Regulation of adipose cell differentiation. I. Fatty acids are inducers of the aP2 gene expression

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Abstract The regulation of the expression of adipose-related genes, i.e., aP2, adipsin, and glycerophosphate dehydrogenase (GPDH) by growth hormone (GH) and polyamines, as well as the role of fatty acids, have been investigated in polyamine-dependent Ob1754 cells and Ob1771 preadipose cells. Growth hormone acts as an obligatory hormone for adipsin and GPDH gene expression but its presence is not required for the expression of the aP2 gene. In fully differentiated Ob1771 cells, impairment of fatty acid synthesis by glucose deprivation leads to an inhibition of the aP2 gene expression, whereas the expression of adipsin and GPDH genes remains unaffected. Supplementation of the culture medium with fatty acids prevents the decrease of aP2 gene expression, and this effect appears primarily due to an increase in the transcriptional level of aP2 gene. ■ The induction of aP2 gene has been examined in early committed, lipid-free Ob1771 cells in which fatty acid synthesis is very low despite glucose supplementation. Long-chain fatty acids ($\geq C_{12}$) are able to activate the aP2 gene. It is concluded that fatty acids or fatty acid metabolites activate the aP2 gene and subsequently modulate its expression.—**Amri, E. Z., B. Bertrand, G. Ailhaud, and P. Grimaldi.** Regulation of adipose cell differentiation. I. Fatty acids are inducers of the aP2 gene expression. *J. Lipid Res.* 1991. **32**: 1449–1456.

Supplementary key words adipose differentiation • growth hormone • polyamines

The adipose conversion of preadipose cells, i.e., Ob17 (1), 3T3-L1 (2), and 3T3-F442A (3) represents a valid model to delineate in vitro the development of adipose tissue in vivo. Under appropriate culture conditions, these cells can differentiate into adipose cells. This process is accompanied by a large shift in the cellular pattern of protein biosynthesis that reflects activation of the transcription of adipose-related genes (4, 5). The differentiation program can be divided into early and late events that are differently regulated. Early events are triggered by growth arrest and characterized by the activation of a set of genes, among which are pOb24 and lipoprotein lipase genes (6, 7). Expression of the terminal differentiation, characterized by the emergence of lipogenic enzymes and subsequent triacylglycerol accumulation, is under multihormonal regulation. Among genes expressed late during differentiation, the aP2, adipsin, and GPDH genes encode proteins known or postulated to play important roles in adipose cell physiology. Glycerol-3-phosphate dehydrogenase provides the glycerol backbone needed for triglyceride synthesis. The cytosolic aP2 protein is postulated to be an adipocyte lipid-binding protein as suggested by its ability to bind fatty acids (8). The function of adipsin is still unclear, but this secreted serine protease could be a putative systemic regulator of energy balance (9). Among hormones required for terminal differentiation, GH plays a critical role (10, 11). Long-term treatment by GH leads in Ob1771 and 3T3-F442A preadipose cells to a specific rise in the intracellular levels of spermidine and to activation of genes related to terminal differentiation (12, 13). Since the inhibition of spermidine synthesis prevents lipid accumulation (13), it could be hypothesized that spermidine acts as one of the messengers in the GH-dependent pathway regulating the process of terminal differentiation. To delineate more precisely the effects of spermidine accumulation on that process, we have investigated the regulation of the expression of aP2, adipsin, and GPDH genes in the variant cell line Ob1754. In these cells no rise in spermidine level takes place despite the presence of GH; however chronic exposure of the cells to a mixture of putrescine and MGBG, a competitive inhibitor of S-adenosyl-methionine decarboxylase, leads to a rise in the cell content of spermidine similar to that occurring during differentiation of GH-treated Ob1771 and 3T3-F442A cells; this rise is accompanied by terminal differentiation (12, 13). The results presented herein appear to exclude a single mechanism for the regulation of the expression of the three genes under consideration. It was found that changes in spermidine intracellular levels are involved in aP2 gene expres-

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sion, whereas GH effects on the expression of adipsin and GPDH genes appear to be independent or partially dependent, respectively, of polyamine metabolism. The expression of the aP2 gene is closely linked to fatty acid supply in differentiating Ob1754 cells as well as in early confluent, lipid-free Ob1771 cells in which fatty acid synthesis is very low. Fatty acids or fatty acid metabolites appear to activate the aP2 gene and subsequently to modulate its expression.

