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Requirement and role of arachidonic acid in the differentiation of pre-adipose cells

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The terminal adipose differentiation of Ob1771 cells, characterized by glycerol-3-phosphate dehydrogenase activity and triacylglycerol accumulation, was studied in serum-free hormone-supplemented medium containing growth hormone, tri-iodothyronine, insulin, transferrin and fetuin. Arachidonic acid was able to substitute for a crude adipogenic fraction isolated from fetal bovine serum but not for growth hormone or tri-iodothyronine. Arachidonic acid was also able to increase in a rapid and dramatic manner cyclic AMP production; moreover it was able to amplify the adipose conversion promoted by other agents elevating cyclic AMP concentrations and to induce inositol phospholipid breakdown. Both phorbol 12-myristate 13-acetate, a protein kinase C activator, and ionomycin, a Ca^{2+}-mobilizing agent, showed potent synergy with agents elevating cyclic AMP concentrations for the promotion of adipose conversion, whereas 8-bromo cyclic GMP and 4α-phorbol 12,13-dibutyrate were ineffective. The triggering of both the cyclic AMP and inositol phospholipid pathways was accompanied by a single round of cell division, and within a few days all the cells became differentiated. Similar results were obtained, after exposure to arachidonic acid, with pre-adipose 3T3-F442A cells and with rat adipose precursor cells in primary culture. The availability of arachidonic acid from intracellular stores and/or of exogenous origin should play a major role for the onset of critical mitoses leading to terminal differentiation in pre-adipose cells.

INTRODUCTION

The adipose conversion of Ob17 cells [1] and other pre-adipocyte cell lines, i.e. mouse 3T3, 10T1/2 and 1246 cells [2–4], represents a valid model for the development of adipose tissue *in vivo* [5,6]. Studies of the time course of the changes in phenotypic markers expressed during adipose conversion have allowed the distinction of early and late events in the expression of the differentiation programme of pre-adipocyte cells [7–9]. The expression of early markers, such as lipoprotein lipase, is dependent upon growth arrest, and precedes DNA synthesis and post-confluent mitoses of early marker-containing cells [10,11]. The expression of late markers such as glycerol-3-phosphate dehydrogenase (GPDH), accompanied by triacylglycerol accumulation, is dependent on DNA synthesis and corresponds to terminal differentiation [12,13]. This situation is in agreement with the data obtained *in vivo* reported for the development of adipose tissue in newborn rodents. These studies have shown that (i) the labelling indices, after pulse-labelling in rat subcutaneous fat-organs with [^{3}H]thymidine, were the highest in partly differentiated cells still deprived of lipid droplets [14], and (ii) the rise in GPDH activity, which is subsequently detected in all triacylglycerol-filled mature fat-cells, was preceded by a decrease in the labelling index of GPDH-negative cells [15]. These data suggest that DNA synthesis, mitoses and terminal differentiation of adipose precursor cells are coupled *in vivo*.

In order to test this hypothesis and to gain a better understanding of this phenomenon, studies of adipose conversion *in vitro* have been performed in serum-free chemically defined medium [16–19]. The differentiation medium defined for Ob17 cells contained insulin, transferrin, growth hormone (GH), tri-iodothyronine (T_3) and fetuin as well as a serum fraction enriched in some unidentified adipogenic factor(s) [17]. Lipoprotein lipase behaved as an early marker of adipose conversion of Ob17 cells both in serum-supplemented [7,8] and serum-free medium [17]; it was expressed at high concentrations in the absence of the serum adipogenic fraction. This fraction proved to be only required for the expression of terminal differentiation and thus could possibly be involved in the promotion of post-confluent mitoses.

In the present paper we report that arachidonic acid, which is present in this serum adipogenic fraction, was able to promote adipose conversion of pre-adipocyte cells. Further investigation on the role of arachidonic acid has shown that it is involved in the control of post-confluent mitoses through its ability to increase intracellular cyclic AMP concentrations and to promote breakdown of inositol phospholipids.

Abbreviations used: GPDH, *sn*-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8); LDH, lactate dehydrogenase (EC 1.1.1.27); GH, growth hormone (somatotropin); T_3, tri-iodothyronine; FGF, fibroblast growth factor; EGF, epithelial growth factor; PG, prostaglandin; 8-Br-cAMP, 8-bromo cyclic AMP; 8-Br-cGMP, 8-bromo cyclic GMP; αPDBu, 4α-phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; IBMX, 3-isobutyl-1-methylxanthine; 12-HETE, 12-hydroxyeicosa-5,8,10,14-tetraenoic acid; HHT, 12-hydroxyheptadeca-5,8,10-trienoic acid.

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EXPERIMENTAL

Materials and chemicals

Fetal bovine serum was obtained from Flow Laboratories (Puteaux, France) and culture media were from GIBCO (Cergy-Pontoise, France). Basic fibroblast growth factor (FGF) from bovine pituitary and mouse epidermal growth factor (EGF) were purchased from Collaborative Research (Lexington, MA, U.S.A.). Bovine GH was obtained through the National Hormone and Pituitary Program (National Institute of Arthritis and Metabolic Diseases, Baltimore, MD, U.S.A.). The growth-promoting kallikrein-like activity from rat submaxillary gland was prepared as previously described and routinely used after precipitation with $(NH_4)_2SO_4$ [20]. Collagenase from *Clostridium histolyticum*, 8-bromo cyclic AMP (8-Br-cAMP) and 8-bromo cyclic GMP (8-Br-cGMP) were products of Boehringer (Mannheim, Germany). Ionomycin and forskolin were products of Calbiochem (San Diego, CA, U.S.A.). 4α-Phorbol 12,13-dibutyrate (αPDBu) was purchased from Interchim (Montluçon, France). Fetuin prepared by the method of Pedersen, bovine serum albumin, bovine insulin, human transferrin, arachidonic acid, 3-isobutyl-1-methylxanthine (IBMX), prostaglandin E_1 (PGE_1), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), T_3, isoprenaline (isoproterenol), phorbol 12-myristate 13-acetate (PMA), tocopherol acetate, sodium ascorbate and sodium selenite as well as other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *myo*-[2-^{3}H]Inositol (16.3 Ci/mmol) as well as reagents for the assay of cyclic AMP and cyclic GMP were purchased from Amersham International (Amersham, Bucks., U.K.).

