,527 was present throughout the experiment. Protein content was used as a measure of cell growth. Values are means ± S.D. ($n = 3$): *significantly different ($P < 0.01$) from control values, i.e. spermine against untreated and MDL-spermine combination treatment against MDL alone, and †significantly different ($P < 0.01$) from spermine treatment alone, by ANOVA with Dunnett's test. Key: □, untreated; ▨, spermine; ▧, MDL-72,527; ⊠, MDL-72,527 + spermine.

cells to spermine there was no change in the GSH content of the medium. No GSSG was detected in medium taken from control or spermine-treated cells (results not shown).

The concentration of BCNU used inhibited glutathione reductase activity by 58.8% in BHK cells after a 30 min period.

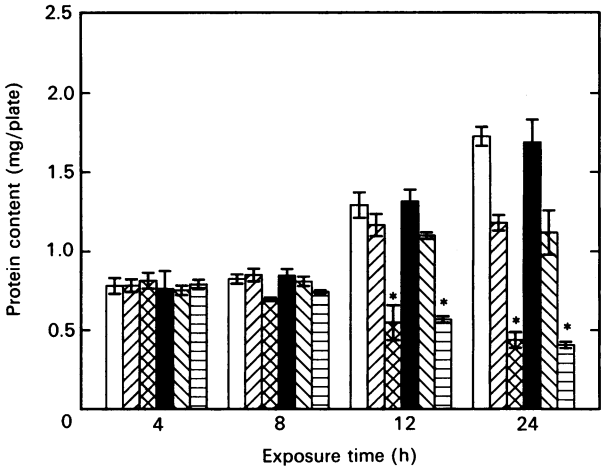


Fig. 3. Effect of BCNU on spermine toxicity in BHK-21/C13 cells

Spermine was added to both untreated and BCNU-pretreated cells (20 μM-BCNU, 30 min). After the 30 min pretreatment period, the cell sheet was washed twice with warm DMEM, and fresh DH_{10} was added with or without spermine. Protein content was used as a measure of cell growth. Values are means ± S.D. ($n = 3$): *significantly different ($P < 0.01$) from control values, i.e. spermine against untreated and BCNU + spermine combination treatment against BCNU alone, by ANOVA with Dunnett's test. Key: □, untreated; ▨, 1 mM-spermine; ⊠, 2 mM-spermine; ■, BCNU; ▧, BCNU + 1 mM-spermine; ▤, BCNU + 2 mM-spermine.

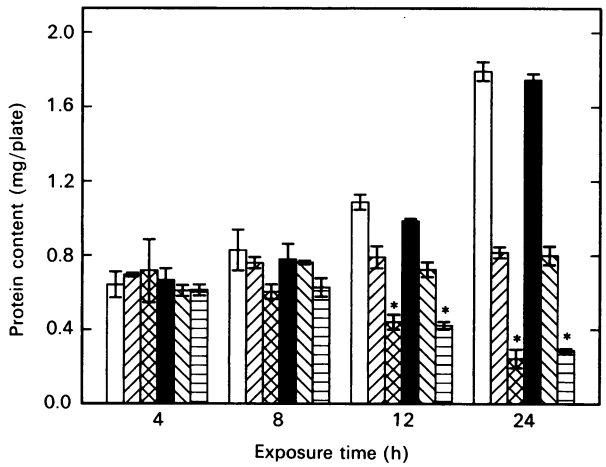


Fig. 4. Effect of AT on spermine toxicity in BHK-21/C13 cells

BHK cells were grown for 16 h before addition of 2 mM-AT. After 10 min the cell sheet was washed twice with warm DMEM, and fresh DH_{10} was added with or without spermine. Protein content was used as a measure of cell growth. Values are means ± S.D. ($n = 3$): *significantly different ($P < 0.01$) from control values, i.e. spermine against untreated and AT + spermine combination against AT alone, by ANOVA with Dunnett's test. Key: □, untreated; ▨, 1 mM-spermine; ⊠, 2 mM-spermine; ■, AT; ▧, AT + 1 mM-spermine; ▤, AT + 2 mM-spermine.