MATERIALS AND METHODS

Cell culture

Ob1754 (12) and Ob1771 (11) cells were plated at a density of 2×10^3 per cm^2 and grown in Dulbecco's modified Eagle's medium supplemented with 8% adult bovine serum, 200 units/ml of penicillin, 50 μg/ml of streptomycin, 33 μM biotin, and 17 nM pantothenate. This medium is termed standard medium. Confluence was reached within 5 days. Differentiation of Ob1754 cells was obtained by chronic addition after confluence of 17 nM insulin, 2 nM triiodothyronine (T$_3$), and 1.2 nM GH, 10 μM MGBG, and 100 μM putrescine. Media were changed every other day. Fatty acids were dissolved in ethanol at a concentration of 50 mM and aliquots were immediately added to standard medium in order to obtain the final fatty acid concentration as indicated. This medium was prewarmed at 37°C for 45 min and then added to the cells after removal of the previous culture medium. The actual concentration of total fatty acids in bovine serum was found to be 1 mM (not shown), corresponding to 80 μM in standard medium. Thus the final fatty acid concentration of fatty acids varied from 110 μM (30 μM added) to 380 μM (300 μM added) in standard medium. Assuming that the serum concentration of albumin is normally about 0.6 mM (14), the fatty acid/albumin molar ratio varied from 2 to 8. Under these conditions, it was found that the actual concentrations of unbound fatty acids varied from 0.2 μM to 10 μM (15).

RNA analysis

RNA were prepared as described by Chomczynski and Sacchi (16). RNA were immobilized on Hybond-N membranes (dot-blot) or electrophoresed on denaturing 1.2% agarose gels containing 1.1 M formaldehyde and transferred to Hybond-N membranes (Northern-blot). Hybridizations were performed as previously described (12) with approximately 10^6 cpm/ml of randomly primed ^{32}P-labeled DNA probes. After washing, the membranes were exposed to Hyperfilm MP Amersham at -70°C with intensifying screens. Results were quantitated by densitometry using an LKB Ultroscan XL laser densitometer. All measurements were performed within the linear response of the integrated peaks as a function of immobilized RNA.

Nuclear transcription assays

Isolation of nuclei and nuclear transcription assays have already been described (11). Incubation of 10^7 nuclei for 30 min at 37°C in the presence of 200 μCi of [α-^{32}P]UTP led to the incorporation of about 10^5 cpm/μg of input DNA. ^{32}P-labeled reaction products (10^7 cpm) were hybridized for 48 h at 65°C to Hybond-C extra membranes containing 10 μg of denatured cDNA probes. The washed membranes were exposed at -70°C to XAR5 Kodak films with intensifying screens. Results were quantitated as described for RNA analysis.

Incorporation of [^{14}C]acetate into lipids

[1-^{14}C]acetate (1 μCi, 56 Ci/mmol) was added to the culture medium. Two hours later, the medium was removed and the cells were rinsed twice with phosphate-buffered saline. Cells were solubilized by addition of 0.1 N NaOH. Protein determinations were performed according to Lowry et al. (17). Lipids were extracted as previously described (18).

Incorporation and metabolism of [^{14}C]hexadecanol

[1-^{14}C]hexadecanol (1 μCi, 10 mCi/mmol) was added to the culture medium. Twenty-four hours later, cells were rinsed and harvested. Lipids were extracted and analyzed as described by Rizzo et al. (19).

Materials

Culture media were obtained from Gibco (Cergy-Pontoise, France). Recombinant human growth hormone was a kind gift from KabiVitrum (Stockholm, Sweden). [^{14}C]Hexadecanol, bovine serum, fatty acids, and other chemical products were purchased from Sigma Chimie (France). Other radioactive materials, random priming kit, and Hybond membranes were from Amersham (France).

