Effects of polyamine levels on the degradation of short-lived and long-lived proteins in cultured L-132 human lung cells

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Biogenic polyamines have important regulatory functions in various biological processes and it has also been suggested that they could modulate intracellular protein degradation. For an overall assessment of the role of polyamines in this process, we have investigated the effect that the decrease in intracellular polyamine levels caused by inhibitors of polyamine biosynthesis brings about on the degradation of the pools of short- and long-lived proteins in cultured L-132 human lung cells. Treatment of cells with 100 μ M (2*R*,5*R*)- δ -methyl acetylenic putrescine (MAP), a potent enzyme-activated irreversible inhibitor of ornithine decarboxylase, or with 100 μ M MAP plus 50 μ M *N*-butyl 1,3-diaminopropane, a specific inhibitor of spermine synthase, caused a similar decrease (65–70% of control) in the total intracellular

levels of polyamines, although they affected the concentrations of spermidine and spermine differently. The effect of the two treatments on protein degradation was essentially the same. In polyamine-depleted cells we observed an inhibition of degradation in long-lived proteins of 16% ($P < 0.05$), with a significant increase in the half-life ($t_{1/2}$) of this pool from 100.5 to 120.1 h. This was concomitant with an increase of 26% ($P < 0.05$) in degradation in short-lived proteins, with a significant decrease in the $t_{1/2}$ of this pool from 0.85 to 0.67 h. Recovery of polyamine levels by the addition of 50 μ M spermidine to polyamine-depleted cells resulted in a restoration of the degradation rates in both pools of proteins. The way(s) by which polyamines could modulate proteolysis are discussed.

INTRODUCTION

Polyamines (putrescine, spermidine and spermine) are naturally occurring polycations that have an important regulatory role in a wide variety of biological processes, including the modulation of membrane properties and functions [1–3], the interaction with macromolecules influencing the rate of biochemical reactions [4–6], the development of tumours [7], and apoptosis [8].

Intracellular levels of these compounds, as well as the activities of the enzymes involved in their synthesis, are known to be regulated in response to growth factors, hormones and tumour promoters [9,10], and the association of polyamine biosynthesis with cell proliferation has been reported repeatedly [11,12]. It has been well established that they stimulate cell growth by increasing the rates of synthesis of DNA, as well as of proteins, by increasing the stability of mRNA species and stimulating the translation process [7,13,14]; a possible role of these compounds modulating intracellular proteolysis has also been suggested [15]. They have been reported to inhibit acidic and neutral proteases in oat leaves [16], neutral proteases in alfalfa leaf extracts [17] and the ATP-dependent proteolytic pathway in reticulocyte lysates [18]. Studies *in vitro* have also revealed that polyamines markedly increase the resistance to proteinases of some polypeptides, most probably by inducing changes in their conformational state rather than by affecting proteinase activity [19], but a physiological role of polyamines in modulating proteolysis *in vivo* remains to be clarified.

In the present study we investigate the effect that the decrease of intracellular polyamine levels causes on the degradation rates of the pools of short-lived and long-lived proteins in

exponentially growing cultures of human embryo lung cells (L-132). Intracellular polyamine levels were decreased in these cells by using the following inhibitors of polyamine biosynthesis: the suicide ornithine decarboxylase (ODC) substrate α -difluoromethylornithine (DFMO), the potent enzyme-activated irreversible inhibitor of ODC, (2*R*,5*R*)- δ -methyl acetylenic putrescine (MAP), and the specific inhibitor of spermine synthase, *N*-butyl-1,3-diaminopropane (N-BDAP).

EXPERIMENTAL

Chemicals

[¹⁴C]Leucine, [³H]leucine and [³⁵S]methionine were purchased from Amersham International (Little Chalfont, Bucks., U.K.). Putrescine, spermidine, spermine (hydrochloride salts) and aminoguanidine were purchased from Sigma Chemical Co. DFMO, MAP and N-BDAP were generously provided by Marion Merrell Dow Research Institute (Strasbourg, France).

Cell culture

L-132 cells (line derived from normal human embryonic lung; ATCC no. CCL 5; Flow Laboratories, Irvine, Scotland, U.K.) were grown in monolayer (25 cm² flasks; A/S Nunc, Roskilde, Denmark) at 37 °C in standard growth medium [minimal essential medium supplemented with Earle's salts, 10% (v/v) foetal bovine serum, 100 i.u./ml penicillin and 100 mg/ml streptomycin] under

Abbreviations used: CI, confidence interval; DFMO, α -difluoromethylornithine; MAP, (2*R*,5*R*)- δ -methyl acetylenic putrescine; N-BDAP, *N*-butyl-1,3-diaminopropane; ODC, ornithine decarboxylase; TCA, trichloroacetic acid.

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air/CO₂ (19:1). Cells were seeded at 5×10^4 cells/ml in standard growth medium, and inhibitors of polyamine biosynthesis were added, at the time of plating, at the concentrations and for the times shown in the legends (usually 100 μ M MAP and/or 50 μ M N-BDAP). In some experiments, exogenous putrescine, spermidine or spermine (50 μ M) was added to the medium for 24 h, after the initial treatments. In these cases, media were supplemented with 1 mM aminoguanidine to inhibit serum amine oxidase activity [20]. During treatments, the culture medium was replaced every 48 h. An additional replacement or reseeded of cells was made as indicated. Cell growth was measured by cell counting in a haemocytometer, and viability of cells was assessed by the Trypan Blue exclusion test.

