Journal of Cellular Biochemistry 85:651–661 (2002)

Evidence for Selective Induction of Phosphoenolpyruvate Carboxykinase Gene Expression by Unsaturated and Nonmetabolized Fatty Acids in Adipocytes

Eric Duplus,[1] Martine Glorian,[1] Joan Tordjman,[1] Rolf Berge,[2] and Claude Forest[1]*

[1]INSERM Unit 530, Centre Universitaire des Saints-Pères, 45 rue des Saints-Pères, 75006, Paris, France
[2]Department of Clinical Biochemistry, Haukeland Hospital, University of Bergen, N-5021 Bergen, Norway

Abstract Polyunsaturated fatty acids (PUFAs) and 3-thia fatty acids are hypolipidemic and decrease insulin resistance in Type II diabetic animals. To exert such an action, these FAs could decrease adipose tissue lipolysis or increase esterification. Glyceroneogenesis is an important metabolic pathway in adipocytes for re-esterification of FAs originating from lipolysis and in hepatocytes for triacylglycerol synthesis during fasting. Cytosolic phosphoenolpyruvate carboxykinase (PEPCK) plays a key role in this pathway. Here we show that the PUFA docosahexaenoic acid (DHA) stimulates PEPCK mRNA in glucose-deprived adipose tissue explants from fed rats and in 3T3-F442A differentiated adipocytes. This effect is maximum at 3 h, stable up to at least 11 h of treatment, and affects the transcription of the gene. PEPCK mRNA half-life is not affected. Among a series of adipocyte transcripts, only the adipocyte lipid binding protein mRNA is also increased by DHA, although later than the PEPCK mRNA and at a much lower extent. DHA has no effect on PEPCK gene expression in the H4IIE hepatoma cells in which this gene is responsive to other inducers like cAMP. This lack of effect is not due to a failure of DHA to act in H4IIE cells since it induces the carnitine palmitoyltransferase 1 (CPT-1) mRNA. Therefore, the DHA effect appears to be cell-selective. Results of experiments using either tetradecylthio acetic acid and α-bromopalmitate, two nonmetabolized FAs, or a series of inhibitors of FA metabolism show that the FA effect on PEPCK mRNA is not due to a product of its metabolism. Hence, polyunsaturated and nonmetabolized FAs stimulate adipose PEPCK, therefore potentially enhancing glyceroneogenesis and reducing FA output. This mechanism could participate in the hypolipidemic action of PUFAs. J. Cell. Biochem. 85: 651–661, 2002.

Key words: fatty acids; phosphoenolpyruvate carboxykinase; transcription; β-oxidation; adipose tissue; liver; PPAR

Fatty acids (FAs) are the major metabolic substrates, which have profound influence in human health. Most particularly, saturated and unsaturated long-chain FAs have many antagonistic properties. For instance, diets rich in long-chain saturated FAs (LCFAs) favor hypertriglyceridemia, adipocyte hypertrophy, atherosclerosis, insulin resistance, and Type II

Abbreviations used: 8-CPT-cAMP, 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate; Br-palm, α-bromopalmitate; AA, arachidonic acid; ALBP/aP2, adipocyte lipid binding protein; BSA, bovine serum albumin; CPT-1, carnitine palmitoyltransferase; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle medium; DR1, direct repeat-1; DRB, 5,6-dichloro-1β-D-ribofuranosylbenzimidazole; FA, fatty acid; FAAR, fatty acid activated receptor; FAS, fatty acid synthase; FCS, fetal calf serum; Glut4, glucose transporter 4; HSL, hormone-sensitive lipase; LCFA, long-chain fatty acid; L-FABP, liver fatty acid binding protein; LPL, lipoprotein lipase; NDGA, nordihydroguaiaretic acid; NEFA, nonesterified fatty acid; PEPCK, cytosolic phosphoenolpyruvate carboxykinase (EC 4.1.1.32); PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; PUFA, polyunsaturated fatty acid; RXR, retinoid X receptor; TDGA, tetradecylglycidic acid; TTA, tetradecylthioacetic acid; TTP, tetradecylthiopropionic acid; Type II-DM, Type II diabetes melitus; UCP2, uncoupling protein 2; VLDL, very low density lipoprotein.

Grant sponsor: "Ministère de l'Education Nationale et de la Recherche" (to MG, ED, and JT); Grant sponsor: Association Française de Nutrition-Société de Nutrition de Langue Française (to MG); Grant sponsor: ≪ Institut Danone ≫ (to ED); Grant sponsor: INSERM (to CF); Grant sponsor: CNRS (to CF); Grant sponsor: Fondation pour la Recherche Médicale (to CF); Grant sponsor: Association pour la Recherche contre le Cancer (to CF).

*Correspondence to: Claude Forest, INSERM U530, Centre Universitaire des Saints-Pères, 45 rue des Saints-Pères, 75006, Paris, France.
E-mail: claude.forest@biomedicale.univ-paris5.fr

Received 31 July 2001; Accepted 23 January 2002
DOI 10.1002/jcb.10175

diabetes mellitus (Type II-DM); whereas diets rich in long-chain polyunsaturated FAs (PUFAs) have opposite effects and are beneficial for health . Moreover, synthetic FAs which are blocked for β-oxidation like the 3-thia FA tetradecylthio acetic acid (TTA) are hypolipidemic in different animal models [Berge and Hvattum, 1994].

Type II-DM is characterized by a state of insulin resistance accompanied with a reduction in insulin secretion and a large increase in plasma NEFAs, the latter being one of the most precocious parameter in the disease [Randle, 1998]. Blood NEFAs mainly originate from the hydrolysis of triacylglycerols (lipolysis) stored in adipose tissue, when the energy requirement is high, for instance during fasting. Therefore, a dysregulation in the processes of adipose tissue FA storage (esterification) and/or triacylglycerol lipolysis could favor Type II-DM. Hence physiopathological repercussions can be expected from the analysis of the mechanisms by which saturated FAs and PUFAs influence adipose tissue metabolism.

Among the routes taken by FAs to alter metabolism, an important one emerged over the past 10 years; they act as signaling molecules involved in regulating expression of a number of genes [Duplus et al., 2000]. To study FA effect on gene expression in adipose tissue, most of the works have been made using cultured adipocyte precursors which, under LCFA stimulation, are entailed to differentiate into mature adipocytes via the process named "adipogenesis" [Grimaldi et al., 1999]. In contrast, studies carried out on mature adipocytes are scarce. A few years ago, we demonstrated that the phosphoenolpyruvate carboxykinase (PEPCK) mRNA was induced by the monounsaturated FA oleate and by PUFAs in mature adipocytes of the 3T3-F442A cell line. Since there is no known post-translational regulation of PEPCK, changes in PEPCK mRNA are directly reflected in modifications of PEPCK activity [Forest et al., 1997]. Besides its well-known involvement in glucose synthesis (gluconeogenesis) in liver, PEPCK plays also an important function in triacylglycerol storage in adipose tissue [Reshef and Shapiro, 1970] and probably also in liver [Kalhan et al., 2001]. It catalyzes the decarboxylation of oxaloacetate to phosphoenolpyruvate, therefore providing glycerol-3-phosphate required for FA esterification. This metabolic pathway, named glyceroneogenesis, is required when the glucose supply to the cell is low, i.e., during fasting, or under low glucose but high lipid or protein diets [Ballard et al., 1967; Reshef et al., 1970; Botion et al., 1995; Kalhan et al., 2001]. In agreement with the physiological role of the enzyme, high PEPCK mRNA level is found in adipose tissue from rats fed a high fat diet [Antras-Ferry et al., 1995]. Hence, because of the role of PEPCK on FA metabolism in adipocytes, the FA effect on the PEPCK gene is physiologically relevant.