RESULTS

Differential regulation by growth hormone and polyamines of the expression of adipose-related genes in Ob1754 cells

When treated with the mixture MGBG/putrescine in the presence of GH, Ob1754 cells accumulated triacylglycerol droplets and acquired the expected morphology of differentiated cells (not shown). These observations illustrate the critical effects of both GH and polyamine treatment on the differentiation of Ob1754 cells since GH alone cannot trigger the specific rise in spermidine concentration observed in cells of the parental Ob17 clonal line. Analysis of the expression of adipose-related gene expression revealed a rather complex picture (**Fig. 1**). As expected, the content of β-actin mRNA, encoding a protein
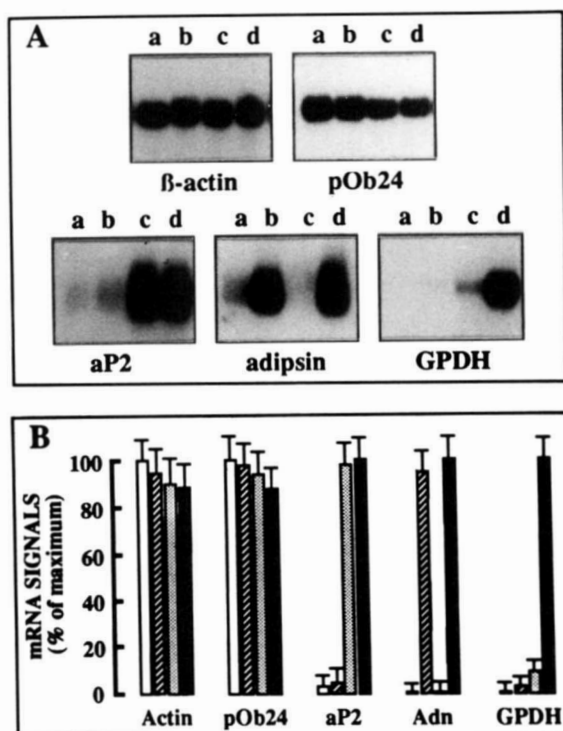
Fig. 1. Differential regulation by growth hormone and polyamines of adipose-related genes in Ob1754 cells. Cells, first grown in standard medium, were exposed after confluence to the same medium supplemented with 17 nM insulin, 2 nM T₃ (a, open columns), and chronically treated with 1.2 nM GH (b, hatched columns), 10 μM MGBG plus 100 μM putrescine (c, punched columns), or a combination of 1.2 nM GH, 10 μM MGBG, and 100 μM putrescine (d, full columns). Eight days later, RNA were prepared and analyzed as described in Materials and Methods. In A, Northern-blot analysis of the various mRNAs; in B, the results of A are given by taking as 100% the maximal signal obtained for each probe and are the mean ± SD of three independent experiments.

not involved in the adipose conversion process, was not significantly changed under any condition. The levels of pOb24 mRNA, which are related to cell commitment rather than to terminal differentiation, appeared to be independent of GH and polyamine treatment. Conversely, the GPDH mRNA accumulated only in cells undergoing the combined treatment. The regulation of aP2 and adipsin gene expression appears to be different since cells exposed to the MGBG/putrescine mixture, in the absence of GH, express high levels of aP2 mRNA but were devoid of adipsin mRNA. In contrast, cells exposed to GH only were expressing adipsin mRNA but not aP2 mRNA. These observations indicate that the regulation of terminal differentiation is not a single process common to a set of genes but instead that activation of each individual gene is controlled by some particular pathway.

Coordinate regulation of fatty acid synthesis and aP2 gene expression in Ob1754 cells

