Role of spermidine in the expression of late markers of adipose conversion

Effects of growth hormone

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Confluent Ob1771 cells treated with an inhibitor of spermidine and spermine synthesis, methylglyoxal bis(guanylhydrazone), were dependent on putrescine addition for the expression of glycerol-3-phosphate dehydrogenase and acyl-CoA synthetase, which behaved as late markers of adipose conversion. A similar dependence was observed with drug-treated Ob17MT18 and 3T3-F442A preadipocyte cells, but not with non-differentiating 3T3-C2 cells. Studies in drug-treated Ob1771 cells at the mRNA level showed that the parallel expression of mRNAs encoding for glycerol-3-phosphate dehydrogenase and an homologue of serine proteinases of M_r 28000 [Cook, Groves, Min & Spiegelman (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 6480–6484] was also dependent on putrescine addition. Double-isotope experiments with [^{14}C]putrescine and [^{3}H]spermidine, as well as analysis of the polyamine content in drug-treated Ob1771 cells under various conditions, demonstrate after putrescine addition that the expression of late markers of adipose conversion was highly correlated with a 2-fold increase in the intracellular concentration of spermidine. No correlation was observed with changes in the intracellular concentrations of putrescine and spermine. Long-term exposure of untreated Ob1771 cells to growth hormone, which led to the expression of late markers of adipose conversion [Doglio, Dani, Grimaldi & Ailhaud (1986) Biochem. J. 238, 123–129] was also accompanied by the same increase in spermidine concentration, which attained values identical with those determined in drug-treated cells supplemented with putrescine. This observation suggests that the permissive effect of growth hormone on the terminal differentiation of adipose cells might be related to changes in the intracellular concentration of spermidine.

INTRODUCTION

The chronological events taking place during the process of adipose conversion have been extensively investigated in preadipocyte cell lines and cell strains (Ailhaud, 1985). The time course of the change in phenotypic markers during adipose conversion of Ob17 cells has shown the following: (i) lipoprotein lipase and monoacylglycerol lipase emerge at confluence before any triacylglycerol accumulation in both serum-supplemented (Murphy et al., 1981; Vannier et al., 1985a) and serum-free medium (Gaillard et al., 1985); (ii) the accumulation of these lipids occurs later and coincides with the acquisition of glycerol-3-phosphate dehydrogenase required for the synthesis of the glycerol backbone (Vannier et al., 1985b); (iii) the emergence of glycerol-3-phospate dehydrogenase is strongly dependent on insulin added chronically to culture media, whereas that of lipoprotein lipase is independent of the presence of this hormone (Amri et al., 1984). These observations supported the existence of an early and a late step in the expression of the differentiation programme of preadipocyte cells. A first direct proof of two distinct steps has been obtained subsequently by using a subclone of Ob17 cells (Ob1754 clonal line), which is totally dependent on polyamines for complete adipose conversion. When treated with an inhibitor of spermidine and spermine synthesis, methylglyoxal bis(guanylhydrazone), confluent Ob1754 cells became totally dependent on putrescine addition for the expression of glycerol-3-phosphate dehydrogenase, which behaved as a late marker of adipose conversion. Under these conditions, the early expression of lipoprotein lipase during growth arrest remained unchanged. Studies at the mRNA level showed that the expression of glycerol-3-phosphate dehydrogenase mRNA was putrescine-dependent (Amri et al., 1986). A second direct proof of two distinct steps in the expression of the differentiation programme has been obtained by using another subclone of Ob17 cells (Ob1771 clonal line). The adipose conversion of Ob1771 preadipocytes was accompanied, during exposure to a growth-hormone-containing medium, by the acquisition of phenotypic markers and the increased accumulation of specific mRNAs. The late expression of the mRNA coding for glycerol-3-phosphate dehydrogenase and that of glycerol-3-phosphate dehydrogenase activity required the presence of growth hormone. By contrast, the early expression of lipoprotein lipase was independent of growth-hormone supplementation (Doglio et al., 1986). Taken together, both sets of data would suggest that growth hormone could play a role by controlling, directly or indirectly, the intracellular concentrations of polyamines, leading in turn to the expression of late markers of adipose conversion. In the present study, we have investigated in Ob1771 cells (a) the critical role of spermidine with respect to the differential expression of lipoprotein lipase and glycerol-3-phosphate dehydrogenase during adipose conversion, and (b) the growth-

hormone-related changes in the intracellular concentrations of spermidine.

EXPERIMENTAL

Cell lines

Ob1771 cells were obtained after subcloning Ob17 cells, as previously described (Amri et al., 1986). Ob17MT18 cells were derived from Ob17 cells by focus formation after transfer of a modified genome encoding only the middle-T protein of polyoma virus (Grimaldi et al., 1984). 3T3-C2 and 3T3-F442A cells were clonal lines established by Green & Kehinde (1976) and obtained through the courtesy of Dr. H. Green (Boston, MA, U.S.A.).

