Polyunsaturated fatty acids inhibit fatty acid synthase and spot-14-protein gene expression in cultured rat hepatocytes by a peroxidative mechanism

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In vivo, polyunsaturated fatty acids (PUFA) inhibit the expression of hepatic genes related to the lipogenic process such as fatty acid synthase and spot-14-protein (S14) genes. In vitro studies have suggested that this was a direct transcriptional effect of PUFA. In hepatocytes, the inhibition of the lipogenic rate by PUFA is not specific, but is linked to a cytotoxic effect due to peroxidative mechanisms. We have investigated whether peroxidation could also explain the inhibitory effect of PUFA on gene expression. Rat hepatocytes were cultured for 24 h with mono-unsaturated or PUFA. PUFA inhibited the expression of fatty acid synthase and S14 genes, and this inhibition was directly related to the number of unsaturations. However, the β-actin and albumin mRNA concentrations were also affected by the most unsaturated fatty acids, suggesting a non-specific effect of PUFA on gene expression. Measurement of lactate dehydrogenase released into the medium indicated a cytotoxicity of PUFA. This was associated with their peroxidation as evaluated by the presence of thiobarbituric acid-reactive substances in the culture medium. The addition of high concentrations of antioxidants abolished lipid peroxidation and lactate dehydrogenase leakage and completely reversed the inhibitory effect of PUFA on gene expression. This suggests (i) that the results obtained previously in cultured hepatocytes in the presence of low concentrations of antioxidants must be interpreted cautiously and (ii) that in vivo, the inhibitory effect of PUFA on lipogenesis-related genes could be indirect through hormonal or metabolic changes or that their effect on gene expression is somehow linked to peroxidative mechanisms.

Key words: antioxidant, cytotoxicity, lipogenesis, peroxidation.

INTRODUCTION

It has been clearly established during the last few years that fatty acids can modulate the transcription of many genes involved in lipid transport and metabolism. Most of these genes, such as those coding for aP2 [1], phosphoenolpyruvate carboxykinase [2], carnitine palmitoyltransferase I [3] and liver fatty-acid-binding protein [4], are activated by both mono-unsaturated and polyunsaturated fatty acids (PUFA). It has been shown that fatty acids are ligands of peroxisome-proliferator-activated receptor (PPAR), a member of the nuclear-hormone-receptor superfamily [5,6]. PPAR regulates the transcription of target genes by binding to DNA sequence elements named the ‘PPAR response element’ (PPAR-RE). A number of PPAR-REs have been identified in various gene promoters, such as aP2 [7] and phosphoenolpyruvate carboxykinase [8].

The inhibition of gene expression by fatty acids is much less documented. Over the past 25 years, numerous studies have demonstrated that high-fat low-carbohydrate diets inhibit the hepatic lipogenic pathway. It was clearly shown that this was due to an inhibition of the expression of the enzymes of this pathway, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), spot 14 protein (S14) and the liver pyruvate kinase (L-PK) [9–12]. This inhibition is induced by the presence of small amount of PUFA in the diet [9,11,13]. The inhibitory effect of PUFA appears to be a liver-specific response, since they are not effective in other lipogenic tissues, e.g adipose tissue and lung. In vitro studies in primary cultures of hepatocytes have confirmed the inhibition of FAS, S14 and L-PK gene expression by PUFA, whereas saturated and mono-unsaturated fatty acids have no effect [12,14,15]. Transfection analysis of the L-PK and S14 promoter in primary cultures of hepatocytes have led to the identification of PUFA responsive elements [12,14]. It should be underlined that these PUFA-sensitive elements are located in key activatory transcriptional regions. Finally, it is noteworthy that the four genes (coding for L-PK, S14, ACC and FAS) which are specifically inhibited by PUFA are also the genes which are activated by glucose [16–19]. Moreover the PUFA responsive element of the L-PK gene is one of the elements involved in glucose transactivation [12].

One of the major problems encountered when using PUFA is their high susceptibility to peroxidation, forming hydroperoxides and other degradation products which are deleterious to the cells [20]. PUFA taken into the body are mostly delivered to liver cells [21]. The liver is thus one of the principal target of PUFA peroxidative effects. In vitro studies in cultured hepatocytes have clearly shown that PUFA taken up by hepatic cells were very sensitive to the peroxidation process [22]. Finally, it has been shown that the inhibitory effect of PUFA on fatty acid synthesis in hepatocytes is linked to the capacity of PUFA to undergo peroxidation [23]. Using primary culture of hepatocytes, these authors clearly demonstrated that PUFA had no inhibitory effects on the lipogenic flux when peroxidation was minimized with the use of high concentrations of antioxidants.

