Polyunsaturated Fatty Acyl Coenzyme A Suppress the Glucose-6-phosphatase Promoter Activity by Modulating the DNA Binding of Hepatocyte Nuclear Factor 4α*

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Glucose-6-phosphatase (Glc6Pase); EC 3.1.3.9) confers on gluconeogenic tissues the capacity to release endogenous glucose in blood. The expression of its gene is modulated by nutritional mechanisms dependent on dietary fatty acids, with specific inhibitory effects of polyunsaturated fatty acids (PUFA). The presence of consensus binding sites of hepatocyte nuclear factor 4 (HNF4) in the −1640/+60 bp region of the rat glucose-6-phosphatase gene has led us to consider the hypothesis that HNF4α could be involved in the regulation of glucose-6-phosphatase gene transcription by long chain fatty acid (LCFA). Our results have shown that the glucose-6-phosphatase promoter activity is specifically inhibited in the presence of PUFA in Hepg2 hepatoma cells, whereas saturated LCFA have no effect. In HeLa cells, the glucose-6-phosphatase promoter activity is induced by the co-expression of HNF4α or HNF1α. PUFA repress the promoter activity only in HNF4α-cotransfected HeLa cells, whereas they have no effects on the promoter activity in HNF1α-cotransfected HeLa cells. From gel shift mobility assays, deletion, and mutagenesis experiments, two specific binding sequences have been identified that appear able to account for both transactivation by HNF4 and regulation by LCFA in cells. The binding of HNF4α to its cognate sites is specifically inhibited by polyunsaturated fatty acyl coenzyme A in vitro. These data strongly suggest that the mechanism by which PUFA suppress the glucose-6-phosphatase gene transcription involves an inhibition of the binding of HNF4α to its cognate sites in the presence of polyunsaturated fatty acyl-CoA thioesters.

Glucose-6-phosphatase (Glc6Pase); EC 3.1.3.9) confers on gluconeogenic tissues, i.e. the liver, the kidney, and the small intestine, the capacity to release endogenous glucose in blood (1, 2). The expression of its gene is increased during diabetes and fasting and normalized upon insulin treatment and refeeding, respectively, in all three gluconeogenic tissues (3, 4). An increase in the Glc6Pase flux (5, 6) and maximal velocity (7) has also been strongly suggested to account for increased glucose production and hepatic insulin resistance in type 2 diabetes mellitus.

The Glc6Pase gene expression is also modulated by nutritional mechanisms dependent on dietary fatty acids. In the liver of rats, Glc6Pase mRNA and protein contents are increased upon high fat feeding (8) and upon elevation in plasma fatty acid levels (9). Under these nutritional conditions, the suppression of hepatic glucose production by insulin is impaired (10). This suggests that a high plasma fatty acid level may contribute increased production of glucose via increased expression of Glc6Pase, resulting in the development of liver insulin resistance (9, 11, 12). In vitro, the treatment of fetal hepatocytes with a high concentration (500 μM) of long chain fatty acids (LCFA), such as oleic and linoleic acids, increases the Glc6Pase mRNA content (13). We have shown that the likely mechanism involves a stabilizing effect on Glc6Pase.