The possible effects of GH and MGBG/putrescine mixture on fatty acid synthesis were next investigated in Ob1754 cells. As shown in **Fig. 2A**, fatty acid synthesis, estimated by rate of [¹⁴C]acetate incorporation into total lipids, was low in cells maintained in medium supplemented with concentrations of putrescine insufficient to induce an increase in spermidine concentration. Fatty acid synthesis increased up to 10-fold as a function of putrescine concentration to reach a plateau in cells exposed to 100 μM putrescine. Exposure to GH did not significantly alter the rate of fatty acid synthesis at any concentration of putrescine. The abundance of aP2 mRNA in Ob1754 cells was low in putrescine-deprived
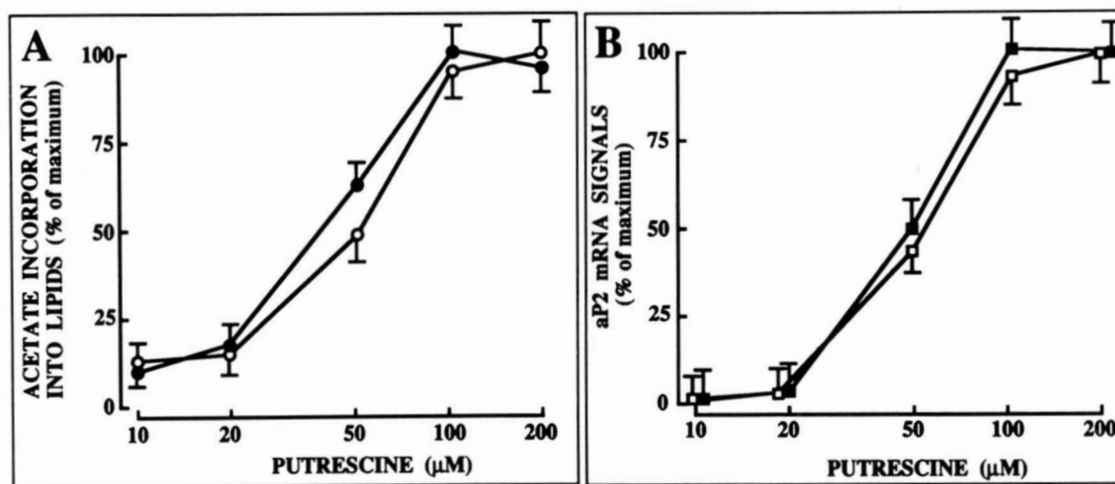
Fig. 2. Effects of polyamine treatment on fatty acid synthesis (A) and aP2 mRNA abundance (B) in Ob1754 cells. Cells grown in standard medium were exposed after confluence to the same medium supplemented with 17 nM insulin, 2 nM T₃, 10 μM MGBG, and various concentrations of putrescine in the absence (O, □) or the presence (●, ■) of 1.2 nM GH. Fifteen days later, [¹⁴C]acetate incorporation into lipids (O, ●) and aP2 mRNA abundance (□, ■) were determined as described in Materials and Methods. The results are presented by taking as 100% the maximal values and are the mean ± SD of four (A) and three (B) independent experiments. In A, the maximal value corresponds to 35,000 dpm of [¹⁴C]acetate incorporated per 2 h per mg of protein. In B, the results were normalized to β-actin mRNA signals.

medium but it increased as a function of putrescine concentration, reaching a maximum at 100 μM putrescine (Fig. 2B). Once again, GH did not affect the expression of aP2 mRNA. A striking parallelism is observed between the rate of fatty acid synthesis and the relative abundance of aP2 mRNA. Taken together these experiments indicate that induction of fatty acid synthesis and expression of aP2 mRNA were similarly dependent upon spermidine accumulation and independent of GH. Keeping in mind the postulated role of aP2 protein as a fatty acid-binding protein, it was hypothesized that fatty acid accumulation in the cells was actually triggering the expression of aP2 mRNA.

Preliminary experiments were performed in Ob1754 cells maintained for one week, in the absence or presence of 30 μM α-linolenate, treated or not with the MGBG/putrescine mixture. Under these conditions, fatty acid synthesis was low in control cells and in α-linolenate-supplemented cells (not shown). The results showed that a ~10-fold increase in aP2 gene expression occurred only in cells that can dispose of fatty acids either from exogenous origin and/or endogenous origin. Interestingly, the combined treatment (α-linolenate plus the MGBG/putrescine mixture) showed no additive effect, suggesting a common pathway. Thus, to strengthen this relationship, the effect of impairing fatty acid synthesis in fully differentiated Ob1771 cells was next investigated.