Polyamine determinations

Polyamines were determined by HPLC. Cell samples were washed twice in cold PBS; an aliquot was removed for polyamine determination. The cells were pelleted and the pellet was extracted with 0.6 M HClO₄ for 30 min at 4 °C and then centrifuged for 10 min at 12000 *g*. Supernatants were dansylated by the method of Flores and Galston [21]. Portions (35 μ l) of dansylated extracts in acetonitrile were applied to a reverse-phase column (250 mm long, 4 mm internal diam.) of Spherisorb ODS-2 (5 mm) and eluted with a programmed 60–90 % (v/v) acetonitrile solvent gradient over 35 min at a flow rate of 1.5 ml/min at 35 °C. An attached fluorescence spectrophotometer equipped with an integrator was used to quantify the dansyl derivatives. The results were expressed as nmol/mg of protein. Protein was determined by the method of Lowry et al. [22].

Measurements of protein synthesis

The effect of polyamine levels on protein synthesis in control and treated cells was determined by the ability of cells to incorporate [³⁵S]methionine (25 μ Ci/ml) as described previously [23]. Radioactivity incorporated into the protein present in the samples was measured by liquid-scintillation counting. Proteins synthesized by cultured cells were analysed by SDS/PAGE and further fluorography.

Measurements of protein degradation

To study the turnover of short-lived proteins, after 72 h of incubation in standard growth medium in the presence or absence of polyamine biosynthesis inhibitors, cells were incubated for 15 min with 10 μ Ci/ml [³H]leucine ('short-pulsed'), washed twice with PBS containing 2 mM leucine and incubated at 37 °C for 10 min, to chase free labelled amino acids from the intracellular pools, before starting protein degradation measurements.

In separate experiments, long-lived proteins were labelled selectively: cells previously grown for 48 h in standard growth medium without or with inhibitors (the former being the control group) were incubated with 1 μ Ci/ml [¹⁴C]leucine for 24 h. Cells were washed twice in PBS containing 2 mM leucine and incubated (in the continued presence of inhibitors in treated cells) for 16 h in standard growth medium plus 2 mM leucine to chase short-lived proteins, before starting protein degradation measurements. In the experiments on the recovery of polyamine levels, and to test whether differences in the level of confluence of cells affected the rates of protein degradation in the long-lived pool, control cells and those previously treated for 48 h were detached from the flasks by mild treatment with trypsin and re-plated at the same starting density (5×10^4 cells/ml), then cultured in standard growth medium with or without inhibitors. After 4 h, 1 μ Ci/ml

[¹⁴C]leucine was added to the medium for 24 h. Cells were washed twice in PBS containing 2 mM leucine, then incubated for 24 h with standard growth medium plus 50 μ M spermidine, inhibitors or no additions, before protein degradation measurements were started.

Measurements of intracellular protein degradation were performed in control and treated cells, as described previously [24]. In brief, 500 μ l portions of the medium were taken during the chase period, and 500 μ l of 10 % (w/v) trichloroacetic acid (TCA) was added to each portion. After 30 min in a ice bath, the samples were centrifuged for 10 min at 5000 *g*; the pellet was then washed with 500 μ l of 5 % (w/v) TCA and centrifuged again. Supernatants from both centrifugations were pooled and the pellets were dissolved in 1 M NaOH and neutralized with HCl. The radioactivities in supernatant and pellet were measured by liquid-scintillation counting. At the end of the chase period, the final radioactivity in the cells was determined as described for protein synthesis. The initial radioactivity was calculated by adding the final radioactivity in the cells to the total radioactivity released into the medium during the chase period. TCA-precipitable radioactivity released into the medium per hour was always less than 1 % of the original radioactivity, and represents protein secretion and/or detachment of cells from the monolayer. Protein degradation at each chase time point (Δ_i) was calculated by dividing the total TCA-soluble radioactivity released at that time into the medium by the initial radioactivity in the cells and is expressed as a percentage. All results are means $\pm$ S.D. for three or more experiments.

Mathematical model for assessing short-lived and long-lived protein degradation kinetics

To establish whether the intracellular protein degradation represented a single pool of proteins (i.e. pure exponentials), we plotted the function

$$\ln[1 - (\Delta_i/100)] = f(t)$$

to test whether they fitted the equation

$$\ln[1 - (\Delta_i/100)] = -kt \quad (1)$$

where Δ = percentage of protein degradation, k expresses the turnover rate V/N (where $V = dn/dt$ is the velocity of protein turnover in the pool, and N is the total protein in the pool), and t is the chase duration.

Eqn. (1) is deduced from

$$n_t^* = n_0^* e^{-kt} \quad (2)$$

which expresses the decay of labelling in a unique pool during the chase duration, assuming steady-state conditions [25,26], where n_t^* is the labelled protein present in the pool at time t of chase, n_0^* is the labelled protein present in the pool at the beginning of the chase, and k is the turnover rate of the pool.

This becomes evident considering that eqn. (2) could be expressed as:

$$[(n_0^* - n_t^*)/n_0^*] = 1 - e^{-kt}$$

and because

$$\Delta_i = 100[(n_0^* - n_t^*)/n_0^*]$$

finally

$$1 - (\Delta_i/100) = e^{-kt}$$

Taking logarithms of the last equation yields eqn. (1). Half-lives ($t_{1/2}$) of the protein pools were calculated from the estimated values for k , by using the equation $t_{1/2} = -\ln 2/k$.