Cell culture

Ob1771 cells [11] were obtained after subcloning of Ob17 cells established from genetically C57BL.6J mice [1,21] and were selected in this study for their higher expression of late markers of adipose conversion, including GPDH, as compared with the original clone. 3T3-F442A cells [2] were kindly provided by Dr. H. Green (Boston, MA, U.S.A.). Stock cultures of Ob1771 and 3T3-F442A cells were maintained in Dulbecco's modified Eagle's medium supplemented with biotin, pantothenate, antibiotics and 10% (v/v) foetal bovine serum as previously described [21].

For experiments in serum-free medium, cells were first inoculated in serum-supplemented medium at a density of 8.5×10^3 cells/cm^2 in 24-multiwell Linbro tissue-culture plates (2 cm^2/well) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air for 20–24 h. Cells were then thoroughly washed twice with a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1, v.v) containing 15 mM-$NaHCO_3$, 15 mM-Hepes buffer, pH 7.4, 33 μM-biotin, 17 μM-pantothenate, 62 μg of penicillin/ml and 50 μg of streptomycin/ml (referred to as basal medium). Cells were grown in 4F medium [16,17], consisting of the basal medium described above supplemented with insulin (5 μg/ml), transferrin (10 μg/ml), a partially purified kallikrein-like activity from rat submaxillary gland (1 μg/ml; ref. [20]) and FGF (25 ng/ml). Under these conditions, cells reached confluence within 3 days. They were subsequently shifted to the same basal medium as above, enriched in either tocopherol acetate (0.5 μM) or sodium ascorbate (100 μM) plus selenite (20 nM) as antioxidants. The medium contained also insulin (5 μg/ml), transferrin (10 μg/ml), GH (2 nM), T_3 (200 pM) and fetuin (500 μg/ml). This medium, referred to as 5F medium, was further supplemented or not as indicated in the Results section. Two medium changes were subsequently performed at days 3 and 7 after confluence. Arachidonic acid, prostaglandins, phorbol esters, forskolin and ionomycin were dissolved in ethanol and added at a 1:100 dilution into culture media. Ethanol concentration, which did not exceed 1%, had no effect on either adipose conversion or short-term cellular responses, i.e. intracellular concentrations of cyclic nucleotides or inositol phosphates.

Cell growth was monitored by cell count determined with a Coulter counter after trypsin detachment. The percentage of lipid-containing differentiated cells was estimated microscopically at days 10–12 post-confluence on at least six separate fields by two independent observers. Mean values are reported; they did not differ by more than 2%.

Purification of the serum-derived adipogenic fraction

The crude fraction was obtained by ethanolic extraction at pH 4.5 of dialysed foetal bovine serum (M_r cut-off of tubing 2000) by the procedure of Löffler *et al.* [22], and solubilized after freeze-drying in 0.15 M-NaCl/1.5 mM-sodium phosphate buffer, pH 7.4. Purification of this fraction was further achieved by extraction with ethyl acetate/cyclohexane (1:1, v/v) followed by chromatography on octadecylsilyl-silica cartridges (Sep-Pak C_{18}; Waters Associates, Milford, MA, U.S.A.). Loading and elution with hexane/chloroform (13:7, v/v) by the procedure of Powell [23] allowed the recovery of an active fraction. All fractions obtained throughout purification were dissolved in ethanol and stored at −20 °C; when used, they were added at 1:100 dilution at each medium change.

Enzyme assays

GPDH and lactate dehydrogenase (LDH) were assayed spectrophotometrically as previously described [24,25] on cell homogenates from two pooled culture wells, obtained by using a Potter–Elvehjem homogenizer (30 strokes), in 20 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol. Assays were carried out in duplicate at day 12 after confluence. Inter-assay variability did not differ by more than 5% and variability among mean values from separate wells maintained under identical culture conditions never exceeded 5%. Protein concentrations were determined by the method of Lowry *et al.* [26] with bovine serum albumin as standard. All enzyme activities are expressed in munits/mg, i.e. nmol of product formed/min per mg of protein.

Cyclic AMP and cyclic GMP assays

Intracellular cyclic AMP and cyclic GMP concentrations were determined with commercial kits by a protein-binding assay and a radioimmunoassay respectively, according to the manufacturer's instructions. Cyclic nucleotides were extracted with 1.2 ml of ice-cold ethanol/5 mM-EDTA (2:1, v/v). After scraping the cell monolayer and centrifugation, 1 ml of the supernatant was dried in a Speed-Vac evaporator. Duplicate samples were solubilized before assays in 150 μl of 50 mM-Tris/HCl buffer, pH 7.5, containing 4 mM-EDTA and assayed at least in triplicate.

