Fatty acids as signal transducing molecules: involvement in the differentiation of preadipose to adipose cells

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Abstract Fatty acids are important metabolic substrates for adipose tissue and act, in preadipose cells, as potent inducers of various proteins directly involved in their metabolism. We have investigated the long-term effects of fatty acids on the conversion process of preadipose Ob1771 cells to adipose cells. Chronic exposure of cells to palmitate led, in a dose-dependent manner, to a strong stimulation of cell differentiation; this effect was confined to terminal events whereas fatty acids did not affect expression of early genes related to commitment of adipoblasts to preadipose cells. Adipogenic action of fatty acids did not require their metabolism as 2-bromopalmitate, which is not metabolized by preadipose cells, was more effective than palmitate in inducing differentiation. The critical role of fatty acids occurred during the first 3 days of the differentiation process and led subsequently to an increase in the number of differentiated cells by means of enhancement of post-confluent mitoses and overexpression of terminal differentiation-related genes. These results thus provide, at the molecular level, a potential link observed in vivo between an increase in fatty acid supply induced by high-fat or high-carbohydrate diets and the hyperplastic development of adipose tissue.—**Amri, E-Z., G. Ailhaud, and P-A. Grimaldi.** Fatty acids as signal transducing molecules: involvement in the differentiation of preadipose to adipose cells. *J. Lipid Res.* 1994. **35:** 930–937.

Supplementary key words adipocyte • differentiation • gene expression

It is now well established that differentiation of cells from preadipose clonal lines, such as 3T3 (1) and Ob1771 cells (2), represents a faithful model to delineate the development of adipose tissue in vivo. Under appropriate culture conditions, these cells differentiate into adipose cells and undergo a large shift of protein biosynthesis reflecting transcriptional activation of adipose-related genes. The differentiation process occurs first by the activation of early genes, such as lipoprotein lipase (LPL) and α2-chain of collagen VI (A2COL6/pOb24), and thereafter by the activation of a set of late genes including those involved in triacylglycerol synthesis, such as adipocyte lipid-binding protein (ALBP), glycerophosphate dehydrogenase (GPDH), and acyl-CoA synthetase (ACS). A burst of proliferation of committed cells, i.e., of early gene-expressing cells, takes place before the expression of late genes. Several adipogenic factors of adipose cell differentiation have been identified so far (for review see ref. 3). Briefly, late events implicate cell surface receptors acting via tyrosine-kinase activity (IGF-I, insulin), protein kinase C activity (growth hormone, $PGF_{2\alpha}$), and protein kinase A activity (prostacyclin) as well as nuclear receptors to glucocorticoids, triiodotyronine (T_3), and retinoids. In addition, we have recently shown that fatty acids from endogenous or exogenous origin play a central role in the control of the expression of genes involved in their own metabolism both in preadipose and adipose cells (4, 5). In preadipose cells where de novo fatty acid synthesis from glucose is nil, exogenous fatty acids are able to activate the genes encoding for ALBP and ACS (5). Once differentiated, either exogenous fatty acids in the presence of glucose-free medium or endogenously synthesized fatty acids in the presence of glucose-supplemented medium are able to modulate the expression of ALBP and ACS genes in adipose cells (4). The effects of fatty acids, which take place primarily at a transcriptional level, are fully reversible upon fatty acid or glucose removal. In this process, fatty acids per se are actually the inducers of gene expression as 2-bromopalmitate, which is neither activated into acyl-CoA nor incorporated into lipids in preadipose cells, is fully active in

Abbreviations: LPL, lipoprotein lipase; A2COL6/pOb24, α2-chain of collagen VI; ALBP, adipocyte lipid binding protein; GPDH, glycerol-3-phosphate dehydrogenase; ACS, acyl-CoA synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAT, fatty acid transporter; Glut-4, glucose transporter-4; HSL, hormone-sensitive lipase; T_3, triiodothyronine; GH, growth hormone; IBMX, isobutyl methylxanthine; PPARs, peroxisome proliferator-activated receptors; VLDL, very low density lipoproteins.

