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STIMULATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE GENE EXPRESSION BY FATTY ACIDS+

Jocelyne Antras-Ferry, Gonzague Le Bigot, Pierre Robin, Danielle Robin, and Claude Forest*

Centre de Recherche sur l'Endocrinologie Moléculaire et le Développement, C.N.R.S, 9, rue Jules Hetzel, 92190 Meudon, France

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We used the 3T3-F442A adipocytes and the FAO hepatoma cells to analyze the effect of oleate on phosphoenolpyruvate carboxykinase (PEPCK) gene expression. In serum-deprived, glucose-free medium, 1mM oleate, bound to albumin in a 6:1 ratio, specifically stimulated PEPCK mRNA. In 3T3-F442A adipocytes, the maximum 5-fold increase occurred in 4 hours then rapidly declined to reach the basal level 20 hours later. This increase was cycloheximide-independent and actinomycin D-dependent, suggesting a direct, transcriptional effect of oleate. FAO cells also responded to oleate with a transient induction of PEPCK mRNA, although the extent of stimulation was lower. Thus, the PEPCK gene provides a useful molecular tool for studying the mechanisms by which fatty acids stimulate gene expression.

Over the past few years, nutrient modulation of gene expression has become a major prospect for a large number of laboratories (1-3). The fact that dietary fat regulates the expression of genes involved in lipid metabolism in liver is now well documented (2-6). Most, if not all, of these genes are under negative control by long-chain fatty acids (2-6). Among these, the S14 gene has been extensively studied (5). It has been shown recently that transcription of this gene is under control by polyunsaturated fatty acids (PUFAs) in cultured hepatocytes (7). Similarly, expression of the stearoyl-CoA desaturase-1 gene is negatively regulated by PUFAs in cultured hepatocytes (8). Much less is known about fatty acid (FA) regulation of gene expression in adipose tissue. The aP2 mRNA, encoding the adipocyte lipid binding protein (ALBP), is induced by long chain FAs in Ob1771, BFC-1 and 3T3-F442A preadipocytes (9-13). Lipoprotein lipase activity and mRNA are decreased by

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* To whom correspondence should be addressed. FAX : (33) 1-45 07 58 90.

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long chain FAs in cultured rat adipocyte precursors (14). There is no clear evidence of FA-mediated gene control in mature adipocytes.

The cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32 ; PEPCK) catalyzes a critical step in liver gluconeogenesis and in adipose tissue glyceroneogenesis, the conversion of oxaloacetate to phosphoenolpyruvate. In liver, in cultured hepatocytes and in selected hepatoma cell lines, PEPCK gene expression is under multihormonal control (15, 16). Glucagon and cAMP, glucocorticoids, triiodothyronine and retinoic acid (RA) stimulate PEPCK gene transcription whereas insulin is inhibitory. In adipose tissue, it is supposed that the function of PEPCK is to permit fatty acid reesterification in order to restrain fatty acid output during lipolysis (17). In that tissue, cAMP stimulates both lipolysis and PEPCK synthesis (18). We showed recently that, in 3T3-F442A adipocytes, the ß-adrenergic agonist isoproterenol, the cAMP analog 8-chorophenylthio-cAMP and RA, stimulate PEPCK gene transcription (19 and Franckhauser S., Antras-Ferry J., Robin P., Robin D., Granner D. K., Forest C., unpublished). These observations prompted us to wonder whether the products of lipolysis, i.e. long chain FAs, could be regulators of PEPCK gene expression in 3T3-F442A adipocytes.

Here we show that the monounsaturated long chain FA oleate transiently stimulates PEPCK gene expression in 3T3-F442A adipocytes and that this induction is likely to be direct and transcriptional. Moreover, we show that the FAO hepatoma cells respond also to oleate although to a lower extent.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and Ham F-12 medium were from Gibco. Fetal calf serum was from Boehringer Mannheim. [α-^{32}P]-dCTP and Hybond-N blotting membranes were from Amersham. Quick Hyb hybridization buffer and random priming kit were from Stratagene. X-ray film was Kodak XAR-5. Essentially Fatty Acid Free Bovine Serum Albumine (cat. # A-6003) and all other products were purchased from Sigma.