Table 1. Induction of adipose conversion by arachidonic acid

Confluent Ob1771 cells were maintained for 12 days in either 5F medium or 5F medium deprived of GH or T_3 (two last lines) and supplemented as indicated. All media were enriched with 0.5 μM-tocopherol acetate as antioxidant. The amount of crude or purified adipogenic serum fractions added per well was equivalent to that extractable from 0.1 ml of foetal bovine serum and found to be maximally effective under these conditions. Specific activities of GPDH were determined as described in the Experimental section. The activity values are reported as means ± S.E.M. for five independent experiments performed with the crude and purified adipogenic serum fractions and as medians ± ranges for two independent experiments performed with arachidonic acid. At day 12 post-confluence, the mean percentage of differentiated cells was estimated to be < 2% in 5F medium and 68%, 42% and 25% in 5F medium supplemented with crude adipogenic fraction, purified adipogenic fraction and 10 μM-arachidonic acid respectively.

Culture conditions	GPDH specific activity (munits/mg)
5F medium	30 ± 10
+ Crude adipogenic fraction	2200 ± 200
+ Purified adipogenic fraction	1350 ± 150
+ Arachidonic acid (1 μM)	100 ± 20
+ Arachidonic acid (5 μM)	350 ± 50
+ Arachidonic acid (10 μM)	800 ± 150
+ Arachidonic acid (10 μM) − GH	90 ± 20
+ Arachidonic acid (10 μM) − T_3	40 ± 10

Measurement of inositol phospholipid breakdown

Growing Ob1771 cells were labelled with *myo*-[2-^{3}H]inositol (2 μCi/well) in 4F medium. After 3 days confluent cells were washed twice and then preincubated at 37 °C in 0.7 ml of 5F medium deprived of fetuin but supplemented with 20 mM-LiCl. After 30 min this medium was discarded and replaced by 0.5 ml of 5F medium containing 20 mM-LiCl and supplemented or not with various agents. Incubations were terminated by substituting the above medium by 0.5 ml of ice-cold 7% (w/v) $HClO_4$. After 30 min at 4 °C the acidic solutions were collected, neutralized with 1 M-Hepes solution and 2 M-KOH and loaded on to Dowex columns equilibrated with 5 mM-ammonium formate buffer, pH 5.0. After washing first with 5 mM- and then with 40 mM-ammonium formate, the fraction containing total soluble inositol phosphates was eluted with 5 ml of 2 M-ammonium formate which was then mixed with 12 ml of Aquassure scintillation cocktail for β-radiation counting.

RESULTS

Arachidonic acid promotes the terminal differentiation of Ob1771 cells

Table 1 shows that addition to 5F medium containing insulin, transferrin, GH, T_3 and fetuin of either a crude or a purified serum-derived adipogenic fraction led to terminal differentiation of Ob1771 cells. This is illustrated by the high GPDH activity, a late marker of adipose conversion, when post-confluent cells were maintained for 12 days in the presence of a maximally effective dose of either fraction, as compared with control cells maintained in 5F medium alone. The crude active fraction, after extraction with ethyl acetate/cyclohexane (1:1, v/v), was chromatographed on octadecylsilyl-silica cartridges according to the extraction procedure for arachidonic acid metabolites [23]. Adipogenic activity was exclusively recovered by elution of the cartridges with hexane/chloroform (13:7, v/v). This fraction was previously reported to contain fatty acids and monohydroxy fatty acids but to be depleted of prostaglandins [23]. (Control experiments with [^{3}H]$PGF_{2\alpha}$ added to fetal bovine serum showed that 47% and <0.2% of the radioactivity were recovered in the crude adipogenic fraction and the purified adipogenic fraction respectively. When the adipose conversion occurred in the presence of the crude adipogenic fraction, depending upon the batch of fetal bovine serum used for the preparation the actual concentration of $PGF_{2\alpha}$ in the culture medium varied from 1.1 to 8 nM.) Analysis of this purified fraction by combined reverse-phase h.p.l.c. and u.v. spectrophotometry indeed showed the presence of hydroxylated polyunsaturated fatty acids, mainly 12-HETE and HHT (approx. 50 nmol of each fatty acid/l of serum). The occurrence of these metabolites was probably the result of their release by platelets during serum preparation. Arachidonic acid was also recovered in this fraction in much higher amounts (2–10 μmol/l of serum).

12-HETE, but not HHT, when added to 5F medium alone or in combination at concentrations 100-fold higher than that corresponding to a maximally effective dose of the purified adipogenic fraction, induced a rather low and irreproducible adipose conversion of Ob1771 cells (results not shown). In contrast, arachidonic acid (1–10 μM) did mimic, at least in part, the 'adipogenic' activity of the serum fraction and induced adipose conversion in a reproducible manner, leading to the expression of GPDH activity (Table 1). The promoting effect of arachidonic acid was not restricted to cells of the Ob1771 clonal line. It was also observed in 5F medium for 3T3-F442A cells. In this latter case, the percentage of differentiated cells, quite significant in 5F medium alone [17], was approximately doubled in the presence of 10 μM-arachidonic acid (R. Négrel, D. Gaillard & G. Ailhaud, unpublished work). Likewise, when supplemented with fetuin and 10 μM-arachidonic acid, a similar doubling in the number of differentiated cells was obtained for adipose precursor cells of rat subcutaneous or periepididymal adipose tissue (R. Négrel, D. Gaillard & G. Ailhaud, unpublished work), which were reported to differentiate within 6 days in the sole presence of insulin, transferrin and T_3 [18].