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inducing the expression of ALBP and ACS genes (6). The results showing that fatty acids can act as signal transducing molecules has shed a new light on previous studies performed in vivo to investigate the nutritional control of adipose tissue development. These studies had shown that high-fat or high-carbohydrate diets lead to hypertrophy and hyperplasia of adipose tissue in adult rats. This phenomenon was independent of strain, sex, and adipose deposit (7). In high-fat diet-fed rats, the excessive development of adipose tissue appeared to be due to proliferation of adipose precursor cells rather than to the filling process of pre-existing, partly differentiated cells (8). In order to gain some insights in the possible links existing between high-fat or high-carbohydrate diet and the acquisition of new fat cells, we have investigated in vitro the effects of natural and nonmetabolized long-chain fatty acids on the terminal differentiation of preadipose cells. It is shown that *i)* fatty acids exert potent effects on the terminal differentiation process by increasing both the post-confluent proliferation of preadipose cells and the level of expression of terminal differentiation-related genes; *ii)* fatty acids per se are sufficient to produce their effects; and *iii)* a short exposure time to fatty acids is sufficient to induce subsequent late events leading to triacylglycerol accumulation.

MATERIALS AND METHODS

Cell culture

Ob1771 cells (9) were plated at a density of 2×10^3 per cm^2 and grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 200 units/ml of penicillin, 50 μg/ml of streptomycin, 33 μM biotin, and 17 μM pantothenate. This medium is termed standard medium. Confluence was reached within 5 days. Differentiation of Ob1771 cells was obtained by chronic addition, after confluence, of 17 nM insulin and 2 nM T_3 (defined as standard differentiation medium). In some experiments, 8% bovine serum was used in lieu of 8% fetal bovine serum. 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 15 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 0.9 mM (not shown), corresponding to 72 μM in standard medium. Thus the final fatty acid concentration of fatty acids varied from 82 μM (10 μM added) to 172 μM (100 μM added) in standard medium. Assuming that the serum concentration of albumin is normally about 0.6 mM, the fatty acid/albumin molar ratio varied from 1.7 to 3.8. Under these conditions, it was found that the actual concentrations of unbound fatty acids varied from 0.1 μM to 1 μM (10). When indicated, fatty acids were removed from the culture medium by washing the cells at 37°C with the standard differentiation medium (two washes of 15 min each). Cell counting was performed using a Coulter counter ZBIC (Coultronics, France).

RNA analysis

RNA were prepared as described by Chomczynski and Sacchi (11). RNA were immobilized on Hybond-N membranes (dot-blot) or electrophoresed on denaturating 1.2% agarose gels containing 1.1 M formaldehyde and transferred to Hybond-N membranes (Northern-blot). Hybridizations were performed as previously described (9) 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. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA signals were used as internal standard.

Enzymatic assays

Cells were homogenized using a cell disrupter in 20 mM Tris-HCl buffer, pH 7.5. GPDH and ACS activities were assayed as previously described (12). Enzyme activities are expressed in milliunits/mg of protein, i.e., in nmol of product formed/min per mg of protein. Protein and DNA were determined as previously described (12) using bovine serum albumin and calf thymus DNA as standard, respectively.

Materials

Culture media were obtained from Gibco (Cergy-Pontoise, France). Recombinant human growth hormone was a kind gift from KabiVitrum (Stockholm, Sweden). 2-Bromopalmitate and 2-bromooctanoate were from Aldrich Chimie (France). Bovine serum and other chemical products were purchased from Sigma Chimie (France). Radioactive materials, random priming kit, and Hybond membranes were from Amersham France.

RESULTS

Effect of palmitate on adipose cell differentiation

To investigate the long-term effects of fatty acids on adipose cell differentiation, confluent preadipose Ob1771 cells maintained in standard differentiation medium were chronically exposed to various concentrations of palmitate. Ten days after confluence, GPDH and ACS activities were determined as indicators of terminal differentiation.

At that time, cells maintained in standard differentiation medium only expressed high levels of both activities, i.e., 485 and 3.9 mU/mg of protein for GPDH and ACS, respectively. As shown in **Fig. 1A,** chronic exposure to palmitate resulted in a net increase of both enzyme activities, indicating a dramatic enhancement of adipose cell differentiation. At 100 μM added palmitate, a 9-fold increase in the GPDH and ACS specific activities could be observed. The expression of various genes related to early events of differentiation, such as that of LPL or A2COL6/pOb24 genes and to late events of differentiation such as that of GPDH, ACS, or ALBP, has been used to characterize further the effects of fatty acids on the differentiation process. For that purpose, RNAs from cells chronically exposed to increasing concentrations of palmitate, were analyzed by Northern blotting. The results of Fig. 1B indicate that the cellular contents of LPL and pOb24 mRNAs were only moderately affected by fatty acids. By contrast, mRNAs related to terminal differentiation accumulated strongly in cells exposed to high concentrations of palmitate (8- to 9-fold induction above control for GPDH, ACS and ALBP mRNAs at 100 μM added palmitate). Taken together, these experiments indicated that chronic exposure to palmitate led to an enhancement of the expression of the terminal differentiation related genes.