In our previous studies, the most potent FA tested was the docosahexaenoic acid (DHA, C22:6, ω3) [Antras-Ferry et al., 1995]. This result appeared of great interest, since ω3 PUFAs administered in vivo are the most efficient FAs in reducing plasma NEFA levels in Type II-DM models [Storlien et al., 1996; Raclot and Oudart, 1999]. Moreover, DHA is poorly oxidized in the mitochondria and in the peroxisomes [Madsen et al., 1998, 1999; Berge et al., 1999]. The effect of ω3 PUFAs on plasma NEFAs could be explained by a related increase in PEPCK.

We already showed that FAs stimulate the transcriptional rate of the PEPCK gene in a direct manner, i.e., without the need for ongoing protein synthesis [Antras-Ferry et al., 1994, 1995]. Whether post-transcriptional effects occur remain an opened question that we decided to address in the present study. Furthermore, FAs are actively metabolized in cells. Hence, not only FAs per se but also products of FA metabolism can act as a relay to affect gene expression. Since potential FA-regulated genes are co-expressed in a given tissue, the question arises as to whether they are regulated in a coordinate manner. Last but not the least, the same gene can be expressed in various tissues where different regulations can occur depending on the cell context. The present work was engaged to address these issues.

We show that DHA and nonmetabolized FAs induce PEPCK gene expression in adipocytes, with no post-transcriptional effect. DHA effect is direct and affects the transcription of the gene. Its metabolism is not required. We demonstrate also that PEPCK is the sole investigated gene which responds to FAs by a rapid and potent induction of expression. Surprisingly, DHA induces PEPCK mRNA in adipocytes but not in H4IIE hepatoma cells, which express and regulate PEPCK in response to hormones. Therefore, FA control of PEPCK gene transcription appears to be cell-selective.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham-F12 medium, new born calf serum, and fetal calf serum (FCS) were from Life Technologies (Cergy-Pontoise, France). [α-^{32}P]-dATP, Hybond-N$^+$ blotting membranes and X-ray films were from Amersham (Les Ulis, France). Random priming kit and QuikHyb hybridization solution were from Stratagene (Amsterdam). Docosahexaenoic acid (DHA, C22:6) and arachidonic acid (AA, C20: 4) were from Cayman Chemical (Ann Arbor). Essentially FA-free bovine serum albumin (BSA) (A-6003); 5,6-dichloro-1β-D-ribofuranosyl benzimidazole (DRB) and all other products were purchased from Sigma (L'Isle d'Abeau Chesnes, France). TTA and tetradecyl thiopropionic acid (TTP) were prepared as previously described.

Cell Culture and Treatment

Cells were cultured at 37°C in a humidified atmosphere of 10% CO_2, 90% air. 3T3-F442A cells were grown and differentiated in Dulbecco's modified Eagle's medium (DMEM) containing glucose (25 mM), penicillin (200 IU/ml), streptomycin (50 mg/L), biotin (8 mg/L), and pantothenate (4 mg/L) with 10% newborn calf serum. At confluence of the cells, newborn calf serum was changed to 10% FCS, and insulin (20 nM) was added to the medium. H4IIE hepatoma cells were grown in Ham's F-12 medium containing glucose (9 mM), penicillin (200 IU/ml), and streptomycin (50 mg/L), sodium bicarbonate (3.7 g/L) and 10% FCS. Medium was changed every 2–3 days. Experiments were performed on mature adipose cells (8–10 days after confluence) or sub-confluent H4IIE cells. Twenty-four hours before RNA extraction, cells were placed in serum-free and hormone-free medium in the presence of glucose or not. Treatments with effectors were performed for 1–24 h. When glucose was omitted, medium was enriched with pyruvate (1 mM) and lactate (0.1 mM).

Culture of Adipose Tissue Fragments

Male Wistar rats, bred in our laboratory, were used. They were housed at a constant temperature of 24°C. Rats weighting approximately 200 g were trained for 14 days to a meal-feeding regimen (10:00–12:00 h) on a standard diet as described previously [Antras-Ferry et al., 1995]. They were then either fasted for 24 h or refed for 3 h before sacrifice. Periepididymal fat pads were immediately removed, cut in small pieces, washed in Kreb's buffer (pH 7.4), and treated with different effectors for 4 h in serum-free, glucose-free DMEM containing pyruvate (1 mM) and lactate (0.1 mM).

RNA Extraction and Analysis

RNA from tissue fragments and from cultured cells was extracted by the method of Chirgwin et al. [1979] and of Chomczynski and Sacchi [Chomczynski and Sacchi, 1987], respectively. Total RNA 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. Prehybridization and hybridization of the blots were carried out using the Quick Hyb solution (Stratagene). Membranes were hybridized for 1 h at 68°C with 100 μg/ml sonicated salmon sperm DNA and 10^6 cpm/ml of cDNA labeled with [α-^{32}P] dATP by random priming, according to the manufacturer's instuctions. Membranes were washed twice for 15 min at room temperature in 2 $\times$ SSC, 0.1% SDS and then for 30 min at 60°C with 0.1X SSC, 0.1% SDS. Specific probes used were PC116, FLHSL, aP2, mL5, pFAS18, and CPT I cDNA fragments for, respectively, rat PEPCK, rat hormone sensitive lipase (HSL), mouse ALBP/aP2, mouse LPL, rat FAS, and liver carnitine palmitoyltransferase 1 (L-CPT-1) mRNAs [Spiegelman et al., 1983; Nepokroeff et al., 1984; Beale et al., 1985; Kirchgessner et al., 1987; Esser et al., 1993; Holm et al., 1988]. An oligonucleotide specific for the 18S ribosomal RNA was ^{32}P-labeled and used as a control, as described previously [Plee-Gautier et al., 1996]. mRNA signals were quantified using the Instant Imager (Packard) and were corrected for differences in RNA loading by comparison with the signals generated by the 18S cDNA probe.