Statistical analysis

Comparisons between two sample means were made by unpaired Student's *t* test with analysis of variance. In some cases 95% confidence intervals (CIs) were calculated from the S.E.M. of the estimate. Linear regression analysis was performed to fit degradation kinetic values.

RESULTS

Inhibition of polyamine levels

Polyamine levels in cultures of L-132 cells were decreased by the addition of inhibitors of polyamine biosynthesis to the culture medium. Table 1 shows that maximal depletion of polyamines, using the ODC inhibitors DFMO and MAP, at concentrations compatible with 90–99% cell viability, required approx. 3 days with 100 μ M MAP, or more than 4 days with 1 mM DFMO. Each treatment decreased putrescine and spermidine but the concentration of spermine was hardly decreased. Therefore, in further experiments, treatment with MAP was preferred to that with DFMO. To improve the depletion of spermine levels, we tested N-BDAP, a potent and specific inhibitor of spermine synthase, which, after 3 days at a concentration of 50 μ M, caused a marked decrease in the spermine content accompanied by a strong increase in the concentration of spermidine. The combined treatment of cells with 100 μ M MAP plus 50 μ M N-BDAP decreased the intracellular concentrations of putrescine, spermidine and spermine to approx. 0%, 58% and 14% of those in control cells respectively. Because other combinations of the two inhibitors resulted in a lower depletion, when polyamine-depleted cells were required the culture medium included 100 μ M MAP, or 100 μ M MAP plus 50 μ M N-BDAP, 3 days before starting the

experiment. Although these two treatments affected the levels of spermidine and spermine differently, they caused a similar decrease in the total concentration of polyamines (65–70%).

Restoration of the normal polyamine contents of cells by exogenous polyamines

To find adequate conditions to restore normal intracellular polyamine contents, cells that had been treated with 100 μ M MAP for 3 days were washed and incubated in a medium containing one of the polyamines putrescine, spermidine or spermine, each at 50 μ M. After 24 h of incubation the polyamine concentration in cells was measured, revealing that the addition of putrescine restored spermidine content but putrescine and spermidine levels were slightly increased; spermine restored spermine and putrescine levels but spermidine was only 58% of the control; and spermidine restored the polyamine content to 95% of the control value, with a final concentration of the three polyamines approximating those in control cells (Figure 1). Therefore for reversal studies 50 μ M spermidine was added to cells treated with MAP for 72 h, as described above.

Cell growth and protein synthesis

Polyamine depletion resulted, after a lag period, in a decrease in cell growth that was recovered after the restoration of normal polyamine levels in treated cells (Table 2). After 3 days of treatment with 100 μ M MAP or 100 μ M MAP/50 μ M N-BDAP the rate of protein synthesis, as measured by the incorporation of [³⁵S]methionine, was respectively $69 \pm 7\%$ or $50 \pm 9\%$ that of control cells. The synthesized proteins of treated and untreated

Table 1 Polyamine levels in cultured L-132 cells in the presence or absence of DFMO, MAP and/or N-BDAP

L-132 cells were seeded at an initial density of 5×10^4 cells/ml and cultured in monolayer at 37 °C for 24, 48, 72 or 96 h in standard growth medium. Inhibitors of polyamine biosynthesis were added at the time of plating for the times and concentrations shown. Polyamines were determined by HPLC as described in the Experimental section. Values are means $\pm$ S.D. for five or seven experiments. Values in the last column are the total polyamine contents (putrescine + spermidine + spermine) of each culture, expressed as a percentage of the control. Abbreviation: n.d.: not detected (less than 0.1 nmol/mg of protein).