Effects of 2-bromopalmitate on adipose cell differentiation

From the experiments described above, it could be hypothesized that the enhancement of adipose differentiation by palmitate was due to an increase in the concentration of fatty acids or fatty acid metabolites. To test this hypothesis, we investigated the adipogenic effects of 2-bromopalmitate, which is not a substrate in preadipose cells and a poor substrate for lipid synthesis in adipose cells, respectively (6). For that purpose, experiments similar to those of Fig. 1 were performed in the presence of increasing concentrations of 2-bromopalmitate. As shown in **Fig. 2,** 2-bromopalmitate affected the expression of adipose markers in a more complex manner than palmitate. The compound exerted a biphasic effect on the expression of terminal differentiation-related markers at both the mRNA and protein levels. At low concentrations, 2-bromopalmitate exerted adipogenic effects with a maximally effective concentration of 25 μM for ACS mRNA and ACS activity (11-fold induction above control), whereas 50 μM was required for ALBP and GPDH mRNAs (>12-fold induction above control). By contrast, at 100 μM, the 2-bromopalmitate negatively affected the expression of such markers and led to a 4- and 5-fold decrease of GPDH and ACS activities, respectively (Fig.

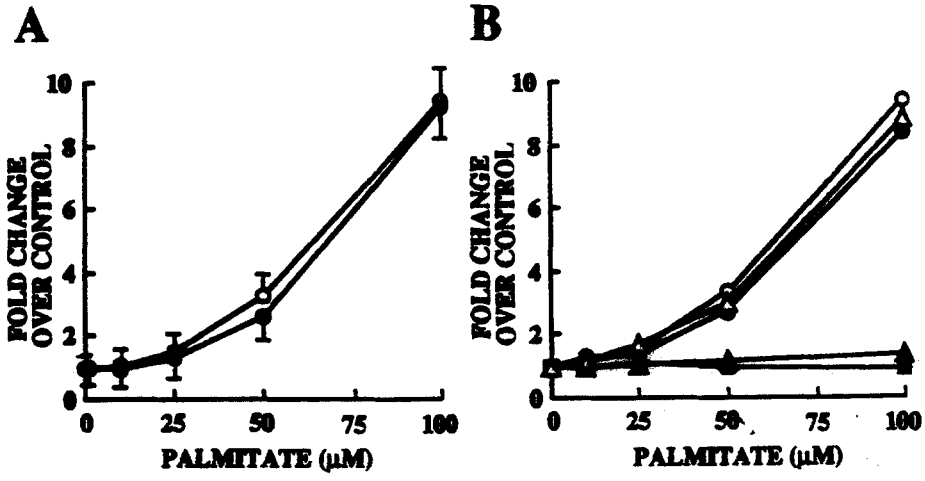

Fig. 1. Effect of palmitate on the expression of late differentiation. Confluent preadipose Ob1771 cells were chronically exposed after confluence to standard differentiation medium supplemented with increasing concentrations of palmitate. A: GPDH (○) and ACS (●) activities were determined at day 10 post-confluence as described in Materials and Methods. The specific activities in cells not exposed to palmitate were taken as 1 and were 485 ± 23 and 3.9 ± 0.2 mU/mg of protein for GPDH and ACS, respectively. These results are expressed as the mean ± SD of four separate experiments. B: RNA was extracted at day 10 post-confluence and analyzed as described in Materials and Methods. After normalization to GAPDH mRNA signals, the values were divided by those obtained for each probe with RNA from cells not exposed to palmitate. Symbols are (▲) LPL mRNA; (■) pOb24 mRNA; (△) ALBP mRNA; (●) ACS mRNA; (○) GPDH mRNA. The curves are representative of three separate experiments. The percentage of changes between triplicate assays did not vary by more than 10%.