RESULTS

DHA-Induction of PEPCK Gene Expression in Rat Adipose Tissue Explants Is Dependent on the Nutritional Status

We demonstrated previously that DHA was the most potent FA inducer of PEPCK gene expression in glucose-deprived 3T3-F442A adipocytes. To determine whether FAs affect

PEPCK mRNA in adipose tissue, we assayed the DHA regulation of this gene in rat periepididymal adipose tissue explants maintained ex vivo. Explants from either fed or fasted rats were incubated with DHA complexed to serum albumin for 4 h in the presence of glucose or not. PEPCK mRNA from fasted rats is not responsive to DHA, whether explants are cultured in the presence of glucose or not (Fig. 1). In contrast, PEPCK mRNA is increased threefold by DHA when explants obtained from fed animals are maintained in the absence of glucose (Fig. 1). Hence, glucose prevents induction as expected [Antras-Ferry et al., 1995]. Therefore, DHA induction of PEPCK mRNA is only observed in adipose tissue explants from fed rats cultured in the absence of glucose.

Adipose tissue contains adipocytes and many other cell types (preadipocytes, macrophages, endothelial cells, fibroblasts...). Consequently, in order to study the mechanism of FA action on PEPCK gene expression specifically in adipocytes, glucose-deprived differentiated 3T3-F442A cells were used throughout the rest of this work.

PEPCK mRNA Is Rapidly Induced by DHA in a Dose-Dependent Manner

A treatment time of 4 h was chosen to determine the concentration-response relationship of DHA action on PEPCK gene expression. At the constant concentration of 40 μM bovine serum albumin, the DHA-induction of PEPCK mRNA was concentration-dependent, with a half-maximum effect (EC50) reached at about 120 μM, and a maximum reached at 320 μM (Fig. 2). At 640 μM, some toxicity is observed, rendering results difficult to interpret (data not shown). Next, we used the maximal concentration of 320 μM to analyze the time-course of DHA action. As shown in Figure 3, a fivefold induction is already observed at 3 h, indicating a very fast effect which is maintained for eight additional hours and decreases thereafter. The basal level is recovered at 24 h.

Moreover, such a strong and rapid stimulation by DHA appears specific to PEPCK mRNA since FAS, LPL, HSL, and ALBP/aP2 transcripts remain unchanged up to 7 h following DHA addition (Fig. 3). On a longer term basis, i.e., at 9 and 11 h, ALBP/aP2 transcript increases transiently and at a lower level than PEPCK

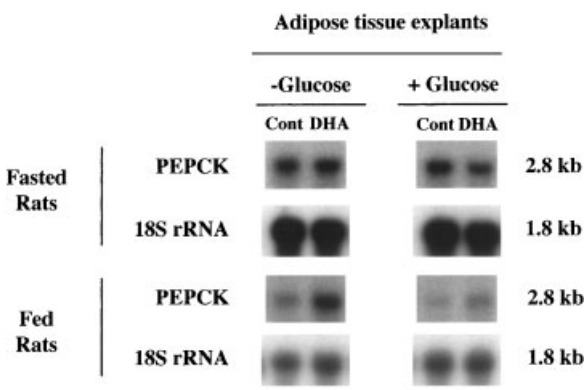

Fig. 1. Effect of DHA and glucose on PEPCK mRNA in adipose tissue fragments from fed or fasted rats. Adipose tissue fragments from fed or fasted rats were incubated for 4 h in serum-free DMEM with BSA (40 μM) and either 25 mM glucose (+ Glucose) or 1 mM pyruvate and 0.1 mM lactate (−Glucose). DHA (320 μM) was complexed to BSA and added (DHA) or not (Cont) in the medium. RNA extraction and analysis are described in Materials and Methods. Results of a typical autoradiogram are shown.

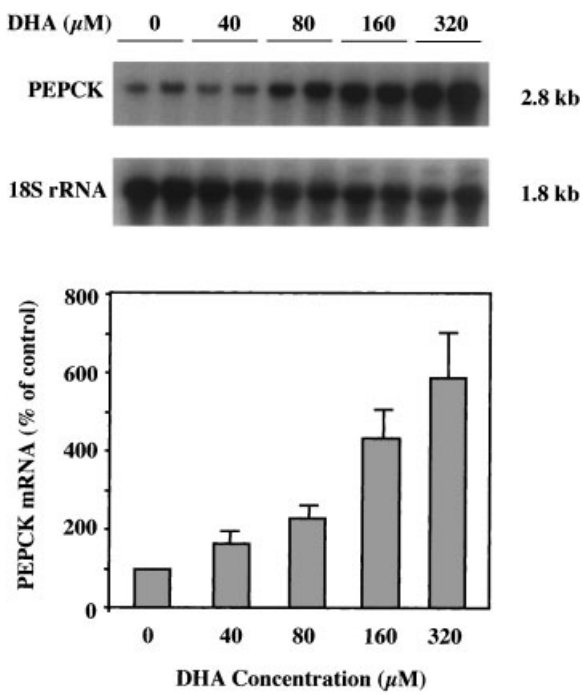

Fig. 2. Effect of DHA concentration on PEPCK mRNA in 3T3-F442A adipocytes. Differentiated cells were maintained for 20 h in serum-free, glucose-free medium. Medium was then renewed with the indicated concentrations of DHA complexed to BSA (40μM). Four hours later, RNA was extracted from two 60-mm dishes and was analyzed as described in Materials and Methods. **Upper pannel**: Results of a typical autoradiogram are shown. **Lower pannel**: Data were quantified and normalized for differences in RNA loading by using the 18S rRNA signal. Data are expressed as the percentage of PEPCK signal from control (BSA-treated cells). Each value represents the mean ± SEM of results obtained from three independent experiments.

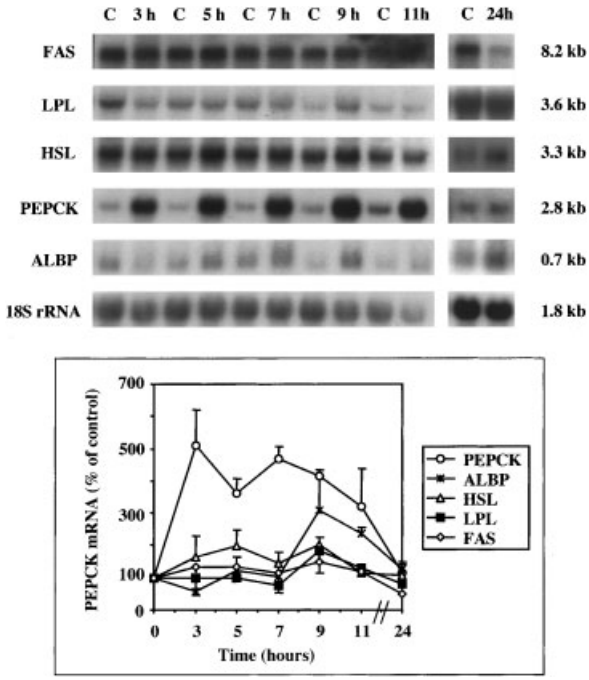

Fig. 3. Time-course of DHA action on the expression of various genes in 3T3-F442A adipocytes. Differentiated cells were maintained throughout the 24 h experiment in serum-free, glucose-free medium. BSA (40 μM), either alone (C) or complexed to DHA (320 μM) was added to the medium for 24, 11, 9, 7, 5, and 3 h before the cells were harvested. RNA was extracted and analyzed as described in Materials and Methods. **Upper pannel**: Results of typical autoradiograms are shown. **Lower pannel**: Data were quantified and normalized for differences in RNA loading by using the 18S rRNA signal. Data are expressed as the percentage of PEPCK signal from control (BSA-treated cells). Each value represents the mean ± SEM of results obtained from three independent experiments.