Addition	Time (h)	Polyamines (nmol/mg of protein)			Total polyamine content	
		Putrescine	Spermidine	Spermine	(nmol/ml)	(% of control)
None	0	8.0 $\pm$ 1.2	14.2 $\pm$ 0.8	16.1 $\pm$ 1.5	38.3 $\pm$ 2.9	100
	24	14.1 $\pm$ 1.5	18.1 $\pm$ 0.7	21.2 $\pm$ 1.8	53.3 $\pm$ 4.7	100
	48	13.5 $\pm$ 1.2	19.0 $\pm$ 1.1	22.0 $\pm$ 1.4	54.6 $\pm$ 5.0	100
	72	10.5 $\pm$ 0.9	15.3 $\pm$ 0.8	18.2 $\pm$ 1.2	44.0 $\pm$ 3.7	100
	96	8.8 $\pm$ 1.3	15.4 $\pm$ 0.7	17.9 $\pm$ 0.8	42.1 $\pm$ 4.1	100
MAP (100 μ M)	24	1.3 $\pm$ 0.2	12.9 $\pm$ 0.5	23.5 $\pm$ 2.0	37.7 $\pm$ 2.0	71.1
	48	0.5 $\pm$ 0.1	6.5 $\pm$ 0.3	21.1 $\pm$ 1.7	28.1 $\pm$ 2.5	51.4
	72	n.d.	n.d.	15.0 $\pm$ 1.2	15.0 $\pm$ 1.2	34.1
	96	n.d.	n.d.	15.6 $\pm$ 1.1	15.6 $\pm$ 1.1	35.3
DFMO (1 mM)	72	1.5 $\pm$ 0.4	7.5 $\pm$ 0.7	16.3 $\pm$ 1.2	25.3 $\pm$ 2.1	57.1
	96	0.5 $\pm$ 0.2	4.7 $\pm$ 0.5	14.5 $\pm$ 0.9	19.7 $\pm$ 1.8	46.8
N-BDAP (50 μ M)	24	14.0 $\pm$ 1.3	21.0 $\pm$ 1.5	10.5 $\pm$ 1.0	45.5 $\pm$ 3.9	85.8
	48	8.5 $\pm$ 0.9	24.0 $\pm$ 1.9	6.8 $\pm$ 0.7	39.3 $\pm$ 3.7	72.1
	72	5.1 $\pm$ 0.7	25.5 $\pm$ 2.1	1.5 $\pm$ 0.4	32.1 $\pm$ 2.8	73.1
MAP (100 μ M) + N-BDAP (50 μ M)	24	1.4 $\pm$ 0.3	22.0 $\pm$ 1.5	11.0 $\pm$ 0.9	34.4 $\pm$ 2.9	64.9
	48	0.5 $\pm$ 0.1	16.1 $\pm$ 1.3	8.5 $\pm$ 0.7	25.1 $\pm$ 1.7	45.9
	72	n.d.	8.8 $\pm$ 0.9	2.6 $\pm$ 0.5	11.4 $\pm$ 0.8	26.2
	96	n.d.	9.5 $\pm$ 1.1	2.1 $\pm$ 0.3	11.6 $\pm$ 0.7	27.6

Figure 1 Polyamine levels in cultured L-132 cells in the presence or absence of MAP: effects of exogenous added polyamines [putrescine (PUT), spermidine (SPD) or spermine (SPM)] on the polyamine content of MAP-treated cells

L-132 cells were seeded at 5×10^4 cells/ml in standard growth medium and grown for 72 h with the indicated treatments (control or 100 μ M MAP); polyamine concentrations were measured as described in the Experimental section. The results for cells incubated for 72 h without or with inhibitor are labelled 'Control 72 h' and 'MAP 72 h' respectively. At that time, the medium was changed and cells were incubated in standard growth medium containing 1 mM aminoguanidine for a further 24 h with (MAP, PUT, SPD or SPM) or without treatment ('Control 96 h'). The results for cells treated for 72 h with 100 μ M MAP, and for 24 h with 100 μ M MAP, are labelled 'MAP 96'. The results for cells treated for 72 h with 100 μ M MAP and for 24 h with 50 μ M putrescine, 50 μ M spermidine or 50 μ M spermine, are labelled 'MAP72 + PUT', 'MAP72 + SPD', 'MAP72 + SPM' respectively. Cells were harvested and analysed for polyamine content at the end of the 24 h incubation period. Values are nmol of each polyamine/mg of protein, averaged from four to six replicate determinations. Error bars are S.D.

cells seemed to be the same, as judged by SDS/PAGE and fluorography (results not shown).

Effect of polyamines on the degradation of short-lived and long-lived proteins

Degradation of long-lived proteins

L-132 cells were cultured with or without inhibitors of polyamine biosynthesis in the culture medium (100 μ M MAP or 100 μ M

Figure 2 Effect of polyamine depletion on the degradation of long-lived proteins in L-132 cells

L-132 cells were seeded at 5×10^4 cells/ml in standard growth medium, then grown for 72 h with the indicated treatments (control, 100 μ M MAP or 100 μ M MAP/50 μ M N-BDAP). Long-lived proteins were labelled and protein degradation was calculated as described in the Experimental section. Symbols: ●, control cells; ○, 100 μ M MAP; ▲, 100 μ M MAP/50 μ M N-BDAP. Each point is the mean $\pm$ S.D. for four different experiments with duplicate samples.

MAP/50 μ M N-BDAP). Long-lived proteins were pulse-labelled and protein degradation was calculated as described in the Experimental section. Figure 2 shows the kinetics of protein degradation for control and polyamine-depleted cells. It is apparent that treatment of cells with 100 μ M MAP (depleting cells of putrescine and spermidine) or with 100 μ M MAP/50 μ M N-BDAP (depleting cells of putrescine and spermine) resulted in both cases in a similar and significant decrease ($P < 0.05$) in the degradation rate by approx. 15% after 6 h of chase, compared with control cells. There were no significant differences ($P > 0.05$) in protein degradation between treatments with MAP and with MAP/N-BDAP. Restoration of the normal polyamine content in MAP-treated cells resulted in the recovery of the

Table 2 Effects of MAP and MAP/N-BDAP on cell growth of L-132 cells: reversal of polyamine depletion by spermidine

L-132 cells were seeded at an initial density of 5×10^4 cells/ml and cultured in monolayers at 37 °C for 24, 48, 72 or 98 h in standard growth medium. Inhibitors of polyamine biosynthesis were added at the time of plating for the times and concentrations shown. Polyamines were determined by HPLC as described in the Experimental section. Values are means $\pm$ S.D. for three or four experiments. Results in the last column are for cells cultured for 72 h with 100 μ M MAP that were washed and incubated for 24 h in standard medium containing 50 μ M SPD. *Significant differences ($P < 0.05$) between control and treated cells; †significant differences ($P < 0.05$) between treatment with MAP and treatment with N-BDAP or SPD.