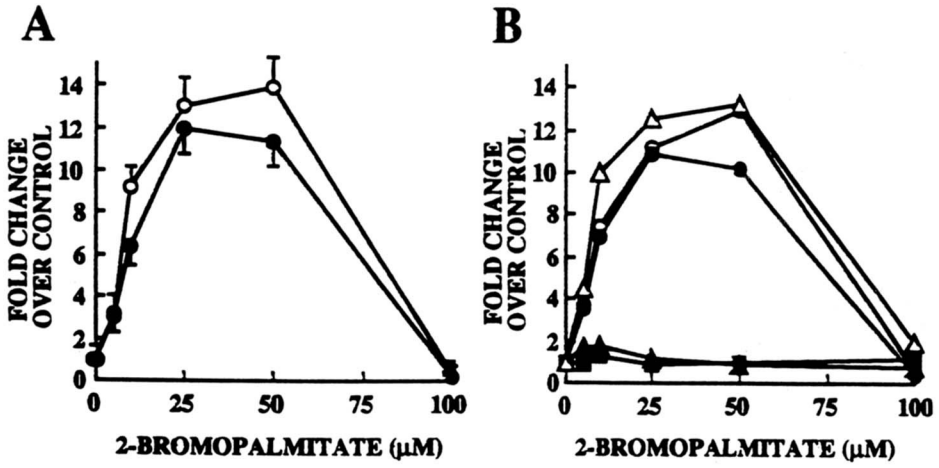

Fig. 2. Effect of 2-bromopalmitate on the expression of late-differentiation markers. Confluent Ob1771 cells were chronically exposed after confluence to standard differentiation medium supplemented with increasing concentrations of 2-bromopalmitate. A: GPDH (○) and ACS (●) activities, in 10-day post-confluent cells not exposed to 2-bromopalmitate, were 493 ± 28 and 4.1 ± 0.2 mU/mg of protein, respectively. The results are expressed as the mean ± SD from four separate experiments. B: The experimental protocol, the presentation of the results, and the symbols are similar to those of Fig. 1B. The curves are representative of three separate experiments. The percentage of changes between triplicate assays did not vary by more than 10%.

2A). As previously described for palmitate, the expression of genes related to early events of adipose cell differentiation, i.e., LPL and A2COL6/pOb24, was not significantly affected by 2-bromopalmitate (Fig. 2B and **Fig. 3**). By contrast, exposure to 2-bromopalmitate led to an enhancement of the terminal differentiation process as illustrated by the potent and general effect of the compound on the accumulation of mRNAs encoding late markers of adipose differentiation (for review, see ref. 3), such as ALBP, ACS, GPDH, Glut-4, HSL, adipsin, and FAT mRNAs (Figs. 2 and 3). The adipogenic action of 2-bromopalmitate appeared also evident at the morphological level. As shown in **Fig. 4** and as previously described (2), when cells were maintained for 16 days after confluence in standard differentiation medium, differentiation occurred in 30–40% of the cells, which appeared to be clustered (Fig. 4A). Chronic exposure to 2-bromopalmitate resulted in a net increase in the differentiated cell number (Fig. 4B). Under these culture conditions, the percentage of differentiated cells was estimated to be more than 90%.

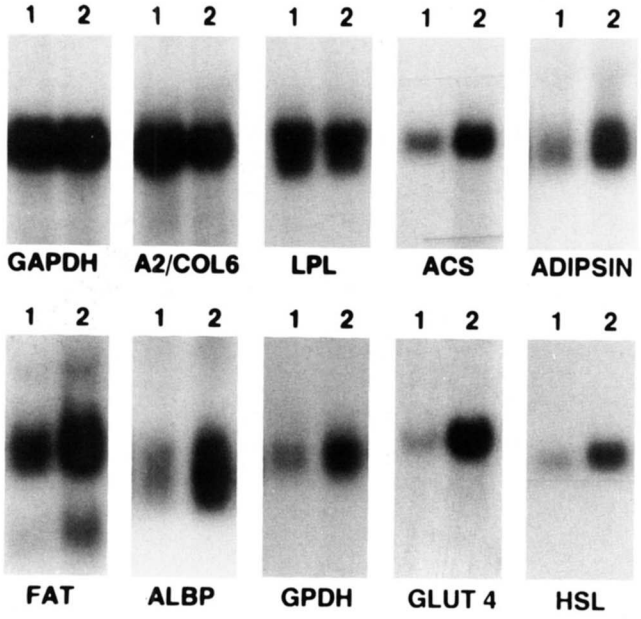

Fig. 3. Northern-blot analysis of adipose-related mRNAs of Ob1771 cells exposed or not to 2-bromopalmitate. Cells were maintained from confluence to day 16 in standard differentiation medium in the absence (lane 1) or the presence (lane 2) of 25 μM 2-bromopalmitate. RNA (20 μg per lane) was analyzed as described in Materials and Methods. The results are representative of three independent experiments.

It is worth noting that 2-bromopalmitate, as compared to palmitate, exerted its adipogenic effect at lower concentrations (half-maximal effect observed at ~10 μM versus >75 μM). Keeping in mind that the bromylated derivative was significantly less metabolized than the natural fatty acid (6), this observation suggested that the adipogenic action of fatty acids was likely not related to an increase in substrate availability for triacylglycerol synthesis but pointed out their potential role as signal transducing molecules. This point was investigated further.