It is noteworthy that arachidonic acid was only active in the simultaneous presence of GH and T_3 (Table 1), hormones that have already been shown to be required for the terminal differentiation of pre-adipocyte cells in serum-supplemented media [27–30]. During the course of our first experiments, performed in 5F medium supplemented with 0.5 μM-tocopherol acetate as antioxidant, the proportion of differentiated cells in the presence of 10 μM-arachidonic acid that was attained within 12 days ranged from 15 to 25% (Tables 1–3). In further experiments, where tocopherol acetate was replaced by 100 μM-ascorbate plus 20 nM-sodium selenite, the percentage of differentiated cells in the presence of 10 μM-arachidonic acid was actually increased to at least

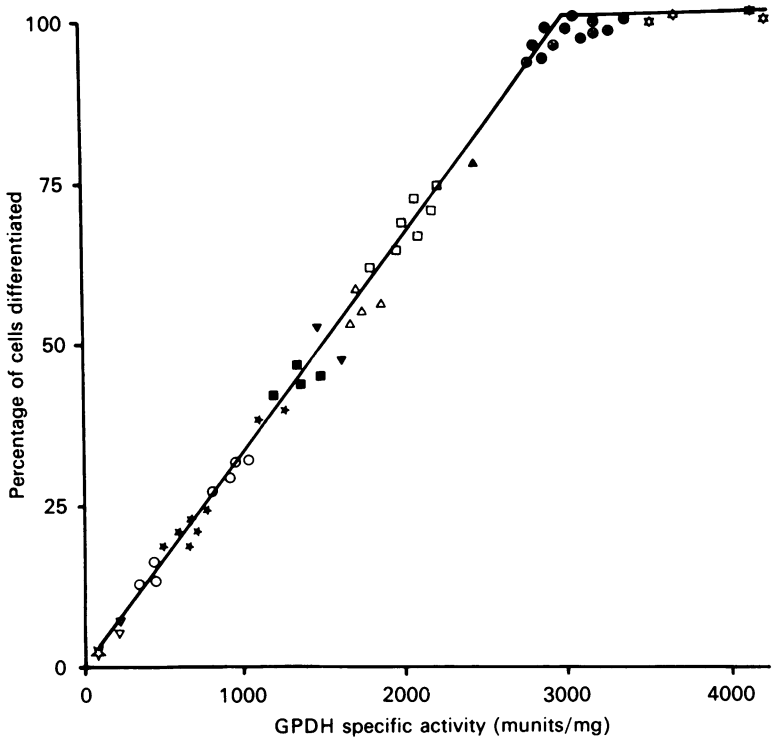

Fig. 1. Relationship between the proportion of differentiated cells, containing triacylglycerols, and the specific activity of GPDH

The specific-activity values of GPDH and the proportions of differentiated cells determined under various culture conditions were plotted and analysed by using a classic linear-regression programme ($r = 0.982$). The different culture conditions are indicated by symbols as follows: 5F medium alone (☆); 5F medium supplemented with the crude (□) or the purified (■) serum-derived adipogenic fraction or with 10 μM-arachidonic acid (★), with 0.5 mM-8-Br-cAMP (○), with 10 μM-arachidonic acid plus 0.5 mM-8-Br-cAMP (⊛), with 100 μM-IBMX or 10 μM-forskolin (same symbol: △), with 10 nM-PMA (▽), with 10 nM-PMA plus 100 μM-IBMX or plus 10 μM-forskolin (same symbol: ✿), with 10 μM-arachidonic acid plus 100 μM-IBMX (▲) or with 0.5 mM-8-Br-cAMP plus 10 nM-PMA (▼).

35% (Table 2). As shown in Fig. 1, independently of the nature of the differentiation-triggering agent, a correlation was observed between the proportion of differentiated triacylglycerol-containing cells that were easily detected microscopically and the specific activity of GPDH measured at day 12 ($r = 0.982$): a specific activity of 3000 munits/mg or more was found to correspond in a reproducible manner to 98–100% differentiated cells present as a monolayer.

Modulation of cyclic AMP production by arachidonic acid and adipose conversion

Arachidonic acid has been described in numerous cellular systems to modulate intracellular concentrations of cyclic nucleotides [31,32]. The possible relationships between changes in the content of cyclic nucleotides and the differentiation induced by arachidonic acid were first investigated by measuring the cyclic AMP and cyclic GMP contents of Ob1771 cells exposed or not for 5 min to 10 μM-arachidonic acid in the presence of 100 μM-IBMX. The cyclic GMP content (5 ± 1 pmol/mg of cell protein) determined in control cells maintained in 5F medium remained unchanged when arachidonic acid was added. In contrast, the cyclic AMP content was dramatically increased from 80 ± 20 to 1600 ± 200 pmol/mg of cell protein after a 5 min exposure to 10 μM-arachidonic acid. As illustrated by the dose–response curve presented in Fig. 2, a good correlation was observed between the increase in GPDH specific activity and the enhancement of cyclic AMP production induced by arachidonic acid.

The possible involvement of cyclic AMP as an intracellular messenger of adipose conversion was further investigated by examining, in the absence of arachidonic acid, the behaviour of cells chronically exposed to agents reported to modulate cyclic AMP. Data in Table 2 show clearly that long-term addition to 5F medium of forskolin or PGE_1 as adenylate cyclase-activating agents, IBMX as a phosphodiesterase inhibitor or 8-Br-cAMP as a stable analogue of cyclic AMP led to the expression of high GPDH activity. In contrast, chronic addition of 8-Br-cGMP was ineffective. The dose-dependent increase in GPDH specific acitivity induced by 8-Br-cAMP is presented in Fig. 3. This curve indicates that this agent exerted a maximal effect upon terminal differentiation when added in the millimolar range of concentration. Taken together, these data are consistent with the fact that arachidonic acid was active on adipose conversion through its ability to enhance cyclic AMP production.