Cell culture

3T3-F442A and FAO cells were cultured at 37° C in a humidified atmosphere of 10% CO_2 90% air. 3T3-F442A cells were grown and differentiated in DMEM containing 10% fetal calf serum, penicillin (200 IU/ml), streptomycin (50 mg/l), biotin (8 mg/l) and pantothenate (4 mg/l). When 3T3-F442A cells were confluent, insulin (2×10^{-8} M) was added to the medium to favor triglyceride accumulation. FAO cells were cultured in Ham F-12 medium containing 5% fetal calf serum, penicillin (200 IU/ml) and streptomycin (50 mg/l). Experiments were performed on mature adipose cells grown for 8 days after confluence and on subconfluent FAO cells.

RNA extraction and analysis

After extraction by the method of Chomczynski and Sacchi (20), total RNA (20 µg) was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and blotted onto a nylon membrane. The integrity and relative amounts of RNA were assessed by methylene blue staining as described (21). Membranes were then hybridized in Quick Hyb using cDNA labeled with [α^{32}P] dCTP by random priming according to the manufacturer's recommendation. Membranes were washed twice for 15 min at

room temperature in 2 x SSC (20 X SSC is 3 M NaCl, 0.3 M sodium citrate, pH = 7), 0.1 % SDS and then for 30 min at 60° C with 0.1 X SSC, 0.1 % SDS. Specific probes used were pPC116, pFAS 18 and pA21 cDNA fragments for respectively the rat PEPCK and FAS mRNAs and the mouse actin mRNA (22-24). The PEPCK mRNA signal was quantified by densitometric scanning and was corrected for differences in RNA loading by a comparison with the signals generated by the actin mRNA.

RESULTS AND DISCUSSION

When glucose is available to adipocytes, the endogenous synthesis of FAs is high. Under these circumstances, the effect that exogenous FAs might exert on gene expression could be blunted. Therefore, we decided to analyze fatty acid action on PEPCK mRNA in 3T3-F442A adipocytes maintained or not in the presence of glucose. Differentiated cells were cultured either in glucose-supplemented (25 mM) serum-free medium or deprived of glucose for 18 hours. In that latter case, the culture medium was enriched with pyruvate (1 mM) and lactate (0.1 mM). A decrease of about 75% in fatty acid synthase (FAS) mRNA occurred after glucose removal whereas PEPCK mRNA remained similar (figure 1). This stimulatory effect of glucose on FAS gene expression corroborates earlier observations (25). Cells were treated for 4 hours with either 1 mM oleate complexed to fatty acid-free bovine serum-albumine (BSA) in a 6:1 ratio or to BSA alone. Oleate specifically stimulated PEPCK mRNA slightly (about 2-fold) in the presence of glucose and strongly (about 5-fold) in its absence (figure 1), suggesting that glucose impairs oleate action. Interestingly, BSA alone induced a 50% decrease in PEPCK mRNA that was also more pronounced when glucose-deprived medium was used (compare lanes 1 and 2 to lanes 4 and 5 in figure 1). The effects of oleate and of BSA were specific to PEPCK as neither FAS nor actin mRNAs were modified. The mechanism of BSA action is unknown. However, the hypothesis that BSA might act as an external trap for FAs released from the intracellular store of triglycerides during lipolysis can be envisioned. In that case, the stimulatory action that endogenous FAs might exert on PEPCK gene expression would be reduced.

We next deciphered the time course of oleate action (figure 2). In response to 1 mM oleate, PEPCK mRNA rapidly increased, peaked at 4 hours with a maximum of about 5-fold above the basal, and rapidly declined to reach the basal level 20 hours later. The observed changes in PEPCK mRNA may be the consequence of an increased rate of gene transcription or of a change in mRNA stability. In order to analyze further the mode of action of oleate, we tested the effects of the inhibitor of transcription, actinomycin D and of the inhibitor of protein synthesis, cycloheximide. Actinomycin D totally prevented the oleate-induced increase of PEPCK mRNA (figure 3). Cycloheximide affected neither the basal nor the oleate-induced level of PEPCK mRNA (figure 3). This lack of cycloheximide effect was not due to a failure of the drug to work as it was able to prevent the dexamethasone-induced decrease in PEPCK mRNA in 3T3-F442A cells (Franckhauser S., Antras-

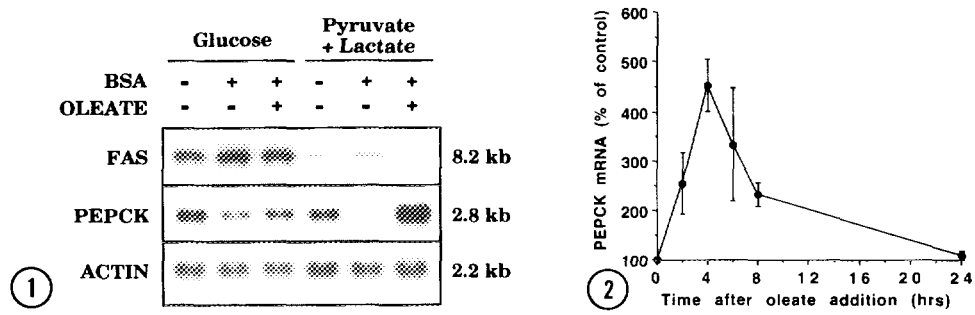

Figure 1. Effects of glucose and oleate on FAS, PEPCK and Actin mRNAs in 3T3-F442A adipocytes.
Differentiated cells were maintained for 20 hours in serum-deprived medium containing either 25 mM glucose or 1 mM pyruvate and 0.1 mM lactate. Cells were then treated for 4 hours with 0.17 mM fatty acid-free BSA complexed or not with 1 mM oleate. Cells from two 60 mm dishes were pooled and RNA was extracted and analyzed as described in Materials and Methods. Results of a typical autoradiogram are shown.

Figure 2. Time course of oleate action on PEPCK mRNA in 3T3-F442A adipocytes.
Differentiated cells were maintained throughout the 24 hour experiment in serum-deprived, glucose-free medium, containing 1 mM pyruvate and 0.1 mM lactate. Fatty acid-free BSA, 0.17 mM either alone or complexed to 1 mM oleate was added to the medium for 24, 8, 6, 4, and 2 hours before the cells were harvested. Cells from two 60 mm dishes were pooled and RNA was extracted and analyzed as described in Materials and Methods. Data, obtained by densitometric scanning, was normalized for differences in RNA loading by using the actin mRNA signal. Data are expressed as the percentage of the PEPCK signal from control, untreated cells. Each value is the mean ± S.E.M. of data collected from three independent experiments.

Ferry J. and Forest C., unpublished observations). Altogether, these results show that the induction by oleate of PEPCK mRNA is independent of ongoing protein synthesis and is likely to be largely due to a stimulation of the transcription rate of the gene.

The PEPCK gene is also expressed and regulated in the hepatoma cells of the H4IIE cell line and of its derivative, the FAO cell line (15, 16, 26). We decided to use the latter cells in order to determine whether the induction by oleate of PEPCK mRNA in 3T3-F442A adipocytes was cell-specific. FAO cells were submitted to a 4-hour treatment with 1 mM oleate in the presence and in the absence of glucose. A maximum of 2-fold stimulation of PEPCK mRNA occurred in both conditions (not shown). Results of time course experiments showed that, like in adipocytes, this increase was transient in FAO cells (figure 4). Thus, oleate stimulates PEPCK gene expression in 3T3-F442A adipocytes and in FAO hepatoma cells although with a much lower magnitude in the latter.

These results constitute the first demonstration that the PEPCK gene is submitted to regulation by FAs. What molecular route could borrow these molecules to affect gene transcription? Although the effect is rapid and transient, the hypothesis that ß-oxidation of FAs is required cannot be ruled out. In the case of the

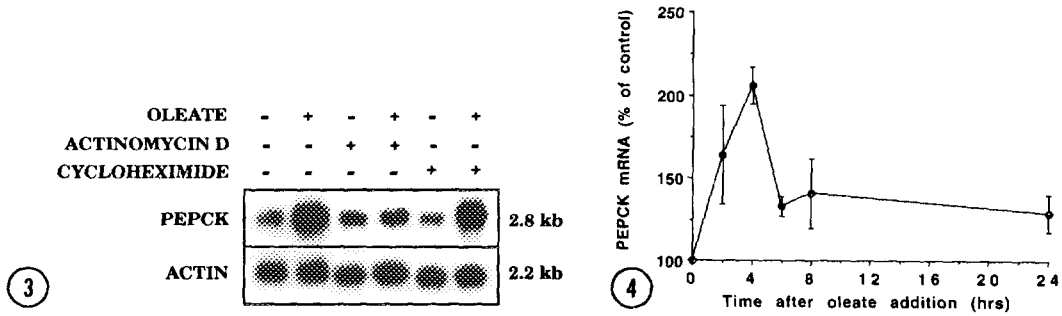

Figure 3. Effects of Actinomycin D and cycloheximide on the stimulation by oleate of PEPCK mRNA in 3T3-F442A adipocytes.
Differentiated cells were maintained for 20 hours in serum-deprived, glucose-free medium containing 1 mM pyruvate and 0.1 mM lactate. Cells were then preincubated for 30 minutes with either 4 μg/ml actinomycin D or 10 μM cycloheximide. Fatty acid-free BSA, 0.17 mM alone or complexed to 1 mM oleate was then added in the same media and the cells were incubated for 4 hours. Cells from two 60 mm dishes were pooled and RNA was extracted and analyzed as described in Materials and Methods. Results of a typical autoradiogram are shown.

Figure 4. **Time course of oleate action on PEPCK mRNA in FAO hepatoma cells.**
Subconfluent FAO cells were maintained throughout the 24 hour experiment in serum-deprived medium, containing 10 mM glucose. Fatty acid-free BSA, 0.17 mM either alone or complexed to 1 mM oleate was added to the medium either at time 0 or for 24, 8, 6, 4, and 2 hours before the cells were harvested. Cells from two 60 mm dishes were pooled and RNA was extracted and analyzed as described in Materials and Methods. Data, obtained by densitometric scanning, was normalized for differences in RNA loading by using the actin mRNA signal. Data are expressed as the percentage of the PEPCK signal from control, untreated cells. Each value is the mean ± S.E.M. of data collected from three independent experiments.

aP2 gene, α-bromopalmitate or oleyl alcohol, two molecules that cannot be directly oxidized, are potent stimulators (12, 13). If the same situation prevails for the PEPCK gene, then FAs themselves would be regulators. FAs have been shown to activate expressed rat Xenopus laevis peroxisomal proliferator activated receptor (xPPAR) (27) or chimeric receptors consisting of the PPAR and the glucocorticoid receptor (28). The PPARα and the retinoid X receptor ß (RXRß) can heterodimerize and cooperatively stimulate the peroxisomal acyl-CoA oxidase gene promoter (29). It has been shown recently that a novel member of the PPAR family called mPPARγ2 heterodimerize with RXRα to activate the adipocyte-specific aP2 enhancer in cultured fibroblasts (30). This activation is potentiated by fatty acids, 9-cis RA and peroxisome proliferators (30). Interestingly, an element homologous to the ARE6 element of the aP2 enhancer is present around -1.9 kb of the 5' flanking region of the PEPCK gene (31). Moreover, in adipocytes and in hepatocytes, the PEPCK gene promoter is under transcriptional activation by RA (19, 32). In H4IIE hepatoma cells, RA action is mediated through the binding of the retinoic acid receptor α (RARα) and a liver nuclear factor identical to or closely related to RXRα to a retinoic acid receptor element (RARE) located between positions -451 and -433 bp relative to

the transcription start site (33). This RARE or the ARE6 consensus element are two potential targets for FAs to stimulate PEPCK gene transcription.

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