Time dependence of the adipogenic effects of 2-bromopalmitate

Ob1771 cells maintained from confluence (day 0) in standard differentiation medium were exposed for various periods of time (1 day to 10 days) to 25 μM 2-bromopalmitate, and GPDH and ACS activities were deter-

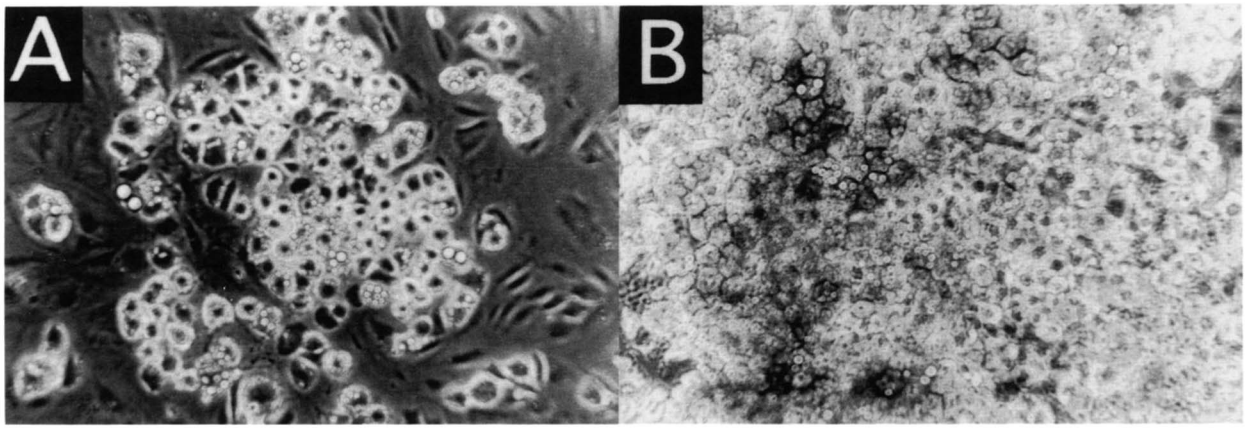

Fig. 4. Effect of 2-bromopalmitate on morphological differentiation of Ob1771 cells. Cells were grown to confluence in standard medium and maintained from confluence to day 12 in differentiation medium in the absence (A) or the presence (B) of 25 μM 2-bromopalmitate (magnification × 400).

mined at day 10 post-confluence. As shown in **Fig. 5,** treatment for the first 3 days following confluence was sufficient to provoke a near maximal induction of both enzyme markers determined 7 days later. Shorter treatments, i.e., from confluence to day 1 or day 2, produced only partial effects. Interestingly, 2-bromopalmitate appeared to be more efficient in inducing expression of late markers at an early stage rather than at a late stage of differentiation as exposure between days 5 and 8 led to a weak induction of GPDH and ACS activities determined at day 10 (Fig. 3) or at later days (not shown). These observations suggest that fatty acids exert their adipogenic effects by acting at early steps of the differentiation process which, in turn, control terminal events.

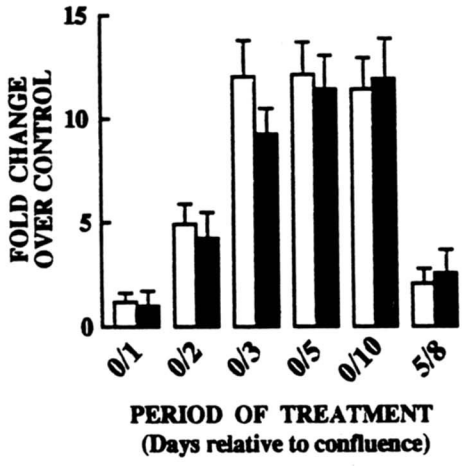

Fig. 5. Effect of exposure time to 2-bromopalmitate on the enzyme activities of late markers. Ob1771 cells were maintained after confluence (day 0) in the standard differentiation medium supplemented with 25 μM 2-bromopalmitate for the indicated period of time. GPDH (open columns) and ACS (filled columns) activities were determined at day 10 after confluence and were expressed by dividing each value by the value obtained for cells never exposed to the fatty acid derivative (483 ± 21 and 4.0 ± 0.2 mU/mg of protein for GPDH and ACS, respectively). The results are expressed as the mean ± SD of four separate experiments.