Dual effect of arachidonic acid on adipose conversion of Ob1771 cells

In addition to its role as an effector of cyclic AMP production, arachidonic acid was also able to amplify the adipose conversion promoted by other agents elevating cyclic AMP concentrations (Table 2). This effect of

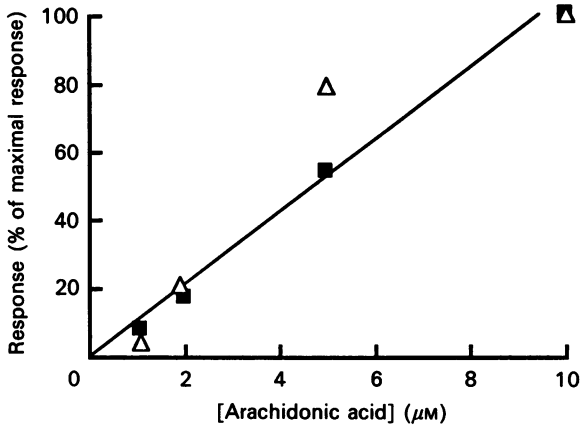

Fig. 2. Correlation between short-term cyclic AMP production and long-term GPDH expression induced by arachidonic acid

After preincubation in 5F medium (5 min) containing 100 μM-IBMX, confluent Ob1771 cells were exposed for 5 min to the same medium with or without supplementation with increasing concentrations of arachidonic acid as indicated. The intracellular cyclic AMP content was assayed as described in the Experimental section. Mean values from triplicate determinations in two separate experiments are expressed as percentages of the response obtained at 10 μM-arachidonic acid (1600±200 pmol/mg of protein) after subtracting the mean basal value in the absence of arachidonic acid (80±20 pmol/mg of protein). The specific activity of GPDH was determined on 12-day post-confluent cells maintained by 5F medium supplemented or not with the indicated concentrations of arachidonic acid. Mean values from duplicate determinations in two separate experiments are expressed as percentages of the response obtained at 10 μM-arachidonic acid (680±50 munits/mg) after subtracting the mean basal value of control cells maintained in 5F medium (30±10 munits/mg). The relationship between cyclic AMP content (△) and GPDH specific activity (■) was analysed by using a classic linear-regression programme ($r = 0.963$).

Table 2. Induction of adipose conversion by agents elevating cyclic AMP concentrations and amplification by arachidonic acid

Confluent Ob1771 cells were maintained for 12 days under the culture conditions as indicated and assayed for GPDH as described in the Experimental section. Culture media were enriched with tocopherol acetate in Expts. 1 and 2 and with sodium ascorbate plus selenite in Expts. 3 and 4. The values are the means for two separate determinations that did not differ by more than 5% in Expts. 1 and 2 and the medians ± ranges for two independent series of cells in Expt. 3 and for 12 independent series of cells in Expt. 4. The percentages of differentiated cells in the presence of 10 μM-arachidonic acid plus 0.5 mM-8-Br-cAMP (Expt. 1), 1 μM-forskolin or 200 nM-PGE_1 (Expt. 3) were close to 100%. The combination of 0.5 mM-8-Br-cAMP and 10 μM-arachidonic acid in 5F medium supplemented with 100 μM-sodium ascorbate and 20 nM-selenite (Expt. 4) also gave rise to 100% of differentiated cells in 12 independent series of cells.

	GPDH specific activity (munits/mg)	
Addition...	None	10 μM-Arachidonic acid
Culture conditions		
Expt. 1		
5F medium	23	475
+8-Br-cAMP (0.5 mM)	368	3110
+8-Br-cGMP (0.5 mM)	25	440
Expt. 2		
5F medium	30	530
+Isoprenaline (1 μM)	60	1090
Expt. 3		
5F medium	73±20	1200±300
+IBMX (100 μM)	1676±150	2300±210
+Forskolin (1 μM)	1060±120	3970±120
+PGE_1 (200 nM)	500±85	4030±90
Expt. 4		
5F medium + 8-Br-cAMP (0.5 mM)	900±150	3800±400

arachidonic acid was observed with 8-Br-cAMP, isoprenaline, IBMX, forskolin and PGE_1. Under three different conditions, i.e. 0.5 mM-8-Br-cAMP, 1 μM-forskolin or 200 nM-PGE_1 in the presence of 10 μM-arachidonic acid, the whole population of cells became able to differentiate into adipose cells. As expected, the combination of 0.5 mM-8-Br-cGMP plus 10 μM-arachidonic acid did not enhance adipose conversion as compared with that induced by arachidonic acid alone. Data in Tables 2 and 3 show that when Ob1771 cells were exposed to a concentration of 0.5 mM-8-Br-cAMP, i.e. a near maximally effective concentration (see Fig. 3), arachidonic acid was still able to induce amplification of the adipose conversion process even when present at a submaximally effective concentration. This effect seems to be specific for enzyme markers of adipose conversion, since GPDH activity was increased by 10–18-fold whereas LDH activity, not related to lipogenesis, was only increased 1.5–2-fold. These results suggested that arachidonic acid was able to cause additional molecular events beyond those attributable to enhanced intracellular concentrations of cyclic AMP.