Cell culture

Ob1771, 3T3-C2 and 3T3-F442A cells were plated at 1000 cells/cm^2 in 35 mm-diam. dishes (or 60 mm-diam. dishes when stated) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 200 units of penicillin/ml, 50 μg of streptomycin/ml, 33 μM-biotin and 17 μM-pantothenate (defined as standard medium). After confluence (5 days after seeding), this medium was replaced by the differentiation medium, i.e. standard medium supplemented with 17 nM-insulin and 2 nM-tri-iodothyronine, in the presence or absence of methylglyoxal bis(guanylhydrazone) and a polyamine as indicated. Media were changed every other day. Ob17MT18 cells were grown in Dulbecco's modified Eagle's medium supplemented with 3% foetal bovine serum and maintained at confluence in a medium containing a mixture of Dulbecco's modified Eagle's and Ham's F12 media (1:1, v/v), 15 mM-NaHCO$_3$, 15 mM-Hepes buffer, pH 7.4, 33 μM-biotin, 17 μM-pantothenate, 62 mg of penicillin/ml, 50 μg of streptomycin/ml, 5 μg of insulin/ml, 5 μg of transferrin/ml, 0.15 nM-tri-iodothyronine, 1.2 nM-bovine growth hormone and 0.1% foetal bovine serum, in the presence of methylglyoxal bis(guanylhydrazone) and a polyamine (see Fig. 3).

Cell-free extracts and enzyme assays

At 18–24 h after the last change of medium cells were rinsed twice at 37 °C with phosphate-buffered saline, pH 7.4 (140 mM-NaCl, 3 mM-KCl, 8 mM-Na$_2$HPO$_4$, 1.5 mM-KH$_2$PO$_4$). Cell homogenates (from two pooled dishes) were obtained by using a Potter/Elvehjem homogenizer (20 strokes) with 20 mM-Tris adjusted to pH 7.5 with HCl; they were used directly for lactate dehydrogenase, glycerol-3-phosphate dehydrogenase and acyl-CoA synthetase assays and for protein determinations. For lipoprotein lipase assays, samples of homogenates were adjusted to 150 mM-NaCl, 2.5 mM-sodium barbital, pH 7.4, 140 mM-mannitol, 0.9 mM-CaCl$_2$, 0.25 mM-MgCl$_2$, 0.5 M-glycerol, 0.2% Triton X-114, and incubated for 2 h at 4 °C. As described by Bordier (1981), the detergent was removed by heat treatment of the solubilized homogenate for 10 min at 30 °C, followed by sedimentation for 10 min at 12000 g, and the supernatant was used for enzyme assays. Under these conditions, the recovery of lipoprotein lipase was 98–100%, whereas > 95% of the initial amount of detergent was removed. Lipoprotein lipase (EC 3.1.1.34), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), lactate dehydrogenase (EC 1.1.1.27) and acyl-CoA synthetase

(EC 6.2.1.3) activities were determined as previously described (Négrel et al., 1978; Murphy et al., 1981) and are expressed as nmol of product formed/min per mg of protein. Inter-assay variability was less than 5%, and variability among mean values from separate dishes never exceeded 5%. Protein was determined with bovine serum albumin (fraction V; Sigma Chem.) as a standard (Lowry et al., 1951).

Polyamine determination

This was performed as previously described (Amri et al., 1986). When the synthesis of polyamines from exogenous [^{14}C]putrescine and [^{3}H]spermidine was examined in Ob1771 cells, both labelled polyamines were added simultaneously to 4-day confluent cells maintained between day 0 and day 4 in the presence of the differentiation medium with or without 10 μM-methylglyoxal bis(guanylhydrazone); only drug-treated cells were also supplemented with 20 μM- or 100 μM-putrescine. At 1–25 h after addition of labelled polyamines, cell homogenates were prepared and analysed for their polyamine content and radioactivity.

RNA isolation and analysis

RNA was prepared and analysed by Northern blots as previously described (Amri et al., 1986).

Materials

[α-^{32}P]dCTP, tri[9,10-^{3}H]oleoylglycerol, [1-^{14}C]palmitic acid, [1,4-^{14}C]putrescine dihydrochloride, [terminal methylenes-^{3}H(n)]spermidine trihydrochloride and nick-translation kit were purchased from Amersham International (Amersham, Bucks., U.K.). Culture media and bovine serum were from Gibco (Cergy-Pontoise, France), and foetal bovine serum was from Flow Laboratories (Bethesda, MD, U.S.A.). Guanidinium monothiocyanate was obtained from Fluka (Buchs, Switzerland). Other compounds were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Plasmids pA21 and pC8 have been previously described (Amri et al., 1986). A plasmid pAd20 containing a cDNA insert of 940 bases complementary to an homologue of serine proteinases of M_r 28000 (Cook et al., 1985) was kindly given by Dr. H. Green (Boston, MA, U.S.A.). Bovine growth hormone was obtained through the National Hormone and Pituitary Program (NIADKK, Baltimore, MD, U.S.A.).