The time-course of appearances of GPDH and ACS mRNAs were next examined in cells exposed or not to 25 μM 2-bromopalmitate for the first 5 days of the confluent phase. As shown in **Fig. 6A,** for cells maintained in standard differentiation medium, GPDH and ACS mRNAs emerged 1 week after confluence and accumulated thereafter to reach a plateau at day 16. Exposure to the fatty acid derivative provoked an acceleration of the expression of both mRNAs and resulted lately as a ~4-fold enhancement in the content of both adipose-related mRNAs. It is of interest to note that after 3 days of treatment, i.e., at the minimal exposure time required to observe at later days a maximal effect on adipose differentiation (see Fig. 5), GPDH and ACS mRNAs were still undetectable. This lack of expression of terminal differentiation markers at that early time has been confirmed by measurements of GPDH and ACS activities which cannot be detected before day 5 post-confluence in cells maintained in the presence of 25 μM 2-bromopalmitate (not shown).

Using the same series of cells, the effects of the fatty acid derivative were investigated on post-confluent proliferation which is associated with terminal differentiation of Ob1771 cells (13). As shown in Fig. 6B, with respect to control cells, the amount of DNA per dish increased at day 2 and reached a plateau (50% increase) at day 6 post-confluence. In cells exposed to 2-bromopalmitate, the post-confluent proliferation was lengthened until day 10, resulting in a near doubling of the DNA amount per dish. Cell counting performed at day 10 indicated that exposure to 25 μM 2-bromopalmitate for 5 days from confluence led to a 30% increase in the cell number per dish when compared to cells maintained in standard differentiation medium (2.53 × 10^5 versus 1.93 × 10^5 cells per dish).

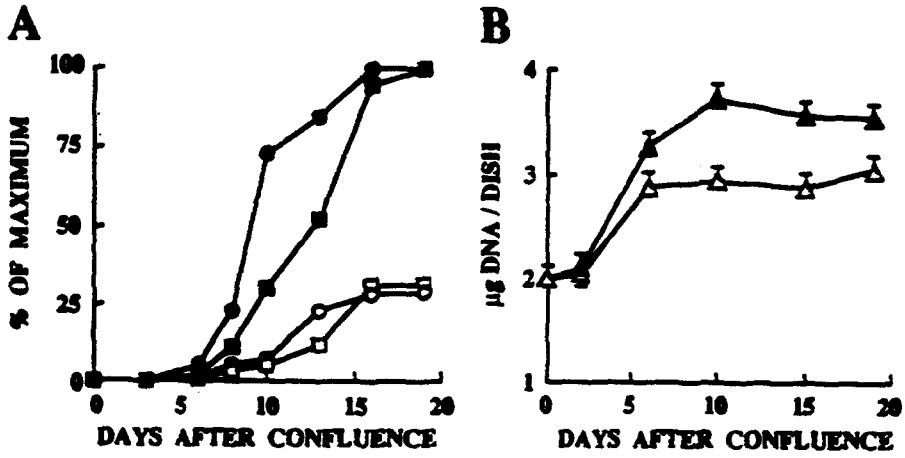

Fig. 6. Time-course of expression of adipose-related mRNAs (A) and post-confluent proliferation (B) in cells exposed or not to 2-bromopalmitate. Ob1771 cells, maintained after confluence (day 0) in standard differentiation medium, were treated (filled symbols) or not (open symbols) with 25 μM 2-bromopalmitate for the first 5 days of the confluent phase. A: RNA was analyzed as in Fig. 1B. Symbols are (●, ○) GPDH and (□, ■) ACS mRNA. The curves are representative of three separate experiments. The percentage of changes between triplicate assays did not vary by more than 10%. B: DNA content per dish was determined for cells treated (▲) or not (△) with 2-bromopalmitate. The results are expressed as the mean ± SD from four separate experiments.

Taken together, these results indicate that treatment of preadipose cells with fatty acids during the first days of the confluent phase improve the terminal differentiation process by increasing both the number of post-confluent mitoses and the expression of adipose-related genes, i.e., the final number of differentiated cells. This effect seems to be specific for long-chain fatty acids; control experiments, not reported here, have shown that octanoate and 2-bromooctanoate were ineffective in regard to the expression of terminal differentiation markers, whatever the concentration used or the time period of treatment.