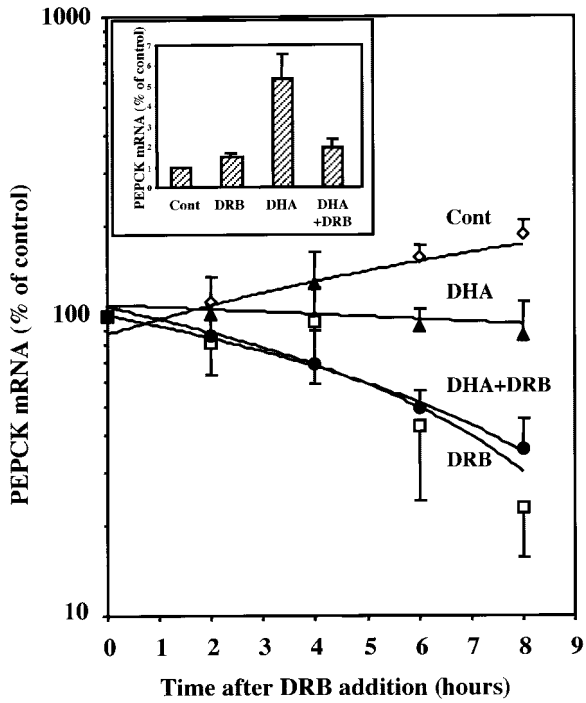

Fig. 4. DHA action on PEPCK mRNA half-life and influence of the transcription inhibitor DRB on DHA induction in 3T3-F442A adipocytes. Differentiated cells were maintained for 20 h in serum-free, glucose-free medium. Medium was then renewed either with BSA (40 μM) alone (Cont) or complexed to DHA (320 μM). Three hours later, DRB (25 μg/ml) was added (DRB; DHA + DRB) or not (Cont; DHA). Cells were harvested at different times relative to DRB addition. Insert: differentiated cells were maintained for 20 h in serum-free, glucose-free medium, then medium was renewed with BSA (40 μM) either alone (Cont) or with 25 μg/ml 5,6-dichloro-1β-ribofuranosyl benzimidazole (DRB) for a 30 min preincubation. DHA (320 μM) was then added in the medium for 4 h with or without DRB. RNA was extracted and analyzed as described in Materials and Methods. Data were quantified and normalized for differences in RNA loading by using the 18S rRNA signal. Data are expressed as the percentage of PEPCK signal from control (BSA-treated cells). Each value represents the mean ± SEM of results obtained from three independent experiments.

mRNA as expected [Amri et al., 1991b]. A 24 h-treatment with DHA results in a decrease in FAS gene expression (Fig. 3) as described earlier [Mater et al., 1998].

DHA Effect on PEPCK Gene Expression Is Transcriptional and Does not Affect mRNA Half-Life

DHA induction of PEPCK mRNA can be the result of an increased transcription rate of the gene and/or of an enhancement in mRNA stability. To address these questions, 3T3-F442A adipocytes were first exposed to the Class II genes transcription inhibitor DRB for 30 min before DHA treatment. DRB has no effect on the basal PEPCK mRNA but does prevent DHA induction (Fig. 4, insert). This result, combined with the observation that the protein synthesis inhibitor cycloheximide does not affect DHA induction of the PEPCK mRNA (not shown), is consistent with a direct and transcriptional action of DHA on the PEPCK gene.

To investigate the possibility that PEPCK mRNA half-life could be affected by FAs, we treated 3T3-F442A adipocytes with 320 μM DHA for 3 h, then with DRB. We followed the decrease over time of the PEPCK mRNA signal by Northern blotting. Adipocyte PEPCK mRNA decreases with identical rates in the presence and absence of DHA, showing that half-life is not affected by the treatment (Fig. 4). Hence, DHA specifically affects the transcriptional rate of the gene.

Involvement of DHA Metabolism in PEPCK mRNA Induction

DHA action can be due to FA per se or to a metabolite. Indeed, DHA like most FAs can be metabolized. As shown in Figure 5, DHA can be either β-oxidized or give rise to peroxidation products. Like arachidonic acid (AA, C20:4), DHA is involved in leukotriene and prostanoid synthesis or interferes with it [Corey et al., 1983; Smith, 1989]. To identify the potential involvement of a DHA-derived metabolite on PEPCK gene expression in adipocytes, inhibitors of the metabolic pathways were used.

The FA tetradecylglycidic acid (TDGA) inhibits the CPT-1 activity in the inner mitochondria membrane leading to inhibition of FA β-oxidation [Skorin et al., 1992]. At 1 and 5 μM, TDGA has no effect on PEPCK mRNA (Fig. 6A). At 25 μM, TDGA induces PEPCK mRNA with the same magnitude as that obtained with 320 μM DHA (Fig. 6A). However, PEPCK gene induction by DHA is enhanced by TDGA at 5 and 25 μM, suggesting that β-oxidation of DHA in mitochondria is not required for the effect. To ascertain the latter conclusion, we used nonβ-oxidizable FAs, the tetradecylthioacetic acid (TTA) [Berge and Hvattum, 1994], and α-bromopalmitate [Grimaldi et al., 1992]. TTA is a saturated FA in which a sulfur atom is substitutive of the three methylene group. In vivo and in vitro, TTA has PUFA-like properties, e.g., hepatic peroxisome and mitochondria proliferation and reduction in the plasma concentration of nonesterified FAs, triacylglycerol, and cholesterol [Skorve et al., 1990]. As shown in Figure 6B, TTA induces PEPCK mRNA in a dose-dependent manner, with a magnitude similar to that obtained with DHA. In contrast, the control 4-thia FA, tetradecylthiopropionic acid (TTP) which is β-oxidizable, stimulates PEPCK mRNA with a much lower magnitude than TTA (Fig. 6B). α-bromopalmitate is an inhibitor of FA oxidation. Figure 6C shows that this modified FA is also a strong inducer of PEPCK mRNA as soon as at 2 h of treatment. Taken together, these results show that β-oxidation is not required for FA action on PEPCK gene expression in adipocytes.