Abbreviations used: FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; S14, spot 14 protein; L-PK, liver pyruvate kinase; LDH, lactate dehydrogenase; TBARS, thiobarbituric acid-reactive substances; DFM, deferoxamine mesylate; DPPD, N,N’-diphenyl-1,4-diphenylenediamine; TS, α-tocopherol acid succinate; BHT, butylated hydroxytoluene; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; AA, arachidonic acid; LNA, linolenic acid; LA, linoleic acid; OA, oleic acid; PPAR, peroxisome-proliferator-activated receptor; PPAR-RE, PPAR-response element; T3, triiodothyronine; CPT I, carnitine palmitoyltransferase I; NF-κB, nuclear factor κB.

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The aim of the present study was to analyse whether the inhibitory effect of PUFA on gene expression can also be due to their peroxidation. We have thus studied the effects of various fatty acids on the expression of two lipogenic-related genes, the FAS and S14 genes, in primary cultures of hepatocytes. The results outlined below established that the inhibitory effect of PUFA observed \textit{in vitro} is linked to their ability to be peroxidized and is probably not specific for lipogenic-related genes.

**EXPERIMENTAL**

**Animals**

Animal studies were conducted according to the French Guidelines for the Care and Use of Experimental Animals. Female Wistar rats (200–300 g body weight) from Iffa-Credo (L’Arbresle, France) were used. They were housed in plastic cages at a constant temperature (22 °C) with light from 07:00 h to 19:00 h for at least 1 week before the experiments.

**Isolation and primary culture of hepatocytes**

Hepatocytes were isolated by the collagenase-perfusion method [24]. Cell viability was assessed by the Trypan Blue-exclusion test and was always higher than 85%. Hepatocytes were plated at a density of 8 × 10^4 cells/dish in 100-mm-diameter Petri dishes in medium M199 with Earle’s modified salts (Gibco/BRL, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% (w/v) BSA, 2% (w/v) Ultroser G (IBF, Villeneuve-la-Garenne, France), 100 nM dexamethasone (Sigma, St. Louis, MO, U.S.A.), 1 nM insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark), 100 μM tritiated thymidine (T3) (Sigma). The plated cells were incubated at 37 °C with atmospheric air/CO2 (19/1). After a 4 h attachment period, cells were maintained overnight in a medium similar to the plating medium but free of Ultroser and BSA and containing 25 mM glucose and 100 mM insulin. After 16–18 h the culture medium was removed and hepatocytes were then cultured for 24 h in the presence of 25 mM glucose, 50 μM fatty-acid-free BSA (Euromedex, Souffelweyersheim, France) and in the presence or absence of 500 μM non-esterified fatty acids and antioxidants in concentrations as specified below.

Before experiments, non-esterified fatty acids (Sigma) were diluted in ethanol at a concentration of 300 mM. Fatty acids were complexed to 5% BSA (735 μM) by stirring for 1 h at 37 °C in the presence or absence of antioxidants, after which this mixture was clear. The fatty acids used in the present study included oleic acid (OA, C18:1, n−9), linoleic acid (LN, C18:2, n−3), linolenic acid (LNA, C18:3, n−6), arachidonic acid (AA, C20:4, n−6) and eicosapentaenoic acid (EPA, C20:5, n−3). The final concentrations of antioxidants were: 2 μM N,N′-diphenyl-1,4-phenylenediamine (DPPD), 75 μM (+)-α-tocopherol acid succinate (TS), 3 μM butylated hydroxytoluene (BHT) and 0.8 μM deferoxamine mesylate (DFM) except when otherwise indicated. These antioxidants were dissolved in DMSO. The final concentrations of DMSO and ethanol in the media were 0.6% (v/v) and 0.15% (v/v) respectively and were identical in control dishes.

**Measurement of lactate dehydrogenase (LDH) leakage**

After 24 h of culture in the presence of fatty acids, the medium was collected and frozen at −20 °C. The medium was analysed for the activity of LDH (EC 1.1.1.27) and the concentration of thiobarbituric acid-reactive substances (TBARS). The leakage of LDH from hepatocytes was used to estimate the viability of hepatocytes after treatment with fatty acids. The activity of LDH was measured at 25 °C. Briefly, 200 μl of medium were used for spectrophotometric assay in 800 μl of 0.2 M Tris/HCl, pH 8.0, containing 0.25 mg/ml NADH. After addition of pyruvate (100 μM final concentration), the linear decrease in absorbance at 340 nm was monitored during 10 min and the activity was computed from the slope of the curve.