Regulation by fatty acids of aP2 gene expression in preadipose cells

Experiments were carried out by exposing cells that expressed high amounts of aP2 mRNA to a glucose-free DME medium containing dialyzed serum and the appro-priate hormones. A 5-fold decrease in the level of [^{14}C]acetate incorporation into lipids, after a 15-h exposure to glucose-free medium, was completely prevented by supplementation of the medium with 10 mM glucose, excluding the possibility that the observed decrease was due to the loss of a dialyzable factor from serum (**Fig. 3A**). Addition of a high concentration of α-linolenate to the culture medium did not significantly affect fatty acid synthesis of cells exposed to glucose-free medium. No alteration in the content of β-actin, GPDH, and adipsin mRNAs was observed under all conditions (Fig. 3B). In contrast, the abundance of aP2 mRNA appeared to be severely diminished after a 15-h exposure to glucose-free medium. This decrease in aP2 mRNA content was completely prevented by addition of glucose or exposure of the cells to a high concentration of α-linolenate. Run-on assays, performed with nuclei isolated from differentiated cells maintained for 15 h in glucose-free medium, revealed a 10-fold decrease in the transcriptional rate of aP2 gene without significant change in β-actin gene transcription. Both glucose and α-linolenate supplementation were equally efficient in sustaining the transcription of the aP2 gene (Fig. 3C). Taken together, these experiments show clearly that, in fully differentiated cells, a high level of aP2 gene transcription can be supported by a fatty acid supply from endogenous or exogenous origin. This requirement appeared to be specific for the aP2 gene since the expression of other adipose-related genes, i.e., adipsin and GPDH genes, was independent of fatty acid supply (Fig. 3B).

Therefore, the role of fatty acids in the process of aP2 gene activation was next investigated in committed, lipid-free Ob1771 cells maintained in glucose-supplemented
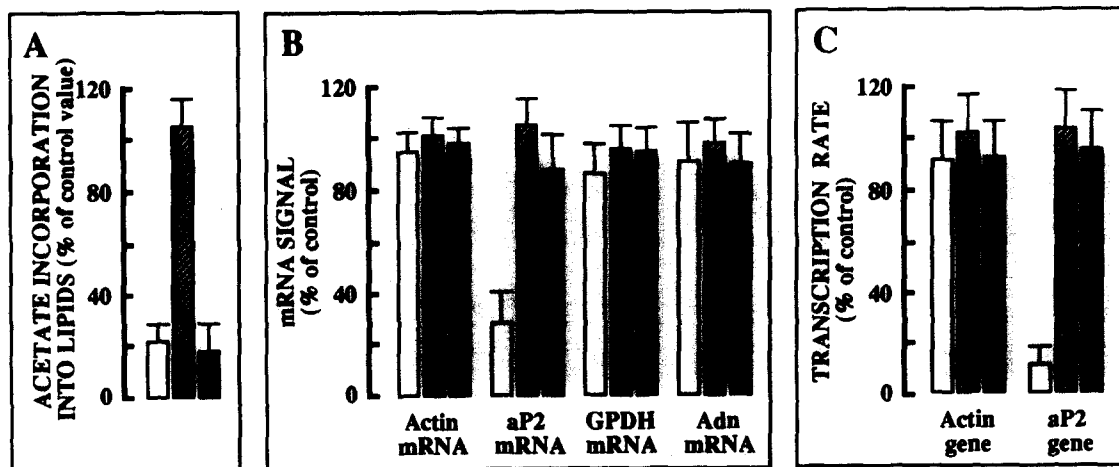
Fig. 3. Effects of glucose and fatty acid supply on fatty acid synthesis (A), adipose-related mRNA abundance (B), and aP2 gene transcription (C) in fully differentiated Ob1771 cells. Ob1771 cells were grown in standard medium. After confluence, cells were exposed to the same medium supplemented with 17 nM insulin, 2 nM T$_3$, and 1.2 nM GH. Twelve days later, the cells were incubated for 15 h in glucose-free medium containing 8% dialyzed fetal bovine serum, 17 nM insulin, and 2 nM T$_3$ (open columns), supplemented with 10 mM glucose (hatched columns) or 200 μM α-linolenate (black columns). [^{14}C]Acetate incorporation into lipids and RNA analysis was performed as in Fig. 2. Transcription rates were determined as described in Materials and Methods. The results are presented by taking as 100% the values obtained with cells continuously exposed to glucose-supplemented medium and are the mean $\pm$ SD of four (A) or three (B and C) independent experiments.

medium since endogenous fatty acid synthesis is very low in early confluent cells and very similar to that determined in growing cells: control experiments performed with 30 μM and 300 μM [^{3}H]α-linolenate and [^{3}H]oleate showed that less than 30% and 20% of the radioactivity was, respectively, recovered within the cells after 24 h.