In the liver, although peroxisome proliferator-activated receptors (PPARs) have emerged as an important factor in the fatty acid regulation at the transcription level, recent evidence indicates that the DNA binding activity and/or the abundance of other factors, such as sterol regulatory element-binding protein 1c (SREBP1c) or hepatocyte nuclear factor 4 (HNF4), may be affected by fatty acids or their metabolites (for reviews, see Refs. 19–21). Interestingly, Hertz et al. (22) have recently reported that the transcriptional activity of HNF4α (previously considered an orphan nuclear receptor) could be differentially modulated by LCFA, depending on their unsaturation level, through the binding of their acyl-CoA thioesters to the ligand domain of HNF4α. For example, saturated LCFA such as C16:0...
(palmitic acid) might activate the transcriptional activity of HNF4α in cellular transfection assays, and its palmitoyl-CoA derivative might enhance the binding of HNF4α to its DNA-binding site in vitro in bandshift assays. On the contrary, PUFA, such as C18:3(n-3) or C20:5(n-3), might inhibit the transcriptional activity of HNF4α, and their respective acyl-CoA derivatives might decrease the binding of HNF4α to its site. The latter data were obtained using a synthetic promoter containing three copies of the HNF4-binding sequence of the human apoCIII gene regulatory region (22). Noteworthy, as consensus sequences for the binding of HNF4α are present in the 5′-flanking region of the rat Glc6Pase gene (23), this has given us, using a natural promoter, the opportunity to examine the original hypotheses: 1) that a differential regulation of Glc6Pase gene expression by saturated and polyunsaturated LCFA could occur at a transcriptional level and 2) that HNF4α might be involved in the mechanism. We studied the effects of LCFA on the Glc6Pase promoter activity in HepG2 hepatoma and HeLa cells. Our results strongly suggest that the Glc6Pase promoter activity is negatively regulated by PUFAs through the modulating effect of their CoA-derivatives on HNF4α activity.

**Experimental Procedures**

Construction of the Reporter Gene Plasmids—The 5′-flanking region of the rat Glc6Pase gene up to nucleotide −1640, relative to the transcription start site, was cloned into the “pGL2 basic” vector (Promega) upstream of a luciferase reporter gene and into the “pGL2 enhancer” vector (Promega), which also contains the SV40 enhancer. The −1640/+109 region of the Glc6Pase gene was amplified by PCR from rat genomic DNA using the primers 5′-AAGCTTAAAGTAACTGAGTGAA-3′ sense and 5′-CCAAAGTCTGGACCCAGTTC-3′ antisense (23) and cloned into the pBluescript SK+ vector to generate the −1640/+109SK+ plasmid. The −1640/+66LUC plasmid was constructed by insertion of pGL2 vector of the Smal/KpnI fragment obtained by digestion of the −1640/+109SK+ plasmid. A series of truncated Glc6Pase promoter-LUC constructs with progressive 5′-end deletions of the Glc6Pase promoter region was generated by either restriction enzyme or PCR using −1640/+109SK+ plasmid as a template (as denoted in Fig. 1). Site-directed mutagenesis of the HNF4α sequences was generated using the GeneEditor™ in vitro site-directed mutagenesis system (Promega) using the antibiotic resistance selection oligonucleotide provided in the kit and specific mutagenic oligonucleotides (denoted in Fig. 7 insets). All PCR-derived constructs and mutations were confirmed by sequencing using the T7 sequencing kit (Amersham Biosciences) to ensure the absence of polymerase errors. Plasmid constructs were purified by the Plasmid Maxi Kit (Jet Star, Genomed).