RESULTS

Effects of polyamine treatment on the activity of glycerol-3-phosphate dehydrogenase in Ob1771 cells

As shown in Fig. 1, the emergence of glycerol-3-phosphate dehydrogenase activity in confluent Ob1771 cells did not require the presence of a polyamine. However, a 2.7-fold increase in activity was observed in the presence of high concentrations of added putrescine or lower concentrations of added spermidine. A different picture emerged when Ob1771 cells were exposed to methylglyoxal bis(guanylhydrazone), a competitive and reversible inhibitor of S-adenosylmethionine decarboxylase. This key enzyme controls the concentration of decarboxylated S-adenosylmethionine, which is normally very low and rate-limiting in the biosynthesis of spermidine and spermine. Methylglyoxal bis(guanylhydrazone) has been reported in many cell systems and
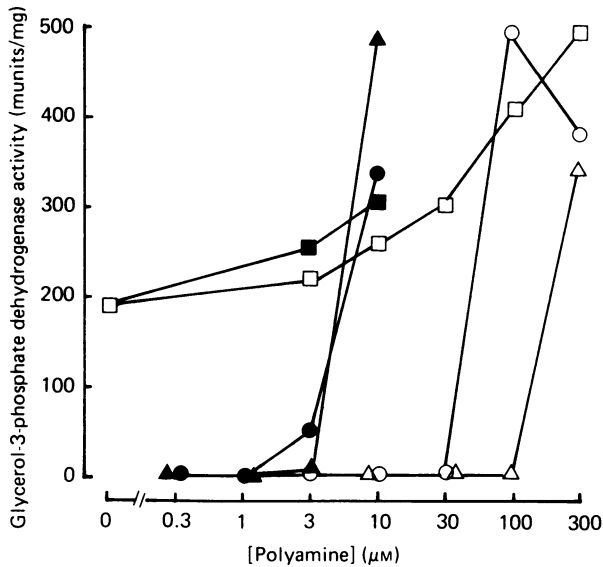
Fig. 1. Effects of polyamine treatment on the expression of glycerol-3-phosphate dehydrogenase activity in Ob1771 cells

Ob1771 cells were grown in standard medium. At confluence, the medium was supplemented with 17 nM-insulin, 2 nM-tri-iodothyronine and various concentrations of putrescine (□, ○, △) or spermidine (■, ●, ▲) in the absence (□, ■) or presence of 10 μM- (○, ●) or 30 μM-(△, ▲) methylglyoxal bis(guanylhydrazone). The glycerol-3-phosphate dehydrogenase activities were determined 11 days after confluence. The curves shown are representative of experiments performed with two independent series of cells. The lactate dehydrogenase activities remained similar under all conditions, and the mean of the specific-activity values was 2850 ± 135 munits/mg of protein.

tissues to increase the activity of S-adenosylmethionine decarboxylase indirectly by increasing the putrescine concentration (Tabor & Tabor, 1976) and directly by increasing the enzyme amount (Shirahata & Pegg, 1985). A complete loss of cell viability made it impossible to perform experiments in the presence of methylglyoxal bis(guanylhydrazone) alone, whereas addition of 3 μM-putrescine or 0.3 μM-spermidine restored complete cell viability. In drug-treated Ob1771 cells, chronic exposure to concentrations of putrescine above 30 μM led to an increase in the glycerol-3-phosphate dehydrogenase activity, the maximal effect being observed at 100 μM (Fig. 1). As expected, exposure of the cells to a higher concentration (30 μM) of methylglyoxal bis(guanylhydrazone) led to a requirement for higher concentrations of putrescine (above 100 μM) to give a similar increase in enzyme activity. Under both conditions spermidine at low concentrations (3–10 μM) could substitute for putrescine (Fig. 1). It is noteworthy that the activity of lactate dehydrogenase remained similar under all conditions (see legend of Fig. 1). The relationships between polyamine concentrations and the activities of enzyme markers were extended to lipoprotein lipase and acyl-CoA synthetase. It should be recalled in this respect that changes in glycerol-3-phosphate dehydrogenase and lipoprotein lipase activities are directly correlated with changes in enzyme concentration (Spiegelman & Green, 1980; Vannier et al., 1982). Fig. 2 shows that, in untreated cells, the maximal rise in activity of lipoprotein lipase and acyl-CoA synthetase observed after putrescine addition did not exceed 1.6-fold, and that of lactate dehydrogenase activity only 1.2-fold (Figs. 2a and 2b). In Ob1771 cells treated with 10 μM-methylglyoxal bis(guanylhydrazone), lipoprotein lipase was clearly expressed in the presence of putrescine and was maximal at a concentration of 30 μM-putrescine. At that concentration, the glycerol-3-phosphate dehydrogenase activity
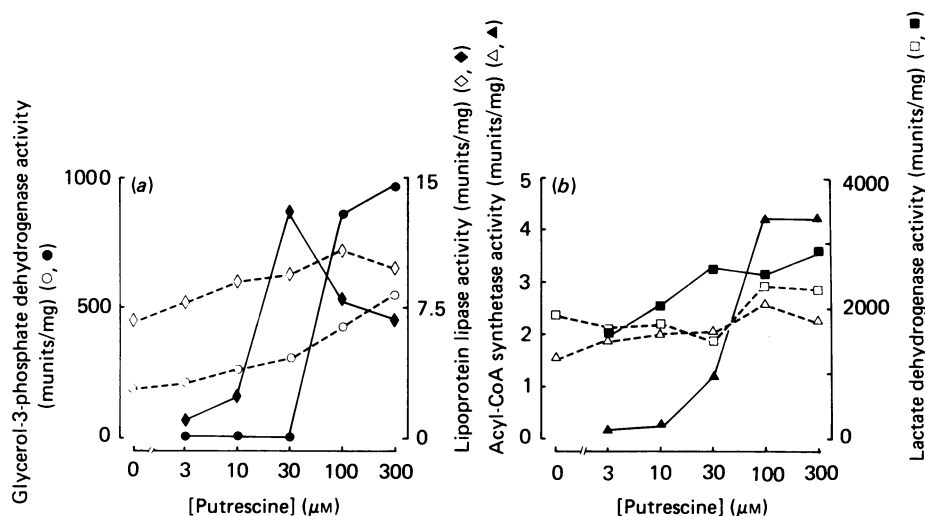