Time (h)	10 ⁻⁵ × Cell density/ml			
	Control	100 μ M MAP	100 μ M MAP + 50 μ M N-BDAP	100 μ M MAP + 50 μ M SPD
0 (seeding)	0.5	0.5	0.5	0.5
24	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.1
48	2.2 ± 0.2	1.8 ± 0.2	1.5 ± 0.3*	1.8 ± 0.2
72	4.9 ± 0.4	3.0 ± 0.2*	2.5 ± 0.4*	3.0 ± 0.2*
96	9.2 ± 0.8	4.7 ± 0.3*	4.2 ± 0.5*	6.1 ± 0.4*†

Figure 3 Effect of exogenous polyamines on the degradation of long-lived proteins in L-132 cells: reversibility of the inhibitory effect of depletion

L-132 control cells and MAP (100 μ M)-treated cells were cultured for 48 h and reseeded at 5×10^4 cells/ml and cultured for 24 h in standard growth medium with or without 100 μ M MAP. During this period, long-lived proteins were labelled as described in the Experimental section. Controls and cells treated for 72 h with 100 μ M MAP were incubated for a further 24 h in presence of no additions (control), 100 μ M MAP or 50 μ M spermidine (SPD) to restore the normal polyamine content. Aminoguanidine (AG; 1 mM) was added to prevent the action of amino-oxidases, and the effect of this compound on the degradation of long-lived proteins was also tested. Symbols: $\bullet$, control cells; $\triangle$, control + 1 mM AG; $\circ$, 100 μ M MAP; $\square$, 100 μ M MAP/1 mM AG; $\blacktriangle$, 100 μ M MAP/50 μ M SPD/1 mM AG. Each point is the mean $\pm$ S.D. for three different experiments with duplicate samples.

Figure 4 Effect of polyamine depletion on the degradation of short-pulsed proteins in L-132 cells

L-132 cells were seeded at 5×10^4 cells/ml in standard growth medium, then grown for 72 h with the indicated treatments (control, 100 μ M MAP or 100 μ M MAP/50 μ M N-BDAP). Cells were 'short-pulsed' and protein degradation was calculated as described in the Experimental section. Symbols: $\bullet$, control cells; $\circ$, 100 μ M MAP; $\blacktriangle$, 100 μ M MAP/50 μ M N-BDAP. Each point is the mean $\pm$ S.D. for five different experiments with duplicate samples.

Figure 5 Effect of the restoration of the normal polyamine profile on the degradation of short-lived proteins in L-132 cells: reversibility of the effect of depletion

L-132 cells were seeded at 5×10^4 cells/ml in standard growth medium, then grown for 72 h with the indicated treatments (control or 100 μ M MAP). Proteins were 'short-pulsed' and protein degradation was calculated as described in the Experimental section. Control and cells treated for 72 h with 100 μ M MAP were incubated for a further 24 h in the presence of no additions (control), 100 μ M MAP or 50 μ M spermidine (SPD) to restore the normal polyamine content. Aminoguanidine (AG; 1 mM) was added to prevent the action of amino-oxidases, and the effect of this compound on the degradation of long-lived proteins was also tested. Symbols: $\bullet$, control cells; $\triangle$, control + 1 mM AG; $\circ$, 100 μ M MAP; $\square$, 100 μ M MAP/1 mM AG; $\blacktriangle$, 100 μ M MAP/50 μ M SPD/1 mM AG. Each point is the mean $\pm$ S.D. for three different experiments with duplicate samples.

kinetics of degradation of control cells (Figure 3). There were no significant differences ($P > 0.05$) in the degradation rates of long-lived proteins between control and polyamine-restored cells. Owing to the presence of aminoguanidine in the culture medium, an additional control with 1 mM aminoguanidine was tested. This compound had no effect on the degradation of long-lived proteins.

Degradation of short-lived proteins

L-132 cells were 'short-pulsed' and protein degradation was measured as described in the Experimental section. Figure 4 shows the kinetics of degradation of the 'short-pulsed' proteins in control and polyamine-depleted cells. The degradation of this pool of proteins in cells that were treated with 100 μ M MAP or with 100 μ M MAP/50 μ M N-BDAP was 1.6-fold ($P < 0.05$) that in control cells, after 4 h of chase. There were no significant differences ($P > 0.05$) in protein degradation rates between the two polyamine-depletion treatments. As with long-pulsed proteins, restoration of the normal polyamine contents resulted in the recovery of the kinetics of degradation of control cells (Figure 5) without statistically significant differences ($P > 0.05$) in the degradation rates between control and polyamine-restored cells. An additional control for the effects of 1 mM aminoguanidine on the short-pulsed protein degradation was also included.

Mathematical analysis of kinetics of degradation in Figures 2 and 4 was performed by testing the function $\ln[1 - (\Delta_t/100)] = -kt$ with the data in these Figures. Owing to the similar effects on protein degradation of both polyamine-depletion treatments,

Figure 6 Plot of equation $\ln(1 - \Delta/100) = -f(t)$ for the pool of long-lived proteins in control and MAP-treated cells

Data from Figure 3 were transformed according to the equation $\ln(1 - \Delta/100) = -f(t)$ (see the Experimental section). Symbols: ●, control cells; ○, 100 μ M MAP. In both cases the values fit well (see r^2 values) with straight lines, indicating that the kinetics of degradation corresponds to single pools with turnover rates, k_1 of $k_1 = -0.00689 \pm 0.00024 \text{ h}^{-1}$ and $k_2 = -0.00581 \pm 0.00024 \text{ h}^{-1}$ for control and MAP-treated cells.

we show only the mathematical analysis for data obtained from the treatment with MAP. Results with N-BDAP were very close and without statistically significant differences.