The potential involvement of peroxidation products was investigated using vitamin E, an antioxidant which inhibits the peroxidative processes. Vitamin E does not prevent DHA stimulation (Fig. 7A). Furthermore, we found no peroxidation products in stimulated-cells (not shown). Conclusively, peroxides are not involved in DHA action in these cells. Last but not the least, nordihydroguaiaretic acid

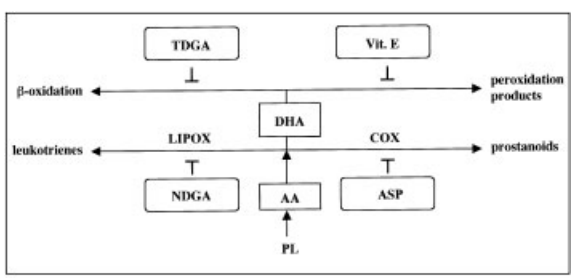

Fig. 5. Metabolic pathways of DHA and action of inhibitors. DHA can be β-oxidized or lead to peroxidation products. Like arachidonic acid (AA) hydrolized from membrane phospholipids (PL), DHA can give rise to prostanoids or leukotrienes synthesized respectively by the cyclooxygenases (COX) or the lipooxygenases (LIPOX). Inhibitors of the different pathways are shown: tetradecylglycidic acid (TDGA), vitamine E (Vit. E), nordihydroguaiaretic acid (NDGA), and aspirin (ASP).

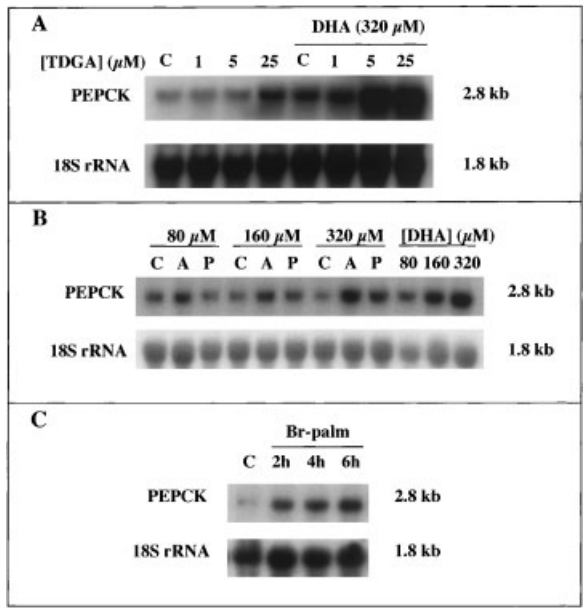

Fig. 6. Influence of TTA and TDGA on DHA action on PEPCK mRNA in 3T3-F442A adipocytes. Differentiated cells were maintained for 20 h in serum-free, glucose-free medium. Medium was then renewed. **A**: Cells were pretreated with BSA (40 μM) either alone (C) or with TDGA at the indicated concentrations for 30 min. DHA (320 μM) was then added for 4 h in the presence or not (C) of TDGA at the indicated concentrations. **B**: Cells were treated with BSA (40 μM) either alone (C) or complexed with TTA (A), TTP (P), or DHA at the indicated concentrations for 4 h. **C**: Cells were treated with BSA (170 μM) either alone (C) or complexed to α-bromopalmitate (Br-palm) for the indicated times. RNA was extracted and analyzed as described in Materials and Methods. Results of a typical autoradiogram are shown.

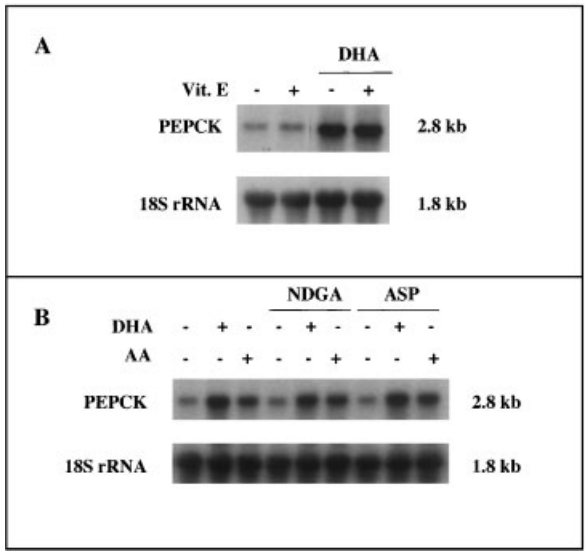

Fig. 7. Influence of vitamin E, NDGA, and aspirin on DHA or arachidonic acid action on PEPCK mRNA in 3T3-F442A adipocytes. Differentiated cells were maintained for 20 h in serum-free, glucose-free medium. Medium was then renewed. **A**: Cells were treated with BSA (40 μM) either alone (−) or complexed to DHA (320 μM) in the presence of 70 μM vitamin E (Vit. E) or not (−). **B**: Cells were pretreated with BSA (40 μM) either alone or with NDGA (60 μM) or 200 μM aspirin (ASP) for 30 min. DHA (320 μM) or 320 μM arachidonic acid (AA) was then added for 4 h in the presence or not of NDGA or ASP. RNA was extracted and analyzed as described in Materials and Methods. Results of a typical autoradiogram are shown.

(NDGA) and aspirin were used to inhibit lipo-oxygenases and cyclo-oxygenases, respectively [Della Loggia et al., 1988]. These two enzyme systems control leukotriene and prostanoid synthesis, respectively. Pretreatment of 3T3-F442A adipocytes with either NDGA or aspirin does not affect DHA-induced increase in PEPCK mRNA (Fig. 7B). The AA also stimulates PEPCK mRNA as expected [Forest et al., 1997] and its effect is not prevented by NDGA or aspirin (Fig. 7B). These results indicate that DHA or AA action is not the result of an interference with prostanoid or leukotriene production.

DHA Induction of PEPCK Gene Expression Does not Occur in Hepatoma Cells

The PEPCK gene is also expressed in hepatocytes where the enzyme is gluconeogenic but also probably glyceroneogenic as expected from recent work [Kalhan et al., 2001]. It has been shown that FA can control the expression of several genes involved in lipid metabolism in the liver [Jump and clarke, 1999]. DHA action on the PEPCK gene was investigated in the H4IIE rat hepatoma cells. The latter cells were treated with DHA or with the cAMP analog, 8-CPT-cAMP for 4 h, either in the presence of glucose or not. As shown in Figure 8, DHA has no effect on PEPCK mRNA in this cell type whether glucose is present or not. In contrast, 8-CPT-cAMP increases PEPCK mRNA in H4IIE cells, as expected [Sasaki et al., 1984], showing that this gene is responsive to other inducers in those cells (Fig. 8). As expected also from earlier studies performed in hepatocytes, DHA induces the CPT-1 mRNA in H4IIE cells, demonstrating that the FA was active in these experiments. Hence, DHA action on the PEPCK