**Cell Culture and Transient Transfection Assays**—HepG2 human hepatoma cells and HeLa human epithelial cervical carcinoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 6% (for HepG2 cells) or 10% (for HeLa cells) fetal bovine serum, 5 mM glutamine, streptomycin (1 μg/ml), and penicillin (1 unit/ml) in a 5% CO2 atmosphere at 37 °C. 1 day before the transfection, 200,000 cells were plated out in 35-mm wells in six-well cell culture plates. The complete medium was refreshed 1 h prior to transfection. Cells were transfected by the calcium phosphate transfection method with 1 μg of the Glc6Pase-LUC plasmid, 0.2 μg of pRSV-CAT to correct for transfection efficiency, and 0.5 μg of a HNF4α expression vector pCR3-HNF4 (obtained from M. Raymondjean and B. Viollet (24) or 1 μg of a HNF1 expression vector pRSV-HNF1 (obtained from M. Yaniv (25)) as indicated. The total amount of DNA (2.2 μg) was kept constant by the addition of pBluescript SK+ plasmid. For transfection of HepG2 cells, calcium phosphate-DNA coprecipitates were formed in Hepes buffer, and for HeLa cells, precipitates were formed in BES buffer (26). The precipitate was removed after 20 h by the addition of EDTA (3.5 mM final) for 1 min, and the cultures were further incubated for another 24 h in normal grown medium. Cells were treated for 6 h by 200 μM fatty acid (Sigma) diluted in ethanol (0.1% final) in the presence of 0.4% bovine serum albumin, in serum-free medium either alone or supplemented with indomethacin (10 μM, Sigma) or NDGA (10 μM, Sigma) or acetylsalicylic acid (aspirin, 200 μM, Sigma). In some experiments, treatment was performed for 24 h in the presence of 5 × 10−4 M Trolox (Sigma). Cells were treated with indomethacin or NDGA or acetylsalicylic acid for 1 h before adding fatty acid. The cells were then washed three times with phosphate-buffered saline and lysed with reporter lysis buffer (Promega). After a 15-min incubation, cells were scraped and centrifuged at 10,000 × g for 5 min at 4 °C to eliminate cell debris. Luciferase activity was determined with a BCLBook luminometer (Promega) using the luciferase assay reagent (Promega). For CAT activity, the cell extract was treated at 60 °C for 10 min to inactivate endogenous deacylase activity, and CAT activity was determined as described by Newmann et al. (27). The levels of luciferase activities were normalized by means of the CAT activities. Statistical analyses were performed using Student’s t test for unpaired data.
Fatty Acyl-CoA Regulation of Glucose-6-phosphatase Promoter

FIG. 2. Polyunsaturated fatty acids inhibit the transcriptional activity of the Glc6Pase promoter in HepG2 cells. HepG2 cells were transiently transfected with the −1320/+60 “basic” construct (1320B, black bars in A) or with the −160/+60 “basic” construct (160B, hatched bars in A) together with pRSV-CAT as a transfection efficiency control. After 24 h of transfection, cells were treated with LCFA at 200 μM for 6 h (A and B) or 24 h (C) in the presence of 0.4% bovine serum albumin. B, cells were treated in the presence of arachidonic acid (C20:4 (n-6)) for 6 h with (or without) either indomethacin (indo; 10 μM), NDGA (10 μM), or acetylsalicylic acid (Aspirin; 200 μM). C, cells were treated for 24 h with arachidonic acid and Trolox (5 × 10⁻⁴ M; Sigma). Note that none among indo, NDGA, aspirin, and Trolox had significant effect when present alone (not shown). LUC activities were normalized to the level of CAT activities and were expressed relative to the activity in the cells incubated in the absence of fatty acids (0.1% EtOH used as vehicle for dilution of fatty acids). The results are the means ± S.E. of at least three independent experiments performed in duplicate. * and **, significantly different from EtOH used as vehicle for dilution of fatty acids. p < 0.05 and p < 0.01, respectively.

Gel Shift Mobility Assays—Double-stranded oligonucleotides used were described in Fig. 3A. Recombinant HNF4α protein was expressed in Escherichia coli from prSET-HNF4 (obtained from M. Raymondjean and B. Viollet) and purified as described by Viollet et al. (24).

End-labeled oligonucleotide probes (0.1 ng, 30,000–50,000 cpm) were incubated for 15 min at 4°C in the binding reaction buffer (10 mM Hepes, 50 mM KCl, 50 mM NaCl, 4 mM spermidine, 0.1 mM EDTA, 2 mM dithiothreitol, 100 μg/ml bovine serum albumin, pH 7.6) in the presence of 50 ng of bacterial recombinant HNF4α protein and 100 ng of poly(dI-dC) (24). Free DNA and DNA-protein complexes were separated on a 5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide, 29:1) in 0.2× Tris borate-EDTA buffer. In competition experiments, the competitor DNA was incubated in the mixture prior to the addition of the probe. The antisera anti-HNF4 used for gel supershifts (28) and/or purified acyl-CoA (Sigma) were preincubated with proteins at 4°C for 15 min before the addition of free probe. After electrophoresis, the gels were dried, and the quantification of the DNA binding complexes was monitored using a PhosphorImager and performed using ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA).