Figure 6 is the plot of eqn. (1) for the pool of long-lived proteins in control and treated cells (100 μ M MAP). In both cases the values fit well (r^2 for control cells is 0.997 ± 0.001 ; r^2 for polyamine-depleted cells is 0.996 ± 0.001) with straight lines, indicating that they correspond to single pools with turnover rates of $k_1 = -0.00689 \pm 0.00024 \text{ h}^{-1}$ and $k_2 = -0.00581 \pm 0.00024 \text{ h}^{-1}$ for control and treated cells respectively. These values are the slopes of the adjusted straight lines shown in Figure 6. The $t_{1/2}$ values for these pools calculated from the k values (see the Experimental section) are 100.5 h (95% CI: 94.1–108.1) for control cells, and 120.1 h (95% CI: 111.2–131.0) for treated cells. The expression $(k_2 - k_1)/k_1$ indicates an inhibition of degradation in long-lived proteins of approx. 16% in polyamine-depleted cells.

Figure 7 is the plot of eqn. (1) for 'short-pulsed' proteins in control and MAP-treated cells. It is evident that those values do not fit well with pure exponentials, indicating that these proteins are not from a single pool (Figure 7A). In fact they fit much better with the kinetics resulting from the overlapping of two pure exponentials [25,26]: one of fast decay (short-lived proteins) and another of slow decay (long-lived proteins), which combine to describe the experimental curve. To test this hypothesis, the results of Figure 7 were analysed in accordance with previous papers [25,26], taking in consideration that the slope of the curve depicted in the Figure will decrease to reach a constant value, as a straight tail-end, that should correspond to the slope calculated above for the pool of long-lived proteins. This approach allowed us to calculate the values corresponding to short-lived proteins by extrapolating back to the ordinate the straight tail-end of the curve, and subtracting the estimated values of this line from those of the experimental curve, as well as to estimate the

Figure 7 Plot of equation $\ln(1 - \Delta/100) = -f(t)$ for the pool of 'short-pulsed' proteins in control and MAP-treated cells: degradation kinetics for long-lived and short-lived proteins

Data from Figure 5 were transformed according to the equation $\ln(1 - \Delta/100) = -f(t)$ (see the Experimental section). Symbols: ●, control cells; ○, 100 μ M MAP. In both cases the values do not fit well with straight lines, indicating that the kinetics of degradation are not from single pools (A). Taking into account that the slope of the curve depicted in the Figure will decrease to reach a constant value, as a straight line, that should correspond to the slope calculated above for the pool of long-lived proteins allows us to calculate the values corresponding to short-lived proteins by extrapolating back to the ordinate the straight tail-end of the curve, and subtracting the estimated values of this line from those of the experimental curve. The obtained values fit well pure exponential kinetics in both cases (B).

contribution of the short-lived pool ($n_{s,0}^*$) and the long-lived pool ($n_{l,0}^*$) to the total label ($n_0^* = n_{s,0}^* + n_{l,0}^*$) measured at zero time as indicated in the Experimental section, in control and MAP-treated cells. The values obtained fit pure exponential kinetics [eqn. (1)] in both control and MAP-treated cells, with k values of $k_1 = -0.817 \pm 0.028 \text{ h}^{-1}$ and $k_2 = -1.032 \pm 0.040 \text{ h}^{-1}$ respectively; $t_{1/2}$ values of 0.85 h (95% CI: 0.79–0.91) and 0.67 h (95% CI: 0.62–0.73) respectively (Figure 7B); and $n_{s,0}^* = 20600 \text{ c.p.m.}$, $n_{l,0}^* = 67600 \text{ c.p.m.}$ in control cells, and $n_{s,0}^* = 19400 \text{ c.p.m.}$, $n_{l,0}^* = 33700 \text{ c.p.m.}$ in MAP-treated cells.

We calculated the proportion of protein belonging to the pools of short- and long-lived proteins, in control and MAP-treated cells, assuming steady-state conditions, by applying the following equations [26]:

$$N_s = n_s^{**} / (1 - 2^{-t/t_{1/2s}})$$

$$N_L = n_L^{**} / (1 - 2^{-t/t_{1/2L}})$$

where: $t_{1/2s}$ and $t_{1/2L}$ are the half-lives of short-lived and long-lived

Table 3 Percentage of short-lived and long-lived proteins in control and MAP-treated cells

L-132 cells were grown in standard growth medium with (MAP-treated) or without (control) the addition of MAP. Cells were incubated for 15 min (0.25 h) with 10 $\mu\text{Ci/ml}$ [^3H]leucine, and protein degradation was measured as described in the Experimental section. Results are means for four different experiments with duplicate samples. $t_{1/2}$ and $t_{1/2}$ are the half-lives of short-lived and long-lived proteins, respectively; n_s^{**} and n_l^{**} are the label in proteins of the short-lived and long-lived pools respectively, just at the end of the pulse time (t); N_s and N_l are the estimated values of labelling of each pool when saturated with label.