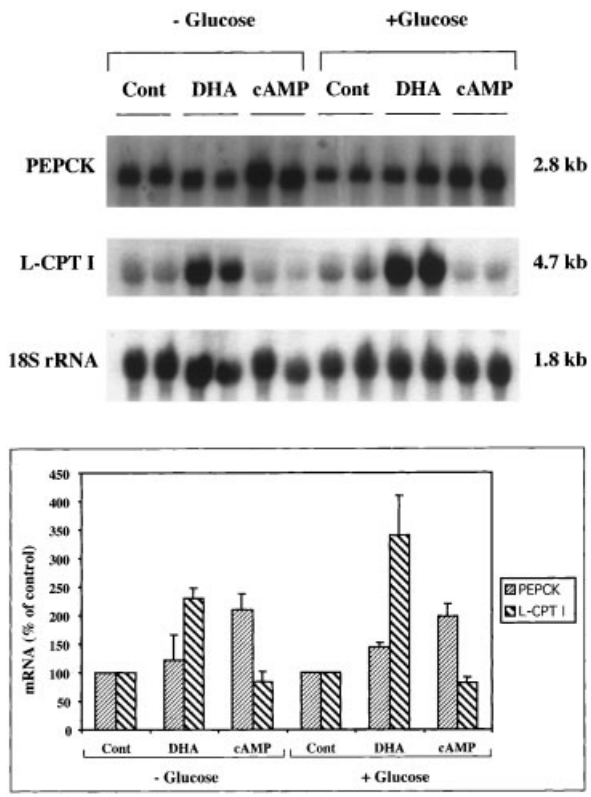

Fig. 8. Effect of glucose and DHA or cAMP on PEPCK and CPT-1 mRNAs in H4IIE hepatoma cells. Sub-confluent cells were maintained for 20 h in serum-free medium with either 9 mM glucose (+ Glucose) or 1 mM pyruvate and 0.1 mM lactate (−Glucose). Medium was then renewed with BSA (40 μM) either alone (Cont) or complexed to DHA (320 μM) or 100 μM 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate (cAMP). RNA was extracted and analyzed as described in Materials and Methods. **Upper panel**: Results of a typical autoradiogram are shown. **Lower panel**: Data were quantified and normalized for differences in RNA loading by using the 18S rRNA signal. Data are expressed as the percentage of PEPCK or L-CPT-1 signal from control (BSA-treated cells). Each value represents the mean ± SEM of results obtained from three independent experiments.

gene appears to be strongly dependent on cell context.

DISCUSSION

DHA induces a robust and rapid increase in PEPCK mRNA in rat periepididymal adipose tissue fragments and in 3T3-F442 differentiated adipocytes. The induction is dose-dependent with an EC50 around 120 μM, in good agreement with NEFA concentrations found in human plasma, varying between 0.1 and 2 mM depending upon the nutritional status and the physiopathological condition of the individual. None of the other transcripts tested in this study is responsive to DHA at early times of treatment, up to 7 h. Only ALBP/aP2 mRNA shows some induction at longer times of treatment, although the magnitude of increase remains far from that of PEPCK mRNA. Others reported previously that rats fed chronically a DHA-enriched diet show, in the post-absorptive state, a decrease in FAS, HSL, LPL, and PEPCK mRNAs specifically in peritoneal adipose tissue compared to the subcutaneous adipose depot [Raclot et al., 1997]. This discrepancy between these results and ours can be explained by (1) a different FA action depending upon the localization of adipose depots and, (2) a long-term in vivo effect compared to a short-term and direct in vitro action in our case. In 1995, we showed that fasted rats refed for 3 h with a high fat diet maintained PEPCK mRNA close to 100% in adipose tissue while a high carbohydrate diet induced a drastic fall [Antras-Ferry et al., 1995]. This result is in good agreement with our present study in which DHA effect is analyzed between 3 and 24 h after treatment.

The ALBP/aP2 transcript is augmented by oleate or by the nonmetabolizable α-bromopalmitate [Grimaldi et al., 1992; Antras-Ferry et al., 1995]. A two- to threefold increase in 4–24 h can be detected only in glucose-deprived cells. Run-on transcription experiments clearly demonstrated the transcriptional nature of the effect [Amri et al., 1991]. However, in the absence of data using inhibitors of protein synthesis, one cannot exclude the occurrence of an indirect effect. A similarly weak increase in LPL mRNA by linoleate or α-bromopalmitate has also been observed in long-term (24 h) treated Ob1771 adipocytes [Amri et al., 1996]. In these experiments, however, LPL gene transcription has not been determined, and a post-transcriptional action of FA cannot be excluded. There is no evident interpretation for the difference between this reported increase in LPL mRNA and our results showing no effect of FAs. The fact that different cell lines and distinct FAs have been used could explain the discrepancy.

Our previous and present results show that FA induction of the PEPCK gene is direct and strictly transcriptional, i.e., does not require protein synthesis and does not change mRNA half-life. Induction of PEPCK activity follows [Forest et al., 1997]. Because of the glyceroneogenic role of PEPCK, such a stimulatory action of FAs is physiologically relevant. First, after a hyperlipidic diet, FAs from LPL-hydrolyzed lipoproteins can be more easily stored as triacylglycerols in adipocytes when glycerol-3-phosphate synthesis is stimulated. Second, the increase in PEPCK activity, which results in glycerol-3-phosphate production during fasting, allows an enhancement in FA re-esterification, thereby controlling the output of FAs originating from lipolysis. PEPCK is also expressed in liver and hepatoma cells. However, it is not responsive to DHA in H4IIE hepatoma cells, in contrast to what is shown in adipocytes. In these cells, PEPCK probably exerts a dual function, gluconeogenic and glyceroneogenic, as in liver [Kalhan et al., 2001]. Hence, a DHA-induced enhancement of glucose and triacylglycerol synthesis in liver, through PEPCK induction during fasting, would augment glycemia and VLDL production, therefore would be contradictory to the observed beneficial effect of DHA on these parameters. Indeed, our results showing that DHA induces adipose PEPCK are consistent with the observation that PUFAs in general and DHA in particular are hypolipidemic, increase glucose disposal, and reduce insulin resistance [Storlien et al., 1998]. The fact that the hypolipidemic TTA also stimulates adipose PEPCK reinforces this point.

The nature of the DHA signal to the PEPCK gene is presently unknown. Because palmitate is not efficient [Antras-Ferry et al., 1995] and because TDGA does not prevent DHA action, the hypothesis that a mitochondrial β-oxidative product of this FA is involved can be inferred. Furthermore, TTA and bromopalmitate are potent inducers although nonβ-oxidizable. DHA could act via the production of peroxidation derivatives as it has been described for the PUFA inhibition of FAS gene expression in hepatocytes [Foretz et al., 1999]. This is not the

case here since vitamin E does not impair DHA stimulation. Furthermore, oleate is a strong inducer of PEPCK, although barely sensitive to peroxidation. One of the modes of action of FAs on gene expression could be *via* an alteration in eicosanoid synthesis. Indeed, DHA has been reported to affect prostaglandin and leukotriene production and to competitively inhibit cyclo-oxygenase from working on arachidonic acid [Corey et al., 1983; Smith, 1989]. However, neither aspirin nor NDGA, which inhibit respectively the synthesis of prostaglandins and that of leukotrienes, have any effect on PEPCK mRNA. They do not modify AA or DHA stimulation of PEPCK gene expression, strongly suggesting that the AA-derived eicosanoids are not involved in FA action in our case. Altogether, these results strongly suggest that the effect of DHA on PEPCK gene transcription is not due to a product of its metabolism but to the FA per se.