Greater inhibition of enzyme activity could not be achieved, owing to the toxicity of BCNU itself at higher concentrations (results not shown). Pretreatment of BHK cells with BCNU did not alter the effect of spermine on cell growth (Fig. 3).

After BCNU treatment there was a 20% decrease in the GSH content of BHK cells. The GSH content of these cells remained significantly lower than in untreated cells up to 24 h after removal of the nitrosourea. BCNU pretreatment had no effect on the GSH content of spermine-treated cells (results not shown).

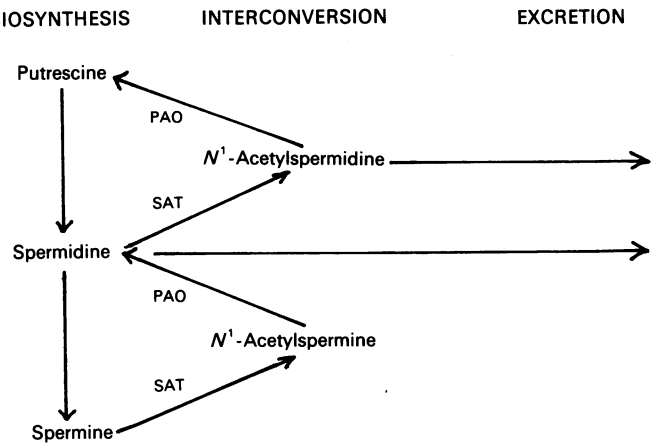
The treatment protocol for AT gave a 94.4% inhibition of catalase activity in BHK cells. Pretreatment of BHK cells with AT also did not alter the toxicity profile of spermine (Fig. 4). AT treatment had no effect on the GSH content of control or spermine-treated cells (results not shown).

DISCUSSION

In the absence of bovine serum amine oxidase, high concentrations of spermine were cytotoxic to BHK cells. This cytotoxic effect on BHK cell growth could be modulated by inhibitors of the amine oxidases responsible for spermine oxidation. Although the possibility that minute quantities of toxic aldehydes may be produced in the medium cannot be excluded completely, we have no evidence of any extracellular metabolism of [^{14}C]spermine in these studies. Therefore the effect of aminoguanidine pretreatment on the toxicity of 1 mM-spermine is likely to be due to the inhibition of intracellular copper-containing amine oxidases. Work by Smith *et al.* [17] also suggested that inhibition of lymphocyte proliferation by polyamines in horse-serum-supplemented medium was due to an amine oxidase activity produced by the cells themselves. They showed that treatment with the hydroxybenzyloxyamine compound NSD-1024, another inhibitor of copper-containing amine oxidases, completely reversed the polyamine-induced inhibition of lymphocyte proliferation in the presence of both foetal-calf and horse serum. In previous reports millimolar concentrations of spermine were shown to inhibit protein synthesis in Walker 256 carcinoma cells grown in horse serum [16], and it was proposed that spermine was interfering with the binding of mRNA to ribosomes. At high concentrations putrescine has also been shown to be bactericidal against the cyanobacterium *Anacystis nidulans* [41]. These workers demonstrated that there was no metabolism of putrescine and that the amine itself inhibited protein synthesis, perhaps by competing with other cations for binding sites on the ribosomes, leading to ribosome dissociation. Putrescine is not a substrate for bovine serum amine oxidase and inhibits fibroblast DNA synthesis only at millimolar concentrations. This inhibition was not prevented by aminoguanidine, suggesting that putrescine metabolism was not involved [6]. In BHK cells [^{14}C]spermine-incorporation studies showed that, at high doses of spermine (2 mM), there was no apparent intracellular oxidation of the amine (results not shown). Together these findings suggest that, when intracellular polyamines are allowed to accumulate to high concentrations, the amine itself, and not an oxidative metabolite, is responsible for the inhibition of growth, whereas at lower doses the intracellular levels may not be sufficient to inhibit cell growth, and oxidation by an aminoguanidine-sensitive enzyme appears to be involved in the response to spermine (Fig. 1).