Fig. 2. Dose–response relationship of putrescine to the activities of lipoprotein lipase, acyl-CoA synthetase and glycerol-3-phosphate dehydrogenase in Ob1771 cells

Confluent Ob1771 cells were maintained in the presence of the differentiation medium (see the Experimental section) supplemented with various concentration of putrescine, in the absence (◇, ○, △, □) or presence (◆, ●, ▲, ■) of 10 μM-methylglyoxal bis(guanylhydrazone). The lipoprotein lipase (◇, ◆), glycerol-3-phosphate dehydrogenase (●, ○), acyl-CoA synthetase (△, ▲) and lactate dehydrogenase (□, ■) activities were determined at day 13 after confluence. The curves shown are representative of experiments performed with at least three independent series of cells.

was not yet detectable (Fig. 2a). The rise in activity of acyl-CoA synthetase was dramatic and parallel to that of glycerol-3-phosphate dehydrogenase (Fig. 2b). However, in contrast with the latter activity (Fig. 2a), a low but significant acyl-CoA synthetase activity was detected in cells exposed to 3 μM-putrescine (Fig. 2b) as well as in exponentially growing untreated cells (results not shown). Once again, the activity of lactate dehydrogenase was not significantly affected by putrescine supplementation, and it is noteworthy that near-maximal values were observed at 3 μM-putrescine, i.e. under conditions where lipoprotein lipase was actually expressed with low activity (Fig. 2).

Effects of putrescine treatment on the activity of glycerol-3-phosphate dehydrogenase in a non-preadipocyte and preadipocyte clonal lines

A requirement of putrescine to express the glycerol-3-phosphate dehydrogenase activity, under conditions where cells were exposed to an inhibitor of spermidine and spermine synthesis, was not specific to the Ob1771 preadipocyte clonal line. As shown in Fig. 3, both Ob17MT18 (Grimaldi et al., 1984) and 3T3-F442A cells (Green & Kehinde, 1976), treated with 10 μM-methylglyoxal bis(guanylhydrazone), showed a similar depend-
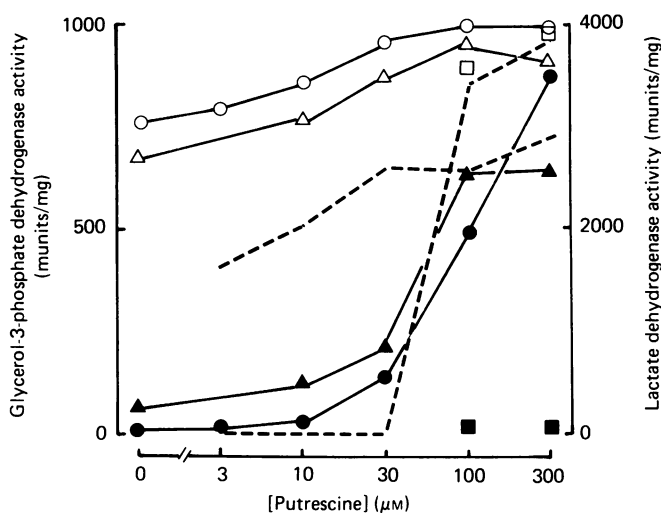

Fig. 3. Dose–response relationship of putrescine to the activities of glycerol-3-phosphate and lactate dehydrogenases in cells of different clonal lines

Confluent cells of 3T3-F442A ($\triangle$, $\blacktriangle$) and 3T3-C2 ($\square$, $\blacksquare$) clonal lines were maintained the the presence of the differentiation medium supplemented with 10 μM-methylglyoxal bis(guanylhydrazone) and various concentrations of putrescine. Ob17MT18 cells ($\bigcirc$, $\bullet$) were grown and maintained at confluence as described in the Experimental section, in the presence of 10 μM-methylglyoxal bis(guanylhydrazone) and various concentrations of putrescine. The glycerol-3-phosphate dehydrogenase and lactate dehydro- lactate dehydrogenase ($\triangle$, $\square$, $\bigcirc$) activities were deter- mined at day 5, 9 and 15 for 3T3-F442A, Ob17MT18 and 3T3-C2 cells respectively. The curves shown by broken lines were taken from Fig. 2 and correspond to glycerol-3-phosphate dehydrogenase and lactate dehydro- genase activities in Ob1771 cells. The curves shown are representative of experiments performed with two indep- endent series of 3T3-C2, 3T3-F442A and Ob17MT18 cells.