One of the proposed mechanisms of PUFA induction of gene transcription is *via* the activation of a nuclear receptor [Rao and Reddy, 1987]. In that respect, the peroxisome proliferator activated receptors (PPARs) are good candidates [Wahli et al., 1999]. To date, three isoforms have been cloned: PPAR α, δ ($=\beta=$ NUC1 = FAAR), and γ, with tissue-selective expression, ligand-specific activation, and ability to heterodimerize with retinoid X receptors (RXR). In in vitro assays, the three PPAR isoforms clearly bind various FAs with some specificity, PUFAs having a good affinity [Forman et al., 1997; Kliewer et al., 1997; Krey et al., 1997]. The PPAR/RXR heterodimer interacts in target genes with peroxisome proliferator response elements (PPREs). PPREs are direct repeat of AGGTCA separated by one nucleotide (DR1) with a 5′ extension of AACT for an increased specificity. Two such DR1-type elements, gAF1/PCK1 and PCK2, have been characterized in the PEPCK gene [Lucas et al., 1991; Tontonoz et al., 1995a]. They are potential targets for DHA induction. Moreover, TTA and α-bromopalmitate are respectively PPARα and PPARδ activators [Raspe et al., 1999; Jehl-Pietri et al., 2000]. However, if one of the PPARs is indeed the relay of DHA stimulation of the PEPCK gene, it is unlikely that PPARα and δ are involved, since PEPCK is not an FA target in H4IIE hepatoma, which express these two PPAR isoforms [Lee et al., 1997]. Studies with primary hepatocytes reinforce this point [Davies et al., 2001]. A PPARγ2 isoform of PPARγ has been cloned by Spiegelman and co-workers [Tontonoz et al., 1994]. We showed recently that the PCK2 element in the PEPCK gene is strictly required to mediate PPARγ-specific ligand induction of PEPCK gene transcription in adipocytes [Glorian et al., 2001]. PPARγ2 is expressed selectively in adipocytes and, therefore, could be the FA-responsive receptor in adipose tissue. This hypothesis is in accordance with the observation that PPARγ is rapidly diminished in adipose tissue of fasted rats in which we show that PEPCK gene expression is not responsive to DHA [Vidal-Puig et al., 1996]. It will be of great value to investigate this point since it raises the question of whether unsaturated FAs exert a selective action on the adipose-specific isoform of PPAR. Anyhow, this striking cell-specific and gene-selective action of DHA on the PEPCK gene places this gene, its product, and glyceroneogenesis on a central role in adipocytes.

ACKNOWLEDGMENTS

MG, ED, and JT were the recipients of a fellowship from the French "Ministère de l'Education Nationale et de la Recherche," MG was a recipient of the 1999 Award from the "Association Française de Nutrition-Société de Nutrition de Langue Française," and ED was a recipient of the 2000 Award from the ≪ Institut Danone ≫. We wish to thank Dr. Florence Chatelain-Egger, Dr. Jean-Paul Pégorier, and Dr. Jean Girard for helpful comments while preparing this manuscript.

REFERENCES

Amri EZ, Bertrand B, Ailhaud G, Grimaldi P. 1991. Regulation of adipose cell differentiation. I. Fatty acids are inducers of the aP2 gene expression. J Lipid Res 32: 1449–1456.

Amri EZ, Teboul L, Vannier C, Grimaldi PA, Ailhaud G. 1996. Fatty acids regulate the expression of lipoprotein lipase gene and activity in preadipose and adipose cells. Biochem J 314:541–546.

Antras-Ferry J, Le Bigot G, Robin P, Robin D, Forest C. 1994. Stimulation of phosphoenolpyruvate carboxykinase gene expression by fatty acids. Biochem Biophys Res Commun 203:385–391.

Antras-Ferry J, Robin P, Robin D, Forest C. 1995. Fatty acids and fibrates are potent inducers of transcription of the phosphenolpyruvate carboxykinase gene in adipocytes. Eur J Biochem 234:390–396.

Ballard FJ, Hanson RW, Leveille GA. 1967. Phosphoenolpyruvate carboxykinase and the synthesis of glyceride-

glycerol from pyruvate in adipose tissue. J Biol Chem 242: 2746–2750.
Beale EG, Chrapkiewicz NB, Scoble HA, Metz RJ, Quick DP, Noble RL, Donelson JE, Biemann K, Granner DK. 1985. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP). Structures of the protein, messenger RNA, and gene. J Biol Chem 260:10748–10760.
Berge RK, Hvattum E. 1994. Impact of cytochrome P450 system on lipoprotein metabolism. Effect of abnormal fatty acids (3-thia fatty acids). Pharmacol Ther 61:345–383.
Berge RK, Madsen L, Vaagenes H, Tronstad KJ, Gottlicher M, Rustan AC. 1999. In contrast with dicosahexaenoic acid, eicosapentaenoic acid and hypolipidaemic derivatives decrease hepatic synthesis and secretion of triacylglycerol by decreased diacylglycerol acyltransferase activity and stimulation of fatty acid oxidation. Biochem J 343 (Pt 1):191–197.
Boden G. 1997. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46:3–10.
Botion LM, Kettelhut IC, Migliorini RH. 1995. Increased adipose tissue glyceroneogenesis in rats adapted to a high protein, carbohydrate-free diet. Horm Metab Res 27: 310–313.
Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299.
Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Anal Biochem 162:156–159.
Corey EJ, Shih C, Cashman JR. 1983. Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotriene biosynthesis. Proc Natl Acad Sci USA 80: 3581–3584.
Davies GF, Khandelwal RL, Wu L, Juurlink BH, Roesler WJ. 2001. Inhibition of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by troglitazone: a peroxisome proliferator-activated receptor-gamma (PPARgamma)-independent, antioxidant-related mechanism. Biochem Pharmacol 62:1071–1079.
Della Loggia R, Ragazzi E, Tubaro A, Fassina G, Vertua R. 1988. Anti-inflammatory activity of benzopyrones that are inhibitors of cyclo- and lipo-oxygenase. Pharmacol Res Commun 20 (Suppl 5):91–94.
Duplus E, Glorian M, Forest C. 2000. Fatty acid regulation of gene transcription. J Biol Chem 275:30749–30752.
Esser V, Britton CH, Weis BC, Foster DW, McGarry JD. 1993. Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I. Direct evidence that a single polypeptide is involved in inhibitor interaction and catalytic function. J Biol Chem 268: 5817–5822.
Forest C, Franckhauser S, Glorian M, Antras-Ferry J, Robin D, Robin P. 1997. Regulation of gene transcription by fatty acids, fibrates and prostaglandins: the phosphoenolpyruvate carboxykinase gene as a model. Prostaglandins Leukot Essent Fatty Acids 57:47–56.
Foretz M, Foufelle F, Ferre P. 1999. Polyunsaturated fatty acids inhibit fatty acid synthase and spot-14- protein gene expression in cultured rat hepatocytes by a peroxidative mechanism. Biochem J 341:371–376.
Forman BM, Chen J, Evans RM. 1997. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci USA 94:4312–4317.
Glorian M, Duplus E, Beale EG, Scott DK, Granner DK, Forest C. 2001. A single element in the phosphoenolpyruvate carboxykinase gene mediates thiazolidinedione action specifically in adipocytes. Biochimie 83:933–943.
Grimaldi PA, Knobel SM, Whitesell RR, Abumrad NA. 1992. Induction of aP2 gene expression by nonmetabolized long-chain fatty acids. Proc Natl Acad Sci USA 89: 10930–10934.
Grimaldi PA, Teboul L, Gaillard D, Armengod AV, Amri EZ. 1999. Long chain fatty acids as modulators of gene transcription in preadipose cells. Mol Cell Biochem 192: 63–68.
Holm C, Kirchgessner TG, Svenson KL, Fredrikson G, Nilsson S, Miller CG, Shively JE, Heinzmann C, Sparkes RS, Mohandas T, Lusis AJ, Belfrage P, Schotz MC. 1988. Hormone-sensitive lipase: sequence, expression, and chromosomal localization to 19 cent-q13.3. Science 241: 1503–1506.
Jehl-Pietri C, Bastie C, Gillot I, Luquet S, Grimaldi PA. 2000. Peroxisome-proliferator-activated receptor delta mediates the effects of long-chain fatty acids on post-confluent cell proliferation. Biochem J 350 (Pt 1):93–98.
Jump DB, Clarke SD. 1999. Regulation of gene expression by dietary fat. Annu Rev Nutr 19:63–90.
Kalhan SC, Mahajan S, Burkett E, Reshef L, Hanson RW. 2001. Glyceroneogenesis and the source of glycerol for hepatic triacylglycerol synthesis in humans. J Biol Chem 276:12928–12931.
Kirchgessner TG, Svenson KL, Lusis AJ, Schotz MC. 1987. The sequence of cDNA encoding lipoprotein lipase. A member of a lipase gene family. J Biol Chem 262:8463–8466.
Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM. 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci USA 94:4318–4323.
Krey G, Braissant O, L'Horset F, Kalkhoven E, Perroud M, Parker MG, Wahli W. 1997. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator- dependent receptor ligand assay. Mol Endocrinol 11:779–791.
Lee MJ, Gee P, Beard SE. 1997. Detection of peroxisome proliferators using a reporter construct derived from the rat acyl-CoA oxidase promoter in the rat liver cell line H-4-II-E. Cancer Res 57:1575–1579.
Lucas PC, O'Brien RM, Mitchell JA, Davis CM, Imai E, Forman BM, Samuels HH, Granner DK. 1991. A retinoic acid response element is part of a pleiotropic domain in the phosphoenolpyruvate carboxykinase gene. Proc Natl Acad Sci USA 88:2184–2188.
Madsen L, Froyland L, Dyroy E, Helland K, Berge RK. 1998. Docosahexaenoic and eicosapentaenoic acids are differently metabolized in rat liver during mitochondria and peroxisome proliferation. J Lipid Res 39:583–593.
Madsen L, Rustan AC, Vaagenes H, Berge K, Dyroy E, Berge RK. 1999. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. Lipids 34: 951–963.

Mater MK, Pan D, Bergen WG, Jump DB. 1998. Arachidonic acid inhibits lipogenic gene expression in 3T3-L1 adipocytes through a prostanoid pathway. J Lipid Res 39: 1327–1334.

Nepokroeff CM, Adachi K, Yan C, Porter JW. 1984. Cloning of DNA complementary to rat liver fatty acid synthetase mRNA. Eur J Biochem 140:441–445.

Plee-Gautier E, Grober J, Duplus E, Langin D, Forest C. 1996. Inhibition of hormone-sensitive lipase gene expression by cAMP and phorbol esters in 3T3-F442A and BFC-1 adipocytes. Biochem J 318:1057–1063.

Raclot T, Oudart H. 1999. Selectivity of fatty acids on lipid metabolism and gene expression. Proc Nutr Soc 58:633–646.

Raclot T, Groscolas R, Langin D, Ferre P. 1997. Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues. J Lipid Res 38: 1963–1972.

Randle PJ. 1998. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes Metab Rev 14:263–283.

Rao MS, Reddy JK. 1987. Peroxisome proliferation and hepatocarcinogenesis. Carcinogenesis 8:631–666.

Raspe E, Madsen L, Lefebvre AM, Leitersdorf I, Gelman L, Peinado-Onsurbe J, Dallongeville J, Fruchart JC, Berge R, Staels B. 1999. Modulation of rat liver apolipoprotein gene expression and serum lipid levels by tetradecylthioacetic acid (TTA) via PPARalpha activation. J Lipid Res 40:2099–2110.

Reshef L, Shapiro B. 1970. The physiological function and regulation of glycerogenesis in adipose tissue. Horm Metab Res 2:136–142.

Reshef L, Hanson RW, Ballard FJ. 1970. A possible physiological role for glyceroneogenesis in rat adipose tissue. J Biol Chem 245:5979–5984.

Sasaki K, Cripe TP, Koch SR, Andreone TL, Petersen DD, Beale EG, Granner DK. 1984. Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. The dominant role of insulin. J Biol Chem 259: 15242–15251.

Skorin C, Necochea C, Johow V, Soto U, Grau AM, Bremer J, Leighton F. 1992. Peroxisomal fatty acid oxidation and inhibitors of the mitochondrial carnitine palmitoyltransferase I in isolated rat hepatocytes. Biochem J 281:561–567.

Skorve J, Asiedu D, Rustan AC, Drevon CA, al-Shurbaji A, Berge RK. 1990. Regulation of fatty acid oxidation and triglyceride and phospholipid metabolism by hypolipidemic sulfur-substituted fatty acid analogues. J Lipid Res 31:1627–1635.

Smith WL. 1989. The eicosanoids and their biochemical mechanisms of action. Biochem J 259:315–324.

Spiegelman BM, Frank M, Green H. 1983. Molecular cloning of mRNA from 3T3 adipocytes. Regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development. J Biol Chem 258:10083–10089.

Storlien LH, Baur LA, Kriketos AD, Pan DA, Cooney GJ, Jenkins AB, Calvert GD, Campbell LV. 1996. Dietary fats and insulin action. Diabetologia 39:621–631.

Storlien LH, Hulbert AJ, Else PL. 1998. Polyunsaturated fatty acids, membrane function and metabolic diseases such as diabetes and obesity. Curr Opin Clin Nutr Metab Care 1:559–563.

Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. 1994. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8:1224–1234.

Tontonoz P, Hu E, Devine J, Beale EG, Spiegelman BM. 1995. PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. Mol Cell Biol 15:351–357.

Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE. 1996. Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. J Clin Invest 97:2553–2561.

Wahli W, Devchand PR, IJpenberg A, Desvergne B. 1999. Fatty acids, eicosanoids, and hypolipidemic agents regulate gene expression through direct binding to peroxisome proliferator-activated receptors. Adv Exp Med Biol 447:199–209.