Relationships between fatty acids and other adipogenic factors

The adipogenic effect of fatty acids is, in many respects, reminiscent of those of growth hormone (GH) and cAMP-elevating agents that exert their effects on adipose differentiation during the first days of the confluent phase (3). To examine the possible interactions between fatty acids and GH, Ob1771 cells were maintained in medium supplemented with GH-poor adult bovine serum, and exposed or not for the first 5 days of the confluent phase to a maximally effective concentration of GH (2.4 nM), to 2-bromopalmitate (25 μM), or to a combination of both agents. Ten days later, RNAs were analyzed for the presence of ALBP, GPDH, and ACS mRNAs. As shown in **Table 1,** and in accordance with our previous reports (9), the level of expression of these mRNAs related to terminal differentiation was very low in GH-poor medium. Growth hormone supplementation led to the emergence of both mRNAs. As anticipated from previous data (4), exposure of the cells to 2-bromopalmitate alone had no effect on GPDH but led to a significant expression of ACS and ALBP mRNAs. Combined treatment with GH and 2-bromopalmitate provoked an additional enhancement in the content of the three adipose-related mRNAs, approximately a 3-fold increase compared to cells exposed to GH only. These results favor the hypothesis that fatty acids and GH could be active at different steps, even if the same transducing machinery was actually involved. A similar hypothesis could be drawn in regard to the mechanisms of action of fatty acids and cAMP-elevating agents. As shown in **Fig. 7,** in the absence of 2-bromopalmitate, isobutyl methylxanthine (IBMX) provoked a 2-fold increase of GPDH activity. At increasing concentrations of 2-bromopalmitate, in the absence or in the presence of IBMX, GPDH activity increased in a dose-dependent manner. In both cases, the maximally effective concentration of 2-bromopalmitate was found to

TABLE 1. Combined effects of growth hormone and 2-bromopalmitate on the expression of terminal differentiation-related genes

	Control	Growth Hormone	2-Bromopalmitate	Growth Hormone + 2-Bromopalmitate
ALBP	0.5	12.81	4.24	30
ACS	0.2	7.19	3.39	24
GPDH	n.d.	6.41	n.d.	18

Cells were maintained in DME medium containing 8% serum. After confluence this medium was supplemented with 17 nM insulin, 2 nM T_3 (control). Cells were treated from day 0 to day 5 with 2.4 nM GH, 25 μM 2-bromopalmitate or a combination of both compounds. RNA was extracted at day 10 and analyzed as in Fig. 1. The results (arbitrary units) are representative of three separate experiments; n.d., not detectable. The percentage of changes between triplicate assays did not vary by more than 10%.

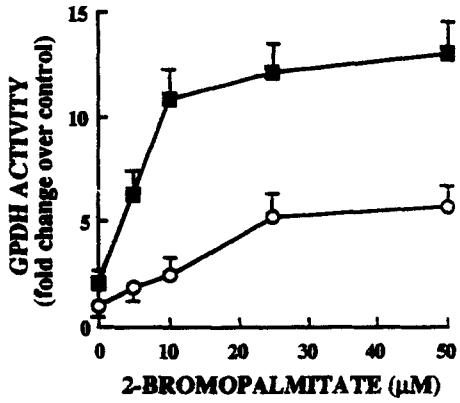

Fig. 7. Combined effects of 2-bromopalmitate and IBMX on GPDH activity. Ob1771 cells were maintained after confluence in medium supplemented with 8% bovine serum, 17 nM insulin, 2 nM T_3, and 1.2 nM GH and treated from day 0 to day 5 with increasing concentrations of 2-bromopalmitate in the absence (○) or in the presence of 100 μM IBMX (■). GPDH activities were determined at day 14 post-confluence and found to be 205 ± 16 mU/mg in control cells. The results are expressed as the mean ± SD of three separate experiments.

be 50 μM with a 5-fold increase above the corresponding control values. It is worth noting that treatment with IBMX led to a shift in the EC_{50} value for 2-bromopalmitate, which decreased from 15 to 5 μM.

DISCUSSION

The results of the present studies demonstrate that fatty acids are involved as triggering agents in the control of adipose cell differentiation. This confirms the previous morphological observations showing that lipid supplementation of the culture medium resulted in an enhancement of triacylglycerol accumulation in adipose cells. The present findings indicate that fatty acids do not affect the first stage of differentiation, i.e., the commitment of adipoblasts to preadipose cells, as the expression of early markers, such as A2COL6/pOb24 and LPL genes, remains unchanged after fatty acid supplementation. In contrast, fatty acids trigger the process of differentiation of preadipose cells to adipose cells by increasing the number of post-confluent mitoses (Fig. 6B) and the expression of terminal differentiation-related genes (Figs. 1, 2, 3, and 6A). In other words, fatty acids trigger the formation of new fat cells and lead, in vitro, to an hyperplastic phenomenon.