ence on putrescine addition. The rise in activity of glycerol-3-phosphate dehydrogenase was already ob- served at 30 μM-putrescine. It became maximal at 100 μM-putrescine for 3T3-F442A cells and was still increasing for Ob17MT18 cells above 100 μM-putrescine. It should be pointed out that in drug-treated 3T3-F442A cells, a low but significant activity of glycerol-3-phosphate dehydrogenase and a high activity of lactate dehydro- genase were observed in the absence of any putrescine supplementation. It should be also pointed out that, in contrast with its effect on preadipocyte clonal lines, putrescine showed no effect on control non-differentiating 3T3-C2 cells (Fig. 3). Not unexpectedly, the results of Fig. 3 indicate also that the activity of lactate dehydrogenase was independent of the cell line and putrescine supplementation.

Metabolism and polyamine contents on Ob1771 cells

Preliminary investigations had shown that Ob1771 cells treated with 10 μM-methylglyoxal bis(guanylhydra- zone) showed a 2-fold increase in their intracellular concentrations of spermidine and spermine when the concentration of exogenous putrescine was increased from 20 μM to 100 μM. Lipoprotein lipase was expressed at both concentrations, whereas glycerol-3-phosphate dehydrogenase was only expressed at 100 μM-putrescine (Amri et al., 1986). This suggested that putrescine was metabolized to spermidine and spermine and that spermidine (and/or spermine) was active in promoting the expression of glycerol-3-phosphate dehydrogenase. In order to show directly that Ob1771 cells were indeed able to metabolize putrescine to other polyamines and to investigate which polyamine(s) was actually involved in the expression of the terminal differentiation programme, untreated and treated Ob1771 cells were exposed simultaneously to [14C]putrescine and [3H]spermidine. The results of Fig. 4(a) show that, in control untreated cells first exposed to tracer amounts of labelled putrescine and spermidine and then analysed 25 h later for their polyamine content, most of the labelled putrescine, after dilution by the pool of endogenous putrescine, was converted into [14C] spermidine and a small proportion into spermine. At the same time, labelled spermidine was metabolized to some extent to spermine and to a smaller extent to putrescine. In treated cells exposed to 20 μM-putrescine, most of the 14C radioactivity was recovered under the peak of putrescine and only a small conversion of [14C]putrescine into [14C]spermidine had occurred (Fig. 4b). By contrast, in treated cells exposed to 100 μM-putrescine, a very significant proportion of the 14C radioactivity (approx. 45%) was recovered as spermidine and a very small proportion as spermine (Fig. 4c). The metabolic transformation of 14C-labelled putrescine into labelled spermidine could be detected as early as 3 h after addition of [14C]putrescine and [3H]spermidine (results not shown). As indicated by the curves in Figs. 4(b) and 4(c), the low metabolic transformation of [3H]spermidine into labelled spermine after dilution by the pool of endogenous spermidine, only became significant at 100 μM-putrescine. This metabolic transformation was already detectable 14 h after addition of [3H]spermidine (results not shown). These observations indicate that the increase in intracellular spermidine seemed to precede the increase in spermine, suggesting that changes in spermidine concentrations could play a critical role in the
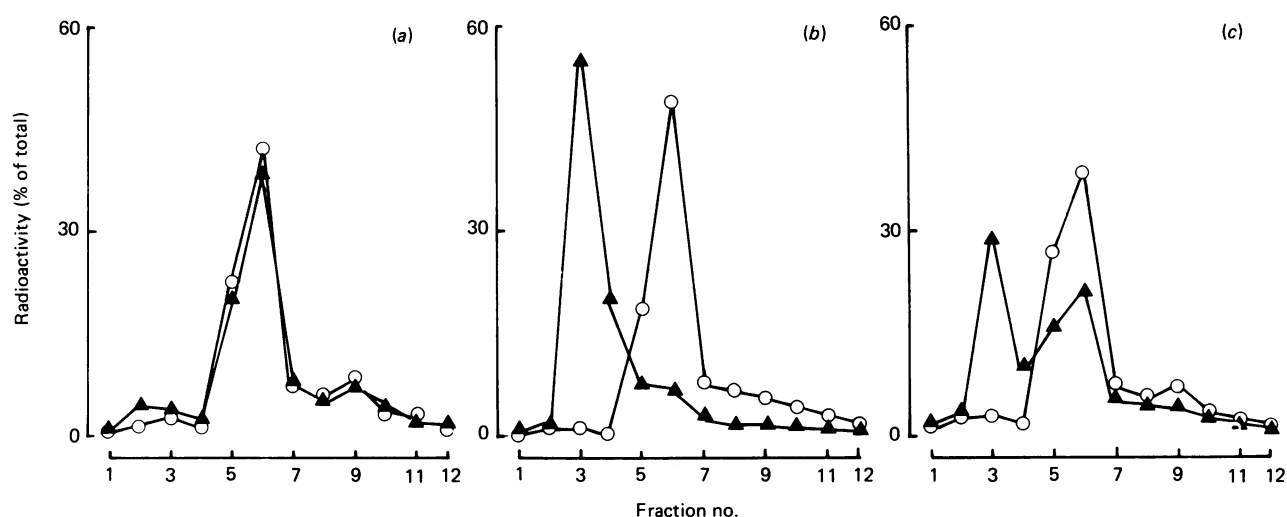
Fig. 4. Synthesis of polyamines in untreated and treated cells