Western Blotting—50 μg of whole cell protein extracts from HeLa cells, HeLa cells transfected by pc3-HNF4 for 48 h, HepG2 cells, or rat liver nuclear extract were separated by SDS-10% polyacrylamide gel electrophoresis and transferred to Immobilon membrane. Immunoblotting was performed as described by B. Viollet et al. (24) using anti-HNF4 antibodies (28) at 1:500 dilution.

RESULTS

The Glc6Pase Promoter Activity Is Inhibited in the Presence of PUFA—All Glc6Pase promoter-LUC constructs conferred a significant promoter activity in HepG2 cells but were almost totally inactive in HeLa cells (Fig. 1). In HeLa cells, the addition of an SV40 enhancer was not sufficient to confer significant promoter activity. Indeed, only a weak LUC activity was observed with constructs of the Glc6Pase promoter containing the SV40 enhancer (−1640/+60E to −80/+60E constructs) (see Fig. 3). This strongly suggested that some hepatic specific factors were required for Glc6Pase promoter activity. A promoter fragment from −80 to +60 bp (minimal promoter construct) containing the TATA box was sufficient to confer significant transcriptional activity in HepG2 cells (Fig. 1). A strong maximal LUC activity was obtained with a construct containing the −500/+60 bp region of the Glc6Pase promoter. In contrast, the −320/+60B and the −730/+60B constructs presented a lower activity, which was very similar to that of the minimal promoter construct (Fig. 1). This suggested that the −320 to −500 bp region might contain enhancer elements and that the −500 to −730 bp might contain suppressive regulatory elements able to counterbalance the inductive action of the upstream region. Other enhancer regulatory elements might exist within the −1320 to −1480 bp region, since both the −1640/+60B and the −1480/+60B constructs exhibited a sim-
FIG. 4. Modulation of HNF4α and HNF1α transactivation activities of the Glc6Pase promoter by long chain fatty acids in HeLa cells. HeLa cells were transiently transfected with the −1640/+60 enhancer construct, a pRSV-CAT (used as a control), and either pRSV-HNF1 α (A, open bars) or pRSV-HNF4α (A, closed bars; B and C, all bars). After 24 h, cells were treated with LCFA at 200 μM for 6 h (A and B) or for 24 h (C), and normalized LUC activity was expressed relative to the activity in the cells incubated in the absence of fatty acids (condition with 0.1% EtOH). B, cells were treated for 6 h with C20:4(n-6) and with (or without) either indomethacin (indo, 10 μM), or NDGA (10 μM). C, cells were treated for 24 h with C20:4 and Trolox (5 × 10⁻⁶ M; Sigma). Indo, NDGA, or Trolox had no significant effect when present alone (not shown). The results are expressed as the means ± S.E. of at least three independent experiments performed in duplicate. + and **, significantly different from EtOH value; p < 0.05 and p < 0.01, respectively.

ilar substantial promoter activity higher than that of the −1320/+60B construct (Fig. 1).

HepG2 cells were then transfected with Glc6Pase promoter–LUC constructs, containing either a long (−1320/+60 bp) or a short (−160/60 bp) promoter fragment, and treated with 200 μM LCFA for 6 h in the presence of 0.4% bovine serum albumin. Saturated (C16:0 and C18:0) and monounsaturated (C18:1(n-9)) had no effect on basal promoter activity (Fig. 2A). In contrast, all PUFAs studied (e.g. C18:2(n-6), C20:4(n-6), and C22:6(n-3)) suppressed the transcriptional activity of both constructs by 30–50% (Fig. 2A). Experiments carried out with other Glc6Pase promoter constructs of variable lengths yielded very similar results (data not shown). These data strongly suggested that the differential effects of saturated and polyunsaturated LCFA on the Glc6Pase gene expression might occur at a transcriptional level.