Treatment	Pulse time, t (h)	n_s^{**} (c.p.m.)	$t_{1/2}$ (h)	$10^{-5} \times N_s$ (c.p.m.)	n_l^{**} (c.p.m.)	$t_{1/2}$ (h)	$10^{-7} \times N_l$ (c.p.m.)	$100N_s/(N_s + N_l)$ (%)	$100N_l/(N_s + N_l)$ (%)
Control	0.25	25300	0.85	1.37	67750	100.5	3.96	0.34	99.66
MAP	0.25	25100	0.67	1.62	33800	120.1	2.34	0.69	99.31

proteins respectively; n_s^{**} and n_l^{**} are the labelled proteins of the short-lived and long-lived pools respectively, just at the end of the pulse time t (0.25 h, in our experiment) [taking into account that measurement started 15 min ($= 0.25$ h) after the end of the pulse, n_s^{**} and n_l^{**} were estimated from the corresponding degradation curves by extrapolating to $t = -0.25$ h]; and N_s and N_l are estimates of the values of labelling that would be reached in each pool when saturated with label ($\lim n^{**} = N$, when $t \rightarrow \infty$).

The proportions of short-lived and long-lived proteins in control and MAP-treated cells are shown in Table 3. These values indicate that short-lived proteins represent approx. 0.34 % of cell proteins in control cells, and 0.69 % in MAP-treated cells.

DISCUSSION

For a global assessment of a possible role of polyamines modulating intracellular proteolysis, measurements of degradation of 'short-lived' and 'long-lived' proteins have been performed in cultures of L-132 cells with or without specific inhibitors of polyamine biosynthesis. Depletion of polyamines from cells is not an immediate and total process and more than one full cell cycle in the presence of appropriate inhibitors is required for attainment of the lowest polyamine levels compatible with cell viability. In the experiments reported in the present paper, it was found that both MAP and MAP/N-BDAP treatments significantly decreased the intracellular levels of all three polyamines. Although these two treatments affect spermidine and spermine differently, they provoked a similar decrease (65–70 %) in the total intracellular concentration of polyamines. In fact the results obtained in the experiments performed to evaluate the effect of polyamine depletion on protein degradation were essentially the same with both treatments. This agrees with the results reported by Nagarajan et al. [27] in SV-3T3 cells, suggesting that, although for some specific functions a particular polyamine has been shown to be preferred [28,29], either spermidine or spermine can support cell growth and protein synthesis. It should be noted that the viability of treated L-132 cells was preserved (more than 90 %) under the conditions used in the experimental procedure, and the removal of inhibitors and the further addition of 50 μM spermidine resulted in a rapid recovery of normal intracellular polyamine levels as well as of the normal rates of growth and protein synthesis, as reported for other cell lines [30].

The measurements of intracellular protein degradation in exponentially growing cultures of L-132 cells reported in this paper have allowed a determination of the turnover rates and the proportions of short-lived and long-lived proteins in these cells, as well as an analysis of how the decrease in polyamines affected these values. The results obtained show that the degradation rate in long-lived proteins was inhibited by approx. 16 % in poly-

amine-depleted cells, with a significant increase ($P < 0.05$) in the half-life of this pool. In short-lived proteins, degradation was increased approx. 26 %, with a significant shortening ($P < 0.05$) of the half-life. We also found that this change affected the proportion of short-lived proteins, which increased from 0.34 % in control cells to 0.69 % in polyamine-depleted cells. It must be noted that the effect of the treatments on protein degradation is not due to the inhibitors themselves (MAP or MAP/N-BDAP) because the presence of these compounds in the medium did not affect protein turnover until polyamine levels were decreased significantly.

If these results are taken as a whole, it seems interesting to discuss a possible interrelationship between the changes reported in this study for the two pools of proteins as a consequence of polyamine depletion. Such a discussion should include a reference to the nature of the proteins belonging to these pools; on this point, several considerations should be taken in account. As we have shown previously [26], under steady-state conditions the pool of short-lived proteins includes only approx. 1–5 % of the total cell protein [31,32], although they can be preferentially labelled because they turn over one to two orders of magnitude faster than those belonging to the long-lived pool [33]. Studies performed to characterize the proteins of both pools suggest strongly that they are substantially the same. At least this is the conclusion when short-pulse-labelled and long-pulse-labelled proteins are examined by SDS/PAGE and further fluorography [34]. In fact it has been proposed that these short-lived proteins would include mostly neo-synthesized peptide chains that do not assume a functional quaternary structure or do not form large macromolecular complexes in their localizations, because they are produced in excess or because they are abnormal and prematurely degraded and do not become integrated into a functional conformation/complex in which they would exhibit a longer half-life. This does not exclude the presence in the short-lived pool of particular peptides with specific short half-lives, such as regulatory and secretory proteins [35].

The increased proportion of short-lived proteins that we report for polyamine-depleted cells could be interpreted as a result of the accumulation of neo-synthesized polypeptides owing to the decrease in the turnover rate of the pool of long-lived proteins that would prevent them from being integrated into their final functional locations at the same speed as under control conditions. It is important to note that polyamines are essential for mRNA translation by increasing the stability of mRNA species and facilitating mRNA–tRNA–ribosome interactions in a specific manner and there is good evidence that depletion of polyamines results in inaccurately translated peptides [14,36]. This could explain the 40 % decrease in the rate of incorporation into the protein pool, as well as the 18 % increase in the rate of label incorporation into the short-lived protein pool in polyamine-depleted cells.