This led us to test the hypothesis that inositol phospholipid hydrolysis, well known to be a source of second messengers [33], could act in concert with the cyclic AMP pathway and, if so, could be stimulated by arachidonic acid. For that purpose, studies on the mobilization of inositol phospholipids were performed as well as studies on the possible role of some arachidonic acid metabolite(s) on the adipose conversion process. In both cases, $PGF_{2\alpha}$ was selected, as it has been reported to be synthesized from arachidonic acid by pre-adipocytes [34–36] and adipocytes [37] and as it is also known to be an agonist of inositol phospholipid breakdown in various cell types [38–40]. EGF was assayed in parallel since, despite the fact that it is a potent mitogen, its inability to induce the mobilization of inositol phospholipids in fibroblastic cells has been reported [41,42]. It is worth mentioning that neither $PGF_{2\alpha}$ nor EGF affected the basal cyclic AMP content in Ob1771 cells. As shown in Fig. 4, arachidonic acid at a concentration of 10 μM was able to induce a slight but significant release of inositol phosphates above control. This mobilization remained

Table 3. Amplification of adipose conversion induced by arachidonic acid in the presence of 8-Br-cAMP or $PGF_{2\alpha}$

Ob1771 cells were maintained for 12 days post-confluence in 5F medium supplemented as indicated. All media were enriched with 0.5 μM-tocopherol acetate. Enzyme specific activities are expressed in munits/mg and amounts of protein in μg/well; their values are the means for two separate determinations that did not differ by more than 5%.

Addition ... / Culture conditions	None			8-Br-cAMP (0.5 mM)			$PGF_{2\alpha}$ (200 nM)			EGF (3 nM)		
	GPDH	LDH	Protein	GPDH	LDH	Protein	GPDH	LDH	Protein	GPDH	LDH	Protein
5F medium	40	1050	52	200	1445	51	130	1275	52	62	1800	127
+ Arachidonic acid (1 μM)	100	1230	48	380	1200	71	400	1625	77	–	–	–
+ Arachidonic acid (5 μM)	420	1520	69	1600	1960	102	1220	2480	102	500	2490	140
+ Arachidonic acid (10 μM)	720	1570	84	2780	2990	120	1250	2260	100	925	2530	135

moderate as compared with that induced by a maximally effective concentration of $PGF_{2\alpha}$ (EC_{50} value 30 nM; results not shown). Nevertheless, this mobilization proved to be reproducible and completely suppressed in the presence of 10 μM-indomethacin as an inhibitor of cyclooxygenase, reaching in that case the values obtained with either control cells or EGF-treated cells. {As previously reported for Ob17 cells maintained in serum-supplemented medium [34], 10 μM-indomethacin was also able to abolish the synthesis of prostaglandins in post-confluent Ob1771 cells (PGE_2 > 6-oxo-$PGF_{1\alpha}$ > $PGF_{2\alpha}$) after prelabelling with [^{3}H]arachidonic acid in 5F medium and then exposure to 5F medium supplemented with 10 μM-arachidonic acid. Additional experiments showed that 1 μM-indomethacin was equally effective in abolishing prostaglandin synthesis.} As illustrated in Table 3 with regard to adipose conversion, the effect of arachidonic acid, present as an agent elevating cyclic AMP concentrations, was amplified when $PGF_{2\alpha}$ was present at a concentration (200 nM) that was maximally effective upon inositol phospholipid breakdown. This amplification phenomenon did not occur in the presence of EGF in arachidonic acid-containing medium, in spite of the fact that EGF elicited a strong mitogenic effect, as illustrated by the increase in the cellular protein content (Table 3) and by microscopic observations. Taken together, these results are consistent with a dual role of arachidonic acid in activating both the cyclic AMP and polyphosphoinositide pathways. These results also suggest that intracellular messengers, generated through the breakdown of inositol phospolipids, amplify the adipose conversion induced by agents elevating cyclic AMP concentrations.

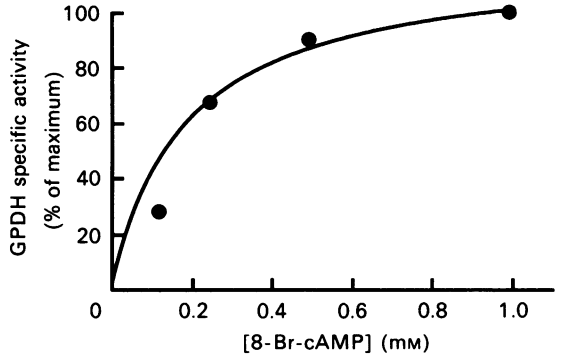

Fig. 3. Dose–response relationship of 8-Br-cAMP to the induction of adipose conversion

Confluent cells were maintained for 12 days in 5F medium supplemented or not with various concentrations of 8-Br-cAMP. All media were enriched with sodium ascorbate and selenite. The specific-activity values of GPDH for cells maintained in 5F medium supplemented with 1 mM-8-Br-cAMP and taken as 100% were 870 and 1250 munits/mg in two independent experiments. Similar dose–response curves were obtained with both experiments.

Amplification of adipose conversion by protein kinase C-activating and Ca^{2+}-mobilizing agents in the presence of effectors of the cyclic AMP pathway

The breakdown of inositol phospholipids as a result of phospholipase C activation is known to provoke subsequently the activation of protein kinase C and the mobilization of Ca^{2+} through the transient formation of diacylglycerol and inositol trisphosphate respectively: both events can be selectively mimicked by a phorbol ester such as PMA for the former and a Ca^{2+} ionophore such as ionomycin for the latter [43]. The actions of both drugs were thus examined in the presence and in the absence of agents elevating cyclic AMP concentrations. Neither PMA (10 nM) nor ionomycin (100 nM), when present alone, was able to trigger adipose conversion to a very large extent (Table 4). A similar situation applied to $PGF_{2\alpha}$ alone, as already observed (see Table 3). In contrast, a potent synergy was observed when either PMA or ionomycin was present simultaneously with a near-maximally effective concentration of 8-Br-cAMP (0.5 mM; see Fig. 3). Chronic treatment with either 10 nM-PMA or 100 nM-ionomycin plus 0.5 mM-8-Br-cAMP led to a percentage of differentiated cells that reached 50% (Table 4). In contrast with PMA, both a phorbol ester of the α configuration (αPDBu), unable to promote protein kinase C activation, and EGF, unable to elicit inositol phospholipid hydrolysis (see Fig. 4), were inactive.