Confluent Ob1771 cells were maintained in the presence of differentiation medium alone (a) or supplemented with 10 μM-methylglyoxal bis(guanylhydrazone) (b and c) and either 20 μM- (b) or 100 μM-(c) putrescine. At day 4 after confluence were added to 4 ml of each medium (changed every other day) 0.1 μCi of [1,4-^{14}C]putrescine and 0.25 μCi of [^{3}H]spermidine per 60 mm-diam. dish, and 25 h later cell homogenates were prepared and analysed for their intracellular contents of labelled polyamines. The retention times were 6, 13 and 19 min at a flow rate of 2 ml/min for putrescine, spermidine and spermine respectively. The percentages of ^{14}C (▲) and ^{3}H (○) radioactivity were determined in each 4 ml fraction by taking as 100% the radioactivity recovered in the pooled fractions for each radioisotope.

Table 1. Intracellular polyamine concentrations and the activities of enzyme markers in confluent Ob1771 cells

Confluent Ob1771 cells were maintained in 60 mm-diam. dishes for 4–9 days in the differentiation medium with the following supplementation: A and A', no addition; B–F, 10 μM-methylglyoxal bis(guanylhydrazone) with, in B, 20 μM-putrescine, in C, 50 μM-putrescine, in D, 100 μM-putrescine, in E, 1 μM-spermine, and in F, 10 μM-spermine. The intracellular polyamine concentrations were determined 4 days after confluence 1 h after a last change of medium. Separate experiments have shown that similar values (±10%) were obtained when determinations were performed between day 3 and day 9 and, at any given day, between 30 min and 24 h after the last change of medium. The enzyme activities were determined at day 9 post-confluence. The values reported (means±s.D.) were obtained on six (A–D) and three (A', E and F) independent series of cells.

Culture conditions	Intracellular polyamine concn. (nmol/mg of protein)			Lipoprotein lipase (munits/mg of protein)	Glycerol-3-phosphate dehydrogenase (munits/mg of protein)	Lactate dehydrogenase (munits/mg of protein)
	Putrescine	Spermidine	Spermine			
A	1.3±0.1	10.8±0.5	1.9±0.2	7.1±1	966±60	2855±196
B	10±1.3	4.2±0.2	0.6±0.1	5.9±0.8	17.5±3	2703±160
C	11.3±1	9.8±0.7	0.9±0.2	7.3±0.9	460±17	2630±106
D	9.3±1	12±0.6	1.3±0.2	9.6±0.75	1448±74	2878±187
A'	0.9±0.1	9.9±0.6	1.3±0.1	6.6±0.8	751±50	2730±160
E	2.4±0.4	4.3±0.25	1.5±0.2	5.2±0.3	19±2	2393±150
F	1.8±0.25	9.8±0.3	2.7±0.3	7.2±0.8	677±16	2130±120

expression of glycerol-3-phosphate dehydrogenase. The relationships between the polyamine concentrations and the activities of lipoprotein lipase and glycerol-3-phosphate dehydrogenase were thus investigated. The results of Table 1 were obtained after exposure of confluent Ob1771 cells to methylglyoxal bis(guanyl-hydrazone) and various concentrations of putrescine or spermine. In these experiments, provided that a selected batch of foetal bovine serum was used, spermine at concentrations below 10 μM proved not to be cytotoxic for Ob1771 cells, at variance with previous observations performed with different batches of foetal bovine serum (Amri et al., 1986). The data of Table 1 indicate clearly

that the expression of lipoprotein lipase was not prevented when the intracellular concentrations of spermidine and spermine were as low as 4.2±0.2 and 0.6±0.1 nmol/mg of protein respectively (condition B). After addition of various concentrations of exogenous putrescine, a 2-fold increase in the intracellular concentrations of spermidine was sufficient to allow the cells to express glycerol-3-phosphate dehydrogenase. Under these conditions, the intracellular concentrations of putrescine remained very similar (conditions B–D). When the concentration of exogenous spermine was increased from 1 to 10 μM, the intracellar concentrations of spermidine increased from 4.3±0.25 to 9.8±0.3

nmol/mg of protein. Under these conditions, lipoprotein lipase-containing cells (condition E) became also able to express glycerol-3-phosphate dehydrogenase (condition F). By contrast with these changes in spermidine concentration, it is of interest to note the lack of correlation between changes in spermine concentration and the expression of glycerol-3-phosphate dehydrogenase, if one compares treated cells exposed to 1 μM-spermine (condition E) with untreated cells (condition A'). Taken together, these observations are in favour of intracellular spermidine playing a critical role, above a threshold concentration, in regulating the expression of the glycerol-3-phosphate dehydrogenase activity.