Induction by fatty acids of aP2 gene expression in committed, lipid-free Ob1771 cells

To study the effect of fatty acids on the activation of aP2 gene expression, 1-day post-confluent Ob1771 cells were used. At this time, corresponding to the first stage of the differentiation program, cells were already committed since they expressed early markers, such as pOb24 mRNA, but were still non-terminally differentiated since they contained very low or undetectable amounts of aP2, GPDH, and adipsin mRNAs (**Fig. 4A**, lane a). As shown in Fig. 4A, exposure to α-linolenate for 24 h led to a concentration-dependent accumulation of aP2 mRNA (12-fold increase at 300 μM α-linolenate). The fatty acid treatment did not affect the expression of GAPDH and β-actin mRNAs, used as internal markers, and that of pOB24 mRNA. Interestingly, fatty acid treatment did not induce the expression of adipsin and GPDH mRNAs when GH was absent from the culture medium (Fig. 4A). As shown in Fig. 4B, the accumulation of aP2 mRNA, which occurs in Ob1771 cells treated for 15 h with 300 μM α-linoleate (lane c versus lane a), was prevented by the addition of actinomycin D to the culture medium (lane d versus lane c). This strongly suggests that transcriptional activation of the gene is the primary factor involved in the abundance of aP2 mRNA when cells are exposed to fatty acids. The induction of aP2 mRNA in early post-confluent cells exposed to fatty acid was accompanied by a parallel accumulation of aP2 protein: a single band of 14.6 kDa was detected by immunoblotting experiments whereas no signal was observed in growing cells or in early post-confluent, untreated cells (not shown). Taken together, these experiments clearly demonstrate that fatty acids were acting as potent and rapid inducers of the expression of aP2 gene and aP2 protein in committed, lipid-free cells.

Relationships between aP2 gene expression and fatty acids

As shown in **Fig. 5**, the potency of a fatty acid to induce aP2 mRNA accumulation was clearly dependent upon the length of the fatty acyl chain. Short-chain fatty acids were ineffective in inducing the accumulation of aP2 mRNA whereas long-chain fatty acids, such as palmitic and stearic acid, appeared to exert more potent effects. The influence of the unsaturation degree of the fatty acyl chain appeared to be less important. However, α- and γ-linolenic acids appeared to be better inducers than linoleic

and oleic acids. Additional experiments have shown that fatty acids, such as arachidonic acid and eicosapentaenoic acid, are not better inducers than linolenic acid, suggesting that metabolites, which could originate from γ- and α-linolenic acid metabolism, were not likely the mediators responsible for the effect on the aP2 gene expression.