The suppression of the Glc6Pase promoter activity by PUFA was not affected in the presence of indomethacin (10 μM), NDGA (10 μM), or aspirin (200 μM) (Fig. 2B). In a similar manner, the PUFA inhibition effect at a longer time point (24 h) in the presence of the antioxidant agent Trolox (50 μM) was in the same order range as that occurring for a 6-h incubation (Fig. 2C). This allowed us to rule out the hypotheses that the PUFA-inhibitory effects could be dependent either on further metabolism through the cyclooxygenase or lipoxygenase pathways or on the deleterious accumulation of peroxidation products (29).

HNF4α Is Involved in the PUFA-induced Inhibition of Glc6Pase Promoter Activity—The involvement of HNF1 in the Glc6Pase gene transcription has been previously documented (30–32). In contrast, the involvement of HNF4α has not yet been described. To assess the putative role of the latter, HeLa cells were co-transfected with Glc6Pase promoter–LUC constructs and eucaryotic expression vectors of either HNF4α or HNF1α as a control (Fig. 3). In HNF4α-expressing HeLa cells, the Glc6Pase promoter activity was markedly induced by about 10 times from the shortest construct (−80/+60 bp), suggesting the presence of at least one HNF4-binding site in this proximal region. This was in agreement with the presence of a potential HNF4-binding site in this region of the rat Glc6Pase promoter (23). In HNF1α-expressing HeLa cells, the Glc6Pase promoter activity was induced by 3–5 times for the constructs containing the −320/+60 up to the −1640/+60 bp regions (Fig. 3). In contrast, HNF1α did not significantly transactivate the Glc6Pase promoter sequence between −160 to +60 bp (Fig. 3B). These results suggested the presence of a first HNF1-binding site localized just upstream the −160 bp of the Glc6Pase promoter. This was in agreement with the presence of the HNF1-binding sequence identified between −220 and −210 bp on the mouse, human, and rat Glc6Pase promoters (30, 31).

The effects of LCFA on the Glc6Pase promoter (−1640/+60 bp) activity were further analyzed in HeLa cells transiently expressing either HNF1α or HNF4α. In HNF4α-expressing HeLa cells, all PUFAs (e.g. C18:2(n-6), C18:3(n-3), C20:4(n-6), C20:5(n-3), and C22:6(n-3)) similarly suppressed the HNF4α-induced Glc6Pase promoter activity by about 50% (Fig. 4A). In contrast, saturated LCFA (C16:0 and C18:0) had no significant inhibitory effect (Fig. 4A). The C18:1(n-9) monounsaturated LCFA had a weak but significant inhibitory action on the Glc6Pase promoter activity (by about 25–30%) (Fig. 4A). In HNF1α-expressing HeLa cells, none among the saturated, monounsaturated, or polyunsaturated LCFA had any effect on the HNF1α-induced Glc6Pase promoter activity (Fig. 4A). Similar experiments were performed with Glc6Pase promoter constructs of variable lengths, which yielded comparable results (data not shown; see also Fig. 7B). As in HepG2 cells, the inhibition effect took place in a comparable manner in the presence of indomethacin or NDGA (Fig. 4B) and of Trolox (Fig. 4C). These results strongly suggested that PUFAs might suppress the Glc6Pase promoter activity via a specific modulatory action on the HNF4α-induced transactivation.