The results obtained with 2-bromopalmitate rule out the possibility that cell hyperplasia is mediated by an increase in fatty acid metabolites and/or substrate availability for lipid synthesis. To the contrary, our results are in favor of fatty acids per se as being the true inducers of terminal differentiation. First, we have previously shown that, in preadipose Ob1771 cells, 2-bromopalmitate is neither metabolized nor incorporated into complex lipids as it is not a substrate for the acyl-CoA synthetase already expressed at that time in preadipose cells and which appears mainly specific for arachidonic acid (6). Conversion of preadipose to adipose cells is associated with the emergence of a new fatty acyl-CoA synthetase, called ACS in this paper. 2-Bromopalmitate could then be activated at about one-third of the rate of palmitate and incorporated into complex and neutral lipids (6). As shown in Figs. 5 and 6, the maximal effect of the bromylated derivative takes place in preadipose cells before the emergence of ACS and thus before its activation into 2-bromopalmitoyl CoA. Second, 2-bromopalmitate was more potent than palmitate in enhancing terminal differentiation (Fig. 2 compared to Fig. 1); this shift in effective concentrations is probably due to the rapid decrease in the actual concentration of palmitate which is extensively activated into palmitoyl-CoA and incorporated into lipids, whereas the utilization of 2-bromopalmitate is less efficient (6). Exposure to high concentrations of 2-bromopalmitate leads to an inhibition of differentiation. This inhibition is not due to toxic effects as the levels of expression of GAPDH, LPL, and pOb24 mRNAs were not affected under these conditions (Fig. 2). We have observed, but not reported here, that a similar inhibition occurs in cells exposed to a high concentration of 2-bromopalmitate for a short period of time (100 μM for 1 day after confluence). The reasons for this inhibition remain unclear but might be a consequence of the effect of fatty acids on glycerolipid synthesis enzymes or unidentified membrane proteins when present at high concentrations (14).

Taken together, these observations suggest that, at the preadipose state, an increase in the intracellular concentration of unprocessed fatty acids plays the role of a signal triggering the proliferation of preadipose cells and the expression of terminal differentiation-related genes. Although the value should be within the micromolar range, it is technically difficult to make a fair estimate of the intracellular concentration of unesterified fatty acid required to enhance adipose cell differentiation. In the case of 2-bromopalmitate, the intracellular concentration (~3 μM) appears to equilibrate with the external concentration of fatty acids that is not bound to serum albumin (6). In any event, in serum-free hormone-supplemented medium, i.e., in the absence of serum albumin, preadipocytes are able to respond at the gene level at a concentration as low as 0.1 μM 2-bromopalmitate (E-Z. Amri, G. Ailhaud, F. Bonino, and P-A. Grimaldi, unpublished data). The intracellular concentration of unesterified fatty acids is of critical importance as natural long-chain fatty acids as well as 2-bromopalmitate are able to modulate the transcription rate of ALBP and ACS genes once the genes are activated. At that stage, as ALBP is present in the cytosol within the millimolar range of concentration, the concentration of active, unesterified fatty acids not bound to ALBP

is postulated to be exceedingly low and actually within the range of other amphipathic carboxylates such as retinoic acid. Retinoic acid is known to be bound intracellularly to cytosolic binding proteins and to act via retinoic acid receptors (15). How fatty acids act to trigger terminal differentiation remains an open question, but it cannot be excluded that *trans*-acting factors recognizing fatty acids are involved in this phenomenon (16). For instance, in Ob1771 cells, a new member of the superfamily of steroid hormone receptor has been cloned and sequenced; it shows 70% homology with members of the peroxisome proliferator-activated receptors (PPARs) and its expression is positively modulated by long-chain fatty acids (E-Z. Amri, G. Ailhaud, F. Bonino, and P-A. Grimaldi, unpublished data). In any event, it is clear that fatty acids act different from other adipogenic agents, i.e., cAMP-elevating agents that activate the PKA pathway (17) and GH which activates the PKC pathway (18). Even though the mechanisms of action of fatty acids remain to be elucidated, fatty acids appear to act as signal transducing molecules in addition to playing the role of an energy source and of being essential membrane components and eicosanoid precursors. Such a new role for fatty acids remains to be assessed in vivo but it can be assumed that, after high-fat and high-carbohydrate feeding, the flux of fatty acids entering preadipose cells from chylomicrons and VLDL, respectively, should be significantly increased. If it were so, both hypertrophy and hyperplasia of adipose tissue could be induced, thus providing a link between adipogenic diets and the excessive development of adipose tissue.

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