In order to raise the intracellular cyclic AMP content through a direct activation of adenylate cyclase or through inhibition of phosphodiesterase, forskolin and IBMX respectively were used. Data in Table 4 indicate

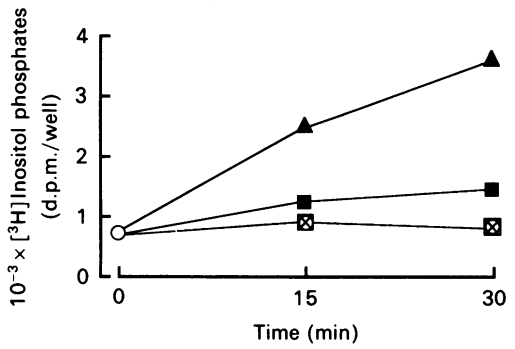

Fig. 4. Promotion of inositol phospholipid breakdown by $PGF_{2\alpha}$ and arachidonic acid

Labelling of growing Ob1771 cells with *myo*-[2-^{3}H]inositol and recovery of total soluble inositol phosphates, at the times indicated, were performed as described in the Experimental section. Cells were incubated in the following media: 5F medium alone (○); 5F medium supplemented with 3 nM-EGF (×), with 200 nM-$PGF_{2\alpha}$ (▲), with 10 μM-arachidonic acid (■) or with 10 μM-arachidonic acid and 10 μM-indomethacin (□). The values are the means for duplicate determinations that did not differ by more than 6%. Similar results were obtained after 15 min incubations performed in three other independent series of cells. 0.5 mM-8-Br-cAMP, 100 μM-IBMX, 200 nM-PGE_1, 10 nM-PMA and 100 nM-ionomycin tested in duplicate in two independent experiments were found to be inactive.

clearly that the adipose conversion induced in the presence of 10 μM-forskolin or 100 μM-IBMX was also amplified in the presence of PMA or ionomcyin: in those cases the specific activity of GPDH reached values of 3000–4000 munits/mg of protein and all the cells were able to differentiate into adipose cells. Taken together, these results indicate that the conditions leading to maximal adipose conversion required both enhancement of intracellular cyclic AMP and stimulation of protein kinase C and/or Ca^{2+} mobilization. The synergistic response on the adipose conversion process induced by $PGF_{2\alpha}$, PMA or ionomycin in the presence of these various agents elevating cyclic AMP concentrations is reminiscent of that induced by arachidonic acid in the presence of the same agents (see Table 2).

Correlation between adipose-cell terminal differentiation and post-confluent mitoses

Since terminal differentiation of early marker-containing cells is dependent on DNA synthesis and post-confluent mitoses [12,13], the evolution of the cell number was determined after confluence in order to establish a relationship, if any, between post-confluent mitoses and arachidonic acid-mediated events. In 5F medium, in which the proportion of differential cells was very low (<2%), the increase in the cell number after confluence was hardly detectable. This was also the case when 5F medium was supplemented with either 10 nM-PMA or 200 nM-$PGF_{2\alpha}$ alone, which were unable to significantly trigger terminal differentiation (see Tables 3 and 4). In contrast, treatment with the combination of 0.5 mM-8-Br-cAMP plus 10 μM-arachidonic acid, which led to 100% of differentiated lipid-containing cells, was accompanied by one doubling in the cell number within 6 days. The simplest interpretation of this observation is that each individual cell exposed to 8-Br-cAMP plus arachidonic acid underwent one cell division before terminal differentiation [13]. If this were not so and if some cells did not divide whereas some others happened to divide a few times, fat-cells would appear as clusters among remaining undifferentiated cells, as observed in serum-supplemented media [12]. In favour of our interpretation is the fact that, in a reproducible manner, culture conditions that led to an increase in the cell number less than 2-fold also led to a proportion of differentiated cells lower than 100%.

DISCUSSION

The present study, performed in serum-free hormone-supplemented medium, has allowed us to characterize intracellular signals involved in the control of terminal differentiation of Ob1771 cells. These signals include both cyclic AMP and second messenger(s) generated through hydrolysis of inositol phospholipids. Addition of agents elevating cyclic AMP concentrations, e.g. forskolin, PGE_1, isoprenaline, IBMX or 8-Br-cAMP, led to a variable but generally rather high proportion of adipose-converted cells. This proportion could be increased up to 100% when $PGF_{2\alpha}$, PMA or ionomycin was included in the presence of the above agents.

The critical role of cyclic AMP in the process of

Table 4. Amplification of adipose conversion by protein kinase C-activating and Ca^{2+}-mobilizing agents in the presence of 8-Br-cAMP, IBMX or forskolin

Confluent Ob1771 cells were maintained in 5F medium or 5F medium containing 8-Br-cAMP (0.5 mM), IBMX (100 μM) or forskolin (10 μM) and supplemented or not as indicated. All media contained sodium ascorbate and selenite. At day 12, GPDH activities were measured as described in the Experimental section. The activity values are medians ± ranges for two independent experiments. The mean percentages of differentiated cells were estimated to be 9%, 12% and 50% in 5F medium supplemented with PMA, 8-Br-cAMP and 8-Br-cAMP plus PMA respectively. The mean percentages of differentiated cells in the presence of IBMX or forskolin alone were increased from 50% to 100% in the simultaneous presence of PMA or ionomycin.