Northern-blot analysis of poly(A)-containing mRNAs from untreated and treated Ob1771 cells

To test whether the expression of glycerol-3-phosphate dehydrogenase was correlated with the expression of its corresponding mRNA (detected with pC8 probe), Northern-blot analyses have been performed (Fig. 5). These analyses also included different mRNAs expressed during adipose conversion, including the mRNA encoding in 3T3-F422A cells for a homologue of a serine proteinase of M_r 28000 (detected with pAd 20 probe); this mRNA is expressed later than the glycerol-3-phosphate dehydrogenase mRNA (Spiegelman et al., 1983). Both mRNAs were expressed in control untreated Ob1771 cells. In treated cells, exposed to 20 μM- or 30 μM-putrescine, both mRNAs remained undetectable. However, when exposed to 50 μM- or 100 μM-putrescine, which are conditions that allowed the expression of glycerol-3-phosphate dehydrogenase (Figs. 1 and 2) and led to a 2-fold increase in the intracellular concentration of spermidine (Table 1), the mRNAs encoding for glycerol-3-phosphate dehydrogenase and the homologue of a serine proteinase accumulated. The concentration of mRNA for actin (detected with pA21 probe) did not differ significantly in any of the conditions used.

Differential effects of growth-hormone treatment on the intracellular polyamine concentration and the activities of enzyme markers of adipose conversion

In order to observe a growth-hormone dependence, bovine serum was used instead of foetal bovine serum, which contains high concentrations of growth hormone (Doglio et al., 1986). The results of Table 2 indicate that the activity of lipoprotein lipase was increased about 2-fold when confluent cells were exposed chronically to a physiological concentration of growth hormone. The activity of glycerol-3-phosphate dehydrogenase was increased by 16-fold under the same conditions. Lactate dehydrogenase, which is not directly related to lipogenesis and which can be used as internal control, had a similar activity in cells maintained in the presence or absence of growth hormone. On long-term exposure to growth hormone, the emergence of glycerol-3-phosphate dehydrogenase was correlated with a 2-fold increase in the intracellular concentration of spermidine, which reached a value almost identical with that determined in control cells exposed to foetal bovine serum and on drug-treated cells exposed either to 50–100 μM-putrescine or to 10 μM-spermine. As the data of Table 2 show, no significant correlation was again observed between the expression of glycerol-3-phosphate dehydrogenase on one hand and the intracellular concentrations of putrescine and spermine on the other hand.
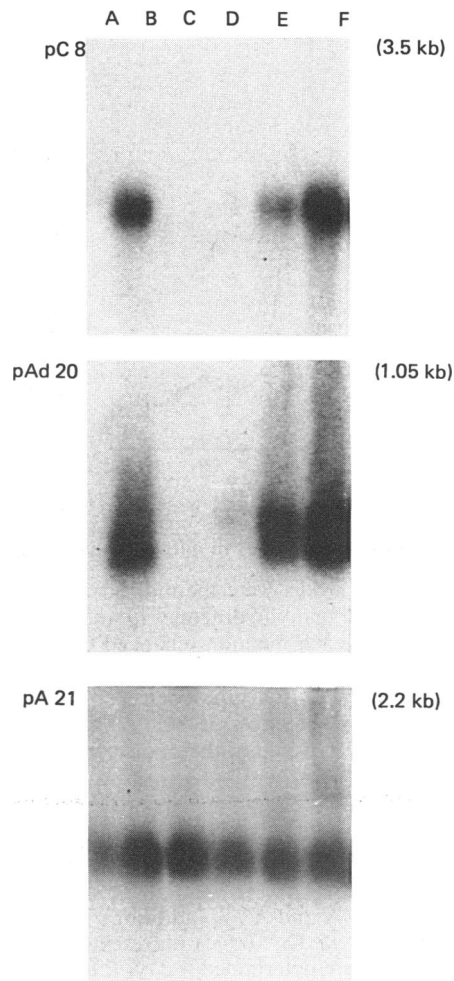

Fig. 5. Northern-blot analysis of poly(A)-containing RNAs

Confluent Ob1771 cells were maintained in 60 mm-diam. dishes under the same conditions as used in Figs. 2 and 4. Poly(A)-containing RNAs were prepared from (A) exponentially growing cells, (B) 9-day post-confluent cells in the absence of any treatment, (C–F) the same cells as in B but chronically treated with 10 μM-methylglyoxal bis(guanylhydrazone) and 20 μM- (C), 30 μM- (D), 50 μM- (E) or 100 μM-putrescine (F). The different cDNA probes used (left) and the length of the corresponding mRNAs in kilobases (kb; right) are indicated.