It has been reported that in cultured cells the selective inhibition of degradation of long-lived proteins can be achieved partly by a variety of agents, including nutritional conditions (serum), growth factors and chemicals [37]. However, very few agents or conditions have been reported to modify the proteolytic rate of short-lived proteins. We have reported [38] that centrifugation forces of 11 800 *g* or more for 30 min markedly inhibited the rate of protein degradation of short-lived proteins without affecting the degradation of long-lived proteins. More recently it has been shown that vanadate inhibits the intracellular degradation of short-lived proteins and accelerates the degradation of long-lived proteins in L132 cells [39]. Our present results indicate that polyamine depletion also affects the degradation of both pools, as with vanadate, although in an opposite direction, and establish the contribution that changes in the turnover rates bring to the overall effect. The 40 % decrease in the rate of incorporation into the long-lived pool in polyamine-depleted cells is in good agreement with the decrease in protein synthesis reported above for treated cells because this pool represents more than 99 % of the cell protein in both control and treated cells. This is comparable with the values reported by others for other cell lines treated with inhibitors of polyamine biosynthesis [40]. It is also interesting to underline that, in spite of the paucity of short-lived proteins in the cell, the relative increase in short-lived proteins from 0.34 % to 0.69 % in polyamine-depleted cells should be kept in mind because the energy consumed in their rapid turnover represents a large percentage of the total energy consumed in this process [26].

Concerning the possible role of intracellular polyamines on protein degradation, there are few studies that have examined this aspect of polyamine function. Special mention is made of the role of polyamines in the proteolysis of the very short-lived protein ODC, the key enzyme in their biosynthesis. It was found that spermidine mediates the degradation of ODC by a non-lysosomal, ubiquitin-independent mechanism [41]; more recently it has been shown that ODC is degraded by the 26 S proteasome complex in a process that requires antizyme, a reversible tightly binding protein inhibitor of ODC activity, but not ubiquitin [42]. It has also been reported that the turnover of ODC can proceed along two different pathways, constitutive and polyamine-dependent [43]. In the polyamine-dependent mechanism, polyamines participate in this process by inducing the antizyme that binds to ODC, inhibits its activity and promotes its degradation. This is a feedback regulation in which excess polyamines induce the destruction of ODC, the enzyme that initiates their biosynthesis.

On a more general role for polyamines on protein degradation, they have been reported to be potent inhibitors of the ATP/ubiquitin-dependent proteolytic system in rabbit reticulocyte lysates, suggesting that they might bind to the ubiquitin-protein conjugates making them inaccessible to proteolytic attack [17]. Because this proteolytic pathway has a major role in the degradation of short-lived, abnormal and denatured proteins [44], it is in good agreement with our results, because the partial depletion of intracellular polyamines causes an increase of the turnover rate of short-lived proteins. This hypothesis is also congruent with previous observations revealing that polyamines *in vitro* markedly increase the resistance to proteinases of the cytosolic precursor of the mitochondrial protein ornithine transcarbamoylase, most probably by inducing changes in its conformational state [18]. Also, polyamines have been involved in modulating the proteolysis by endogenous proteases of nucleolin, a major nucleolar phosphoprotein, interacting with this protein to lead to its preferential degradation to a 60 kDa phosphopeptide [45]. The inhibition has been reported of a highly purified

alfalfa leaf protease by polyamines [16] because in their presence the enzyme undergoes a conformational change with the loss of 16 % α -helix secondary-structure content. In addition, stimulation of Ca^{2+} -dependent proteolysis by polyamines has been described in supernatant fractions from brain tissues and endogenous or exogenous substrates, and it has been shown that the endogenous proteolytic activity stimulated by polyamines is calpain [46]. The way in which polyamines modulate proteolysis in these cases is consistent with the idea that polyamines could modulate the degradative process of proteins interacting with them and modifying their susceptibility to degradation, or with the proteases themselves. These interactions could explain the decrease in the turnover rate of long-lived proteins in polyamine-depleted cells that we report in this paper.

The main difficulty in assessing the significance of effects of polyamines is how to determine their specificity because polyamines influence many processes, making it impossible to ensure whether or not they have a direct effect. This could be the case when considering the effect that polyamine depletion causes on the intracellular pH and ATP content. Polyamine depletion has been reported to result in a decrease in intracellular pH by 0.1–0.4 unit from control values between 7.4 and 7.6 [47]; it has also been shown that the ATP content in polyamine-deficient cells is slightly lower than in normal cells [48]. Changes in both intracellular pH and ATP levels have been related to protein degradation. Because the decrease in ATP inhibits the degradation of both short-lived and long-lived proteins to the same degree, and a decrease in pH increases proteolysis [37], these indirect effects cannot explain the opposite results observed in polyamine-depleted cells in both pools. In addition, polyamines have been reported to influence the phosphorylation of specific proteins [49]. Because phosphorylation might be a degradation signal, polyamines might be required in this process [50].

In conclusion, we show for the first time in cultured L-132 cells that changes in intracellular polyamine levels result in significant changes in rates of proteolysis by inhibiting the degradation of long-lived proteins and increasing the degradation of short-lived proteins. It remains to be clarified whether or not this is a direct effect of polyamines on the proteolytic process(es).

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