Other evidence supporting a direct toxic effect caused by the polyamines themselves is presented in Fig. 2. Here inhibition of PAO potentiated the toxicity of spermine, arguing strongly that spermine is the toxic moiety, and not an oxidative metabolite.

Alternatively, PAO may play an important protective role in BHK cells by aiding the removal of excess spermine, since the intracellular spermine content was higher in MDL-treated cells (Table 2). It has been shown previously that the main excretory products of BHK cells are spermidine and N^1-acetylspermidine [42]. As demonstrated in Scheme 1, inhibition of PAO would effectively inhibit the removal of spermine by this route. Under normal conditions, intracellular levels of the acetyl-polyamines are very low and below the limit of detection by h.p.l.c. analysis. Although there was complete inhibition of PAO over the 24 h exposure period to spermine, there was no accumulation of



Scheme. 1. Routes of polyamine metabolism

Abbreviation: SAT, spermidine/spermine acetyltransferase.

acetylated polyamines within the cells, which would be expected from the pattern of events shown in Scheme 1. Previous workers have shown that inhibition of PAO in HTC cells only led to a build-up of acetylated polyamines after 3 days in culture [43]. These cells are reported to have relatively high PAO activity compared with other cell types [44]. In CHO cells accumulation of N^1-acetylspermidine after PAO inhibition was only observed after stimulation of PAO by heat shock [45]. The basal PAO activity in BHK cells is low (S. H. MacGowan & H. M. Wallace, unpublished work), and this, combined with the short time course of inhibition in the present study, may explain the lack of acetyl-polyamines detected in MDL-72,527 treated cells.

There have been several reports on the role of H_2O_2, oxygen radicals and oxidative stress in polyamine-induced toxicity in the presence of bovine serum amine oxidase. Inhibition of lymphocyte proliferation was independent of exogenously added catalase [24], whereas spermidine toxicity in CHO cells was decreased by the presence of catalase [46], as was the clastogenic effect of spermine in mouse lymphoma cells [47]. Studies using exogenous catalase are difficult to interpret, as the enzyme is a known constituent of serum, and its activity will vary between sera and different batches of the same serum. The inherent ability of the system to break down H_2O_2 will consequently be different. A more effective method of looking at the role of catalase is therefore to inhibit the enzyme with AT.

The results suggest that oxidative stress does not play an important role in spermine toxicity in BHK cells. Inhibition of neither catalase nor glutathione reductase influenced the effect of spermine on BHK cells growth (Figs. 3 and 4), and there was no efflux of GSSG from cells treated with spermine. In livers perfused with t-butyl hydroperoxide the excretion of GSSG has been shown to be related to the oxidative stress produced within the hepatocytes by this agent as the supply of NADPH becomes self-limiting [38,39].

In isolated hepatocytes 90% inhibition of GSSG reductase with 50 μM-BCNU led to a 40% decrease in their intracellular GSH levels [48], which was explained by the probable conjugation of GSH with an alkylating species formed during BCNU degradation [49]. To compensate for this decrease in GSH, the authors allowed the cells to replete their GSH levels by incubation in an amino-acid-rich buffer before their toxicity studies. The lower concentration of nitrosourea used in BHK cells may explain the differences in the extent of GSH depletion in these two systems. We have shown with buthionine sulphoximine that a 20% depletion of GSH in BHK cells does not alter the toxicity of spermine, and therefore we did not follow the protocol for GSH repletion in our study. The degree of inhibition of glutathione reductase by BCNU in our study may not have been sufficient to affect significantly the removal of H_2O_2. In L1210 cells, after complete inhibition of the enzyme by BCNU, 50% of the activity returned within 12 h, which was due to the resynthesis of new protein [50]. The turnover of glutathione reductase in BHK cells is not known, and these points should be considered before a role for glutathione reductase in the production of BHK cells against spermine toxicity is ruled out.

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