DISCUSSION

In the present work, the Ob1771 clonal line has been used to investigate the relationships between the expression of early and late markers of adipose conversion and the intracellular concentrations of polyamines. It is likely that in Ob1771 cells spermidine is the unique polyamine, at concentrations above a critical value, which plays a role in the expression of the glycerol-3-phosphate dehydrogenase and its corresponding mRNA. This conclusion is based on three lines of evidence in drug-treated cells: (i) the ability of spermidine to substitute for putrescine at concentrations one order of magnitude lower (Fig. 1); (ii) the correlation observed at 100 μM-putrescine between the synthesis of spermidine from putrescine (Fig. 4c) and the

Table 2. Intracellular polyamine concentrations in Ob1771 cells exposed to growth-hormone-deficient or growth-hormone-supplemented medium

After growth in the presence of 9% bovine serum, confluent Ob1771 cells in 60 mm diameter dishes were maintained in a medium containing: I, 9% bovine serum, 17 nM-insulin and 2 nM-tri-iodothyronine; II, same medium supplemented with 1.2 nM-growth hormone; III, same medium as I, but in the presence of 9% foetal bovine serum. Under each condition, Ob1771 cells were supplemented only between day 0 and day 4 with 100 μM-3-isobutyl-1-methylxanthine. The intracellular polyamine concentrations were determined 4 days after confluence, 1 h after the last change of medium. Separate experiments have shown that similar values ($\pm 10\%$) were obtained when determinations were performed between day 4 and day 11 and, at any given day, between 30 min and 24 h after the last medium change. The enzyme activities were determined at day 9 post-confluence. The reported values (means $\pm$ S.D.) were obtained on three independent series of cells.

Culture conditions	Intracellular polyamine concn. (nmol/mg of protein)			Lipoprotein lipase (munits/mg of protein)	Glycerol-3-phosphate dehydrogenase (munits/mg of protein)	Lactate dehydrogenase (munits/mg of protein)
	Putrescine	Spermidine	Spermine			
I	1.2 ± 0.2	4.2 ± 0.3	1.3 ± 0.2	5.3 ± 0.2	14 ± 1	2965 ± 240
II	1.5 ± 0.2	9.8 ± 0.9	2.2 ± 0.3	10.4 ± 0.7	220 ± 8	3113 ± 271
III	1.2 ± 0.1	9.3 ± 0.6	1.5 ± 0.2	9.1 ± 1	560 ± 54	2954 ± 236

increase in spermidine (Table 1) on one hand and the emergence of glycerol-3-phosphate dehydrogenase and its corresponding mRNA on the other hand (Table 1 and Fig. 5); (iii) the fact that the emergence of glycerol-3-phosphate dehydrogenase was correlated with changes in spermidine concentration, but not with changes in putrescine or spermine concentrations (Table 1). It is of interest that different preadipocyte clonal lines (Ob17 MT18 and 3T3-F442A), but not a non-preadipocyte clonal line (3T3-C2), when treated with methylglyoxal bis(guanylhdrazone), were able to respond to putrescine addition in a way identical with that of Ob1771 cells (Fig. 3). These results suggest that an increase in spermidine concentrations might also play in these cells a specific role in promoting the expression of a late marker of adipose conversion, i.e. glycerol-3-phosphate dehydrogenase. This hypothesis would be in agreement with another report showing that a potent increase in spermidine concentrations was correlated with the morphological adipose conversion of 3T3-L1 preadipocyte cells (Bethell & Pegg, 1981). The role of polyamines, if any, in the expression of early markers of adipose conversion remains an open question. The curves in Fig. 2 indicate clearly that, at 3 μM-putrescine, treated cells are fully viable and have normal activity of lactate dehydrogenase when lipoprotein lipase is only expressed at low activity; a 3-fold increase in the concentration of exogenous putrescine (from 10 to 30 μM) was sufficient to lead to a 8.5-fold increase in the activity of lipoprotein lipase. The actual concentrations of spermidine were not measured under these conditions, but it should be recalled that, in the polyamine-dependent Ob1754 clonal line, untreated cells that fully expressed lipoprotein lipase had a spermidine content of 2.7 nmol/mg of protein (Amri et al., 1986). This value is lower than that determined in Ob1771 cells treated with 10 μM-methylglyoxal bis(guanylhydrazone) and 20 μM-putrescine (4.2 nmol/mg of protein; Table 1). Thus it can be excluded neither that the expression of early markers of adipose conversion might be controlled by lower concentrations of spermidine nor that putrescine and/or spermine could also play a critical role in that regulation.

In a previous paper (Doglio et al., 1986), we have

shown that the permissive role of growth hormone during adipose-cell differentiation was related to terminal events only and that its effect could be seen at both the protein and the mRNA levels; the late expression of mRNAs encoding for glycerol-3-phosphate dehydrogenase and p422 protein (a myelin-P_2 homologue) and that of glycerol-3-phosphate dehydrogenase activity requires the presence of growth hormone. The results of Table 2 demonstrate that a 4-day exposure of Ob1771 cells to growth hormone increased the spermidine concentration to a value required for the expression of glycerol-3-phosphate dehydrogenase activity. The possibility that shorter times of exposure to growth hormone would be sufficient to give similar effects on spermidine concentrations cannot be excluded, but it should be recalled that exposure of confluent Ob1771 cells to growth hormone for 6 days was needed to induce the full expression of glycerol-3-phosphate dehydrogenase (Doglio et al., 1986). This would suggest that, after binding to growth-hormone receptors, which are present in Ob1771 cells (A. Doglio, E. Amri, P. Grimaldi, C. Dani & G. Ailhaud, unpublished work), some post-receptor event(s) should precede changes in the intranuclear concentration of spermidine.

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