Parallel experiments were performed in the presence of long-chain fatty acid alcohol. The results showed clearly a similar, if not identical, activation of the aP2 gene (Fig. 5). These results were at first suggestive that long-chain aliphatic, hydrophobic molecules per se and not acyl-CoAs were sufficient to behave as true inducers. However, this appears not to be the case since control experiments revealed that the incubation of Ob1771 cells with [^{14}C]hexadecanol led to the accumulation of radioactive material in the lipid extract. Analysis of these radioactive lipids showed that, after a 24-h exposure to 100 μM [^{14}C]hexadecanol, up to 50% of the cell-associated radioactivity was present both as unesterified and esterified fatty acids (polar and neutral lipids).
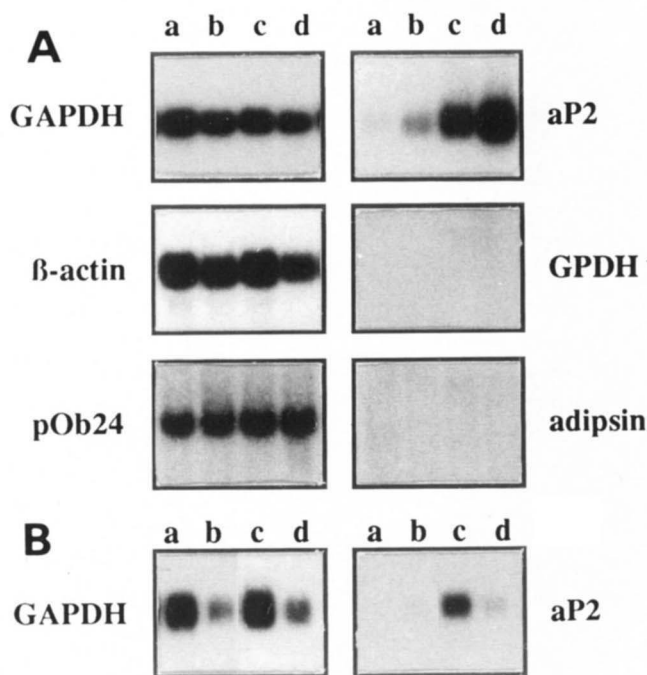
Fig. 4. Northern-blot analysis of RNA from committed, lipid-free Ob1771 cells exposed to α-linolenate. (A) One-day post-confluent cells were maintained for 24 h in standard medium (a) supplemented with 30 μM (b), 100 μM (c), or 300 μM (d) α-linolenate. Twenty μg of RNA was analyzed as described in Materials and Methods. The results are representative of five independent experiments. (B) Inhibition by actinomycin D of the induction of aP2 mRNA by fatty acids. One-day post-confluent ob1771 cells were maintained for 15 h in standard medium (a,b) supplemented with 300 μM α-linolenate (c,d) and exposed (b,d) or not (a,c) to 4 μg/ml actinomycin D. Twenty μg of RNA was analyzed as described in Materials and Methods. Similar results were obtained in two other independent experiments.
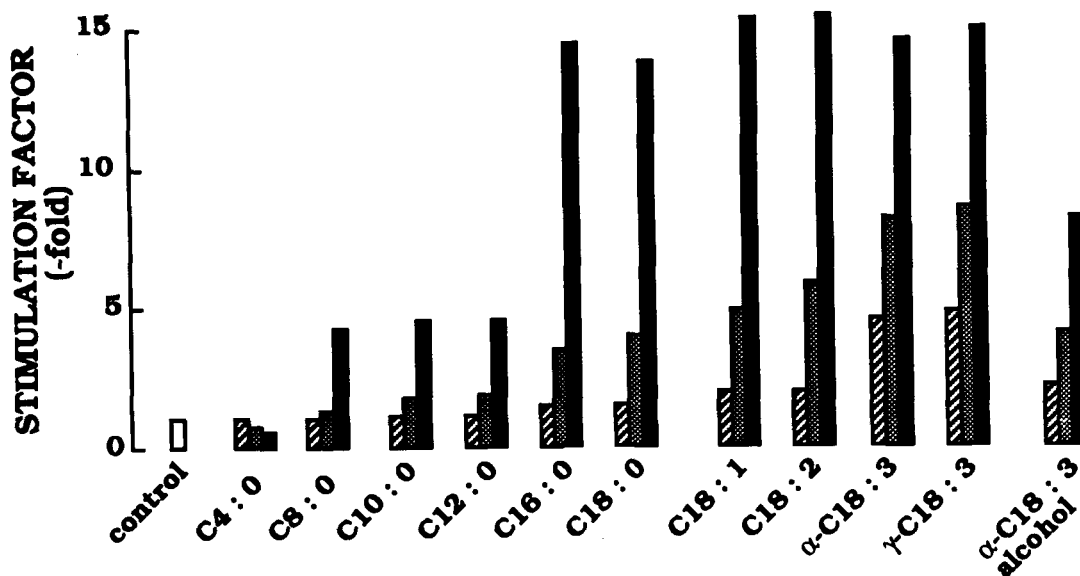
Fig. 5. Relationships between fatty acids and the induction of aP2 gene expression in Ob1771 cells. One-day post-confluent cells maintained in standard medium were exposed for 24 h to increasing concentrations of the various fatty acids as indicated. RNA were then analyzed as described in Materials and Methods. The results are expressed as a fold-stimulation factor as compared to untreated cells (□), in cells exposed to 30 μM (▨), 100 μM ▦), and 300 μM (■) of the various fatty acids as indicated. Similar results were obtained in two other independent experiments.