The amount of HNF4α expressed in the different cell models used above was quantitatively analyzed by Western blotting. Noteworthy, HNF4α was expressed in substantial amount in HepG2 cells (Fig. 5). In contrast, HNF4α was not detected in HeLa cells, whereas the amount expressed in HeLa cells transfected with pCR3-HNF4α was about 2–2.5 times that in HepG2 cells (Fig. 5). It could be noted that the HNF4α level found in HNF4α-transfected HeLa cells was in the same order range as that present in a rat liver extract analyzed in parallel (Fig. 5). Taken together, the data presented in Figs. 2, 4, and 5 were in
Fig. 6. Specific binding of HNF4α to the Glc6Pase promoter. A shows sequences of the probes used in gel shift mobility assays with putative HNF4α binding sites (boldface type, boxed). B, gel shift mobility assays were carried out without (lane 1) or with purified recombinant HNF4α protein expressed in bacteria (lanes 2–6) and double-stranded radiolabeled oligonucleotide probes matching the putative HNF4α-binding site 3 of the Glc6Pase promoter. Competition experiments were performed in the presence of 100 ng of the respective unlabeled Glc6Pase oligonucleotide (lane 3), of an oligonucleotide matching the high affinity HNF4α-binding site of the PEPCK promoter (lane 4), or of an irrelevant oligonucleotide containing a consensus HNF1-binding site (38) (lane 5). An anti-HNF4 antiserum was added in the binding reaction mixture in lane 6; an asterisk indicates the supershift. Specific DNA-protein complexes are indicated by arrowheads. NS, a nonspecific binding. C, gel shift mobility assay was carried out with purified recombinant HNF4α protein expressed in bacteria and a double-stranded radiolabeled oligonucleotide probe containing the sequence of the HNF4α-binding site of the PEPCK promoter. Competition experiments were performed in the presence of 100 or 500 ng of the unlabeled oligonucleotides matching the four putative HNF4α-binding sites of the Glc6Pase promoter.

The functional activity of these HNF4 binding sites in the Glc6Pase promoter was further characterized via deletion or site-directed mutagenesis as indicated in Fig. 7. The −694/+60E construct exhibited the same induction by HNF4α as that of the −730/+60E construct, indicating that the presence of the HNF4α site 4 was not essential for the HNF4α-induced transactivation of the Glc6Pase promoter. The mutation of the single half-site 1a on the −694/+60 fragment decreased the induction by HNF4α by 45%, and the same mutation on the minimal promoter fragment (−80/+60) resulted in a near total loss in the transactivation effect. In contrast, the mutation of the half-site 1b had no effect (Fig. 7A). These results strongly suggested that the site 1a is crucial for the HNF4α transactivation and for the basal promoter activity. The mutation of the site 3a on the −694/+60 fragment decreased the induction by HNF4α by 75% and the same mutation on the minimal promoter fragment resulted in a near total loss in the transactivation effect. In contrast, the mutation of site 3b had no substantial effect on the −694/+60 fragment or additional inhibition effect on the −694/+60 fragment mutated on site 1a (Fig. 7A). These data strongly suggested that the half-site 3a is crucial for the HNF4α-transactivation of the Glc6Pase promoter.

HN4α Sites 1 and 3 Both Confer Inhibition of Transactivation by PUFA—We next sought to determine whether the
HNF4α binding sites were responsible for the PUFA suppression of the Glc6Pase promoter activity. A significant 20–35% of inhibition by C20:4(n-6) of the promoter activity was observed for −730/+60, −694/+60, −694/+60mut3a and -3b, and −80/+60 fragments (i.e., all constructs having a functional site 1) (Fig. 7B). The activity of the construct having an inactivated mutated site 1 (mut1a) and a functional site 3 was also significantly suppressed by 10% by C20:4(n-6) treatment. In contrast, the inactivation of both sites 1 and 3 (−694/+60mut1a + mut3a) resulted in a complete loss of the suppression of promoter activity by C20:4(n-6) (Fig. 7B). These results strongly suggested that individually each of the two sites 1 and 3 alone was able to confer (and that both together were sufficient to fully account for) suppression of transcription of the Glc6Pase promoter by PUFA.

Polyunsaturated Fatty