Culture conditions / Addition...	None	PMA (10 nM)	Ionomycin (100 nM)	$PGF_{2\alpha}$ (200 nM)	αPDBu (10 nM)	EGF (3 nM)
5F medium	50 ± 15	250 ± 50	70 ± 20	100 ± 30	50 ± 10	60 ± 10
+ 8-Br-cAMP (0.5 mM)	400 ± 100	1600 ± 200	1400 ± 100	980 ± 200	380 ± 100	420 ± 100
+ IBMX (100 μM)	1500 ± 200	4000 ± 200	3500 ± 100	–	–	–
+ Forskolin (10 μM)	1350 ± 150	3250 ± 200	3000 ± 300	–	–	–

adipose conversion allows a better understanding of previous studies performed in serum-supplemented media. For instance, addition of IBMX for a few days to confluent pre-adipocyte cells has been reported to accelerate and to increase the frequency of adipose conversion of cells from established pre-adipocyte cell lines [44,45] as well as that of adipose precursor cells in primary culture [46–48]. In these studies, however, a direct involvement of cyclic AMP was not demonstrated. Under our serum-free culture conditions, treatment with 0.5 mM-8-Br-cAMP from day 0 to 3 was sufficient to enhance adipose conversion of Ob1771 cells (results not shown), but a longer treatment from day 0 to day 12 proved to be more efficient, and all of the cells then became able to differentiate in the presence of arachidonic acid. It is thus likely that an appropriate sustained concentration of cyclic AMP is required to recruit a maximal number of cells able to terminate differentiation.

The critical role of a sustained concentration of cylic AMP in the process of adipose conversion seems at variance with previous observations made with 3T3-Ll cells [49] and 3T3-F442A cells [50], which showed that long-term treatment with agents elevating cyclic AMP concentrations in serum-supplemented media decreased both lipid deposition and expression of lipogenic enzymes. In that respect both stimulatory and inhibitory effects of cyclic AMP on the proliferative response of serum-stimulated 3T3 cells have been recently described and related to the control of the cell cycle [51]. According to this latter study, it is likely that, under our serum-free conditions, an appropriate concentration of cyclic AMP is required to induce a G_0–G_1 transition. This would lead in turn to the recruitment of a maximal number of cells able to respond to the various hormonal stimuli by progression through the cell cycle followed by at least one cell division and terminal differentiation. Additional experiments should shed some light on this point.

The positive effect of $PGF_{2\alpha}$ on adipose conversion deserves some comments since it was reported in serum-supplemented media to trigger adipose conversion of 3T3-L1 cells [44] but, in contrast, to inhibit adipose conversion of Ob17 cells [52]. In the present study performed under serum-free conditions, $PGF_{2\alpha}$ was able to act in concert with 8-Br-cAMP in the promotion of adipose conversion. The ill-defined composition of bovine-serum-containing medium as compared with the conditions used in the present study could explain these discrepancies and emphasizes the need to perform studies in chemically defined culture media for the characterization of regulatory factors.

A striking result of our study is that arachidonic acid was a promoter of adipose conversion and that addition of this polyunsaturated fatty acid led to cyclic AMP production and inositol phospholipid breakdown in Ob1771 cells. The highest concentration of arachidonic acid actually used in 5F medium was 10 μM; higher concentrations were often found to be cytotoxic. It must be emphasized that arachidonic acid was only active on the terminal differentiation of Ob1771 cells, since in its absence an early marker of differentiation such as lipoprotein lipase was already expressed at high activities (9 ± 3 munits/mg; see ref. [17]). Clearly, arachidonic acid was not as potent as the crude or the purified adipogenic fraction from fetal bovine serum for adipose conversion (Table 1). This is not surprising since, besides arachidonic acid, which was present in both fractions, other compounds were also present, i.e. 12-HETE and HHT (the present study), glucocorticoids [53] and various prostaglandins including $PGF_{2\alpha}$ able to amplify adipose conversion (Tables 3 and 4). Glucocorticoids have been shown to enhance in serum-supplemented media the adipose conversion of various pre-adipocyte cells [3,53,54] as well as that of rat and human adipose precursor cells in primary culture [48,55]. The effectiveness of glucorticoids in serum-free hormone-supplemented media remains to be studied. Nevertheless it is likely that, in addition to T_3 [27,28], GH [29,30] and insulin-like growth factor I [18], which are acting as obligatory 'adipogenic' hormones, arachidonic acid represents the main constituent of the postulated 'adipogenic activity' present in bovine serum [56].

We postulate that the absence of arachidonic acid from various basal media leads quite rapdily under serum-free conditions to its depletion from intracellular stores and that the intracellular concentration of this polyunsaturated fatty acid becomes limiting for cell multiplication as well as for other cell functions. This proposal is in agreement with the slightly increased doubling time and the lower saturation density obtained in the absence of serum, as compared with those determined in serum-supplemented media, which are reported but ignored in innumerable studies.

In conclusion, by using the serum-free hormone-supplemented medium previously defined, it should be possible to identify the metabolite(s) of arachidonic acid that as second messenger(s) [57] could trigger cyclic AMP production and inositol phospholipid breakdown and thus be involved in the control of terminal differentiation of adipose cells.

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