**Measurement of TBARS concentrations**

Lipid peroxidation was determined from the production of TBARS. The TBARS assay was performed by a modification of the procedure of Minara and Uchiyama [25]. Aliquots of 400 μl of medium were mixed with 3 ml of 1% (w/v) phosphoric acid and 1 ml of 0.6% (w/v) thiobarbituric acid, then heated in a boiling water-bath for 45 min and cooled at room temperature. The TBARS were extracted by addition of 4 ml of butan-1-ol. This mixture was vigorously agitated and centrifuged at 3000 g for 20 min. The absorbance of the upper phase was measured at 532 nm and compared with a range of standards of malonaldehyde bis(dimethylacetal) (Sigma).

**Isolation of total RNA and Northern-blot hybridization**

Total cellular RNAs were extracted using the guanidine thiocyanate method [26] and prepared for Northern-blot hybridization as previously described [27]. Labelling of each probe was performed by random priming (Rediprime labelling kit, Amer sham). Autoradiograms of Northern blots were scanned and quantified using an image processor program. FAS, S14 and β-actin probes were as previously described [28]. The carnitine palmitoyltransferase I probe was kindly given by Professor J. D. McGarry, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.

**Statistical analysis**

Results are expressed as means ± S.E.M. Statistical analysis was performed with Student’s t-test for unpaired data.

**RESULTS**

**Effects of mono- and poly-unsaturated fatty acids on FAS and S14 gene expression in primary cultured hepatocytes**

In order to analyse the effects of various fatty acids on lipogenic-related gene expression, the hepatocytes were cultured in the presence of 25 mM glucose, insulin, dexamethasone and T3 for 18 h to induce a full expression of FAS and S14 genes [28]. Then the medium was replaced for 24 h by a similar one but containing various fatty acids complexed to albumin.

OA and LA had no inhibitory effect on mRNA encoding FAS and S14 (Figure 1A). LNA had a slight inhibitory effect, whereas AA and EPA strongly impaired glucose-induced FAS and S14 mRNA expression (Figure 1A). The inhibitory effect of PUFA in hepatocytes was already detectable in the presence of 100 μM AA (results not shown).

Surprisingly, EPA and, to a lesser extent, LNA and AA, had also a decreasing effect on the concentrations of the mRNA encoding β-actin. In contrast, no effect was observed with OA and LA on the expression of this control gene. A similar pattern was observed with albumin mRNA (results not shown). These observations suggest that the inhibitory effect on mRNA concentrations is specific for PUFA, but is not confined to lipogenic-related genes.
measured concomitantly the peroxidation products released from gated the effects of fatty acids on hepatocyte viability and potentially linked to a peroxidative mechanism, we have investigated the effects of fatty acids on hepatocyte viability and measured concomitantly the peroxidation products released from hepatocytes in the culture medium. Because LDH is a cytosolic enzyme normally absent from the culture medium, we have used LDH activity released in the medium as an index of cytotoxicity.

Whereas OA and LA did not increase LDH leakage, LNA, AA and EPA at a 500 μM concentration significantly increased LDH leakage with a respective potency linked to their number of unsaturation (Table 1). The strongest effect was observed in the presence of 500 μM EPA, with a 3.5-fold increase in LDH leakage into the media. Peroxidation products of unsaturated fatty acids were assessed by measuring levels of TBARS in the culture medium. As shown in Table 1, concentration of TBARS in media also increased with the degree of unsaturation in a manner parallel with that of LDH activity. Both LDH leakage and TBARS concentrations were related to the concentration of PUFA in the culture medium, as shown for EPA (Table 1). In addition, low concentrations of EPA (10 and 50 μM), which do not significantly increase TBARS concentrations (Table 1), do not alter glucose-induced expression of FAS (Figure 1B).

In summary, there is a strong correlation between peroxidative mechanisms in the presence of various fatty acids, their cytotoxicity as assessed by LDH leakage and their potency to inhibit FAS, S14, β-actin and albumin expression, suggesting a causal relationship.

Effects of antioxidants on the repression of FAS and S14 gene expression by PUFA in primary cultured hepatocytes

In hepatocytes it has been shown that the use of high concentrations of antioxidants had protective effects against lipid peroxidation and the subsequent oxidative damage [22,23]. We have therefore examined the effects of antioxidants on the expression of FAS and S14 genes in hepatocytes incubated in the presence of various fatty acids described above. We used a mixture of antioxidants containing a powerful iron chelator, DFM (0.8 mM), and three radical scavengers, namely DPPD (2 μM), TS (75 μM) and BHT (3 μM). In preliminary experiments we checked that the antioxidants did not modify the glucose-induced expression of FAS and S14 genes in hepatocytes incubated in the presence of various fatty acids at a 500 μM concentration and for 24 h was completely inhibited by the addition of these antioxidants. The absence of lipid peroxidation prevented efficiently the LDH leakage (Figure 2A). Moreover, in the presence of antioxidants, PUFA lost their capacity to inhibit FAS, S14 and β-actin expression (Figure 2B), even in the presence of EPA.

Antioxidants have been used in previous studies on the effect of PUFA on gene expression, although at much lower concentrations than in the present study [15]. It is clear from Figure 3 that, in the presence of these low concentrations of antioxidants and 300 μM AA, peroxidative mechanisms and cell cytotoxicity are still present, as well as the inhibition of FAS gene expression. All these phenomena are totally absent even after 48 h of culture if one uses a high antioxidant concentration (Figure 3).

To verify that the presence of antioxidants does not impair general regulatory transcriptional mechanisms, we have used as a positive control the expression of carnitine palmitoyltransferase I (CPT I) gene, since it was shown that it is induced by fatty acids in liver [3]. We have thus measured the mRNA concentration of the CPT I gene in hepatocytes incubated with EPA (500 μM) in the presence of the antioxidant mixture. In these conditions, CPT I mRNA concentration was increased 3-fold in response to EPA (Figure 4), suggesting that the presence of antioxidants in the medium does not preclude gene regulation by fatty acids.
Table 1  LDH activity and TBARS concentrations in the absence or presence of various fatty acids

Hepatocytes were cultured for 16–18 h in the presence of 25 mM glucose and hormones (100 nM insulin, 100 nM dexamethasone and 100 nM T3). Fatty acids (OA, LA, LNA, AA and EPA) complexed with 50 μM albumin were then added for 24 h. At the end of the culture period, LDH activity and TBARS concentrations were determined in the culture medium as described in the Experimental section. The results are presented as the means ± S.E.M. for three independent cultures with triplicates in each culture. * and **, Difference statistically significant for respectively P < 0.05 and P < 0.01 when compared with controls.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>OA</th>
<th>LA</th>
<th>LNA</th>
<th>AA</th>
<th>EPA</th>
<th>EPA</th>
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<tr>
<td>Concentration (μM)</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>300</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>LDH activity (μmol/min per 10⁶ cells)</td>
<td>0.37 ± 0.05</td>
<td>0.33 ± 0.05</td>
<td>0.50 ± 0.04</td>
<td>0.74 ± 0.10*</td>
<td>1.3 ± 0.2**</td>
<td>ND</td>
<td>ND</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1*</td>
<td>1.4 ± 0.2**</td>
</tr>
<tr>
<td>[TBARS] (μM)</td>
<td>2.39 ± 0.05</td>
<td>2.56 ± 0.06</td>
<td>2.60 ± 0.07</td>
<td>7.5 ± 0.9**</td>
<td>11.2 ± 0.3**</td>
<td>2.0 ± 0.3</td>
<td>2.1 ± 0.1</td>
<td>3.16 ± 0.40</td>
<td>9.3 ± 0.5**</td>
<td>13.2 ± 0.9**</td>
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Figure 2  Culture-medium LDH activity and TBARS concentrations and FAS, S14 and β-actin expression in hepatocytes cultured in the presence of various fatty acids and antioxidants

Hepatocytes were cultured for 16–18 h in the presence of 25 mM glucose and hormones (100 nM insulin, 100 nM dexamethasone and 100 nM T3). Cells were then cultured with 25 mM glucose and hormones in the presence of 500 μM various fatty acids complexed to 50 μM fatty-acid-free albumin and the antioxidants. The fatty acids used include OA, LA, LNA, AA and EPA. The final concentrations of antioxidants were: 2 μM DPPD, 75 μM TS, 3 μM BHT and 0.8 μM DFM. After 24 h of culture, the TBARS concentrations (A), LDH activity (B) were determined in the medium. Results are means ± S.E.M. for three independent cultures with triplicates in each culture. Total RNAs were extracted from hepatocytes and analysed for the expression of FAS, S14 and β-actin genes (C). The Northern blot presented is representative of three different culture experiments.
Polyunsaturated fatty acids and expression of lipogenic-related genes

DISCUSSION

This series of experiments demonstrates that the inhibitory effect of PUFA on the expression of FAS and S14 in cultured hepatocytes is not specific and is probably due to lipid peroxidation and cell toxicity.

PUFA added in the diet in low amounts have been shown to inhibit in vivo the expression of several hepatic genes involved in lipogenesis, such as FAS, ACC, S14 protein, L-PK, ‘malic’ enzyme and stearoyl-CoA desaturase-I [15,29]. Further studies have shown that this inhibition occurs at the transcriptional level [10,30,31].

It was concluded from in vitro and in vivo studies (i) that these effects were direct, since they could be reproduced by the sole presence of PUFA in the medium of cultured hepatocytes, (ii) that, in contrast with stimulatory effects, these inhibitory effects on gene transcription were confined to PUFA [14,15], (iii) that PUFA response elements were present in the proximal promoter of the L-PK and S14 gene and associated with, or close to, strong activator elements [12,14], and (iii) that the effects were not mediated by the PPARz [32]. Since these earlier results were obtained, no further mechanism for the transcriptional action of PUFA has been proposed.

Although the in vivo effects on gene expression are extremely clear, an important finding of the present work is that one has to be extremely cautious when extrapolating in vitro studies using PUFA on cultured hepatocytes to the in vivo situation. Indeed, we have been unable to dissociate cytotoxic unspecific effects of PUFA from their action on gene expression. These results are in accordance with those of Mikkelsen et al. reporting that the principal inhibitory action of PUFA on hepatocyte lipogenesis flux is due to cytotoxic effects caused by lipid peroxidation [23]. Cytotoxic effects of PUFA in hepatocytes have also been previously reported by authors in studies addressing their effects on gene expression, but considered as non-significant, since control gene expression was apparently not affected. For instance, in the presence of a low concentration of antioxidants (2.7 μM BHT and 1 μM vitamin E), 300 μM EPA led to a 19 % decrease in total protein content, a 4.5-fold increase of LDH and a 2-fold increase of TBARS concentrations in the medium when compared with 300 μM OA [15]. Lipid peroxidation generates lipid radicals, lipid hydroperoxides and other reactive substances such as malondialdehyde [33]. These products of peroxidation are cytotoxic and can cause cell damage. It has been reported that, in the presence of fatty acids, cultured hepatocytes form products of peroxidation in a concentration- and time-dependent manner. In addition, the formation of peroxidation products by hepatocytes increases with the degree of fatty acid unsaturation [22,23]. Highly unsaturated fatty acids such as docosahexaenoic acid, EPA and AA were considerably peroxidized, whereas OA, a mono-unsaturated fatty acid, was much less susceptible to peroxidation [22].

The in vitro effects of PUFA on gene transcription described previously in the literature could therefore largely be due to peroxidative cytotoxic effects. This could explain why, in contrast with activatory effects of fatty acids on gene transcription for which both mono-unsaturated and PUFA are effective, the inhibitory effects are confined to PUFA.

Another important aspect of the present study is the fact that, in the presence of antioxidants, high concentrations of PUFA are unable to decrease gene expression in cultured hepatocytes, even after 48 h of culture. This suggests that the effects of PUFA in vivo are either indirect, through hormonal or metabolic changes (for instance by altering glucose metabolism itself), or that their effect on gene expression is somehow linked to peroxidative...
mechanisms. In vivo, it is conceivable that hepatocytes are more efficiently protected from the cytotoxic effects of peroxidation inasmuch as peroxidative products do not accumulate as they do in the culture dish. In vivo, peroxidation could then act as a signalling system, as demonstrated for other genes, without compromising cell viability. Reactive-oxygen-species production can lead to changes in gene expression via changes in the protein redox state. For example, transcription factors AP1 and nuclear factor κB (NF-κB) are sensitive to redox regulation. The redox state of critical cysteine residues in transcription factors can affect their ability to bind to DNA and subsequently alter transcription of target genes. A decrease in these critical cysteine residues by thioredoxin and reI protein restores DNA binding [34]. The binding of Sp1, a zinc-finger transcription factor, and upstream stimulatory factor ('USF'), a basic-domain helix–loop–helix leucine-zipper transcription factor, to their cognate DNA elements is sensitive to redox regulation [35–38]. Moreover, reactive oxygen species can modulate the mitogen-activated protein kinase cascade and the transcription factors that are controlled by these kinase cascades, such as AP-1 and NF-κB [34].

Further investigations will be required to explore this potential signalling mechanism by which PUFA could act on gene expression in vivo in the liver.

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