DISCUSSION

The results of the studies performed with Ob1754 cells, a variant of Ob17 clonal line requiring putrescine supplementation for terminal differentiation (12), suggest the existence of a tight correlation between fatty acid supply, from endogenous or exogenous source, and the regulation of the expression of aP2 gene (Fig. 2). In order to extend these observations to cells of the parental Ob17 clonal line, fully differentiated Ob1771 cells were maintained in glucose-free culture medium under conditions where RNA and protein synthesis were only decreased by 12 and 20%, respectively, as compared to cells maintained in glucose supplemented-medium. It is unlikely that the observed effects were due to changes in the ATP content and energy charge of the cells if one recalls the following. *i*) DME glucose-free medium contains 1 mM pyruvate present as carbon and energy source and 4 mM glutamine present as nitrogen source. *ii*) The results of Fig. 3 show that the content in β-actin, GPDH, and adipsin mRNAs remained unchanged in glucose-free compared to glucose- or α-linolenate-supplemented medium. Owing to the half-life of 15, 4, and 30 h for β-actin, GPDH, and adipsin mRNA, respectively (4, 20, 21), one would have expected some decrease in their respective content and this appears not to be the case. *iii*) Results reported in Fig. 4 were obtained with committed, lipid-free Ob1771 cells exposed to DME glucose-supplemented medium. Taking advantage of the fact that endogenous fatty acid synthesis was still very low in early confluent cells, the effect of exogenous fatty acid could be therefore easily observed.

It is important to point out that α-linolenate was not only able to modulate an increase in the content of aP2 mRNA in fully differentiated cells (Fig. 3) but actually to activate the aP2 gene in cells containing no detectable levels of aP2 mRNA and aP2 protein (Fig. 4 and not shown). In both cases, either using nuclei from cells treated or not with fatty acids (Fig. 3) or using actinomycin D-treated cells (Fig. 4B), the effect of α-linolenate appears to take place at a transcriptional level. Both saturated and unsaturated long-chain fatty acids were active. It does not seem that the efficacy of induction is a mirror of fatty acid solubility since fatty acids $\leq C_{12}$ were weak effectors as compared to fatty acids $\geq C_{16}$. However, regarding the more potent effect of linolenate compared to oleate, it cannot be excluded that this may reflect the concentration of monomeric molecular species (22). It could be hypothesized that the specificity of long-chain acyl-CoA synthetase from mouse adipose cells, which could be similar to that described for the rat liver enzyme active on C_{8}–C_{20} fatty acids with an optimum at C_{16}–C_{18} (23), may be responsible for the observed effects.

Based upon its in vitro ligand-binding properties, it has been proposed that aP2 is both a fatty acid- and retinoic acid-binding protein (8, 24). It should be pointed out that, in the concentration range of 10 nM to 100 μM, retinoic acid failed to induce the aP2 mRNA accumulation in preadipose Ob1771 cells. This observation could indicate that the ability of a molecule to bind to aP2 is not sufficient to induce aP2 gene expression, but the lack of effect of retinoic acid could also well be a consequence of

its inhibitory effect on the expression of the overall process of adipose conversion (25).

The regulation by fatty acids of aP2 gene, which plays an important role in the metabolism of fatty acids, seems to parallel that reported for carbohydrates in the case of pyruvate kinase gene (26, 27) and stearoyl-CoA desaturase gene (28) as well as for sterols in the case of HMG-CoA synthetase, HMG-CoA reductase, and apoB, E receptor genes (29, 30). However, in the case of pyruvate kinase and stearoyl-CoA desaturase genes, no distinction could be made between an effect mediated by insulin per se or an effect mediated by a specific increase in one, i.e., fatty acids, or other metabolites stimulated by insulin. Fatty acids are known to participate in various signalling processes. In some instances, fatty acylation of proteins has been suggested as an event involved in second messenger action (31). Fatty acids have been also suggested as intermediates in controlling the proliferation and/or differentiation of mammary cells (32, 33), whereas in preadipocytes long-term exposure to high concentration of fatty acids leads to an increase of both basal and isoproterenol-stimulated lipolysis (34) and to a decrease of the number and affinity of the insulin-receptor (35). Clearly, further investigations will be required to delineate the mechanisms involved in the activation and modulation of the aP2 gene by fatty acids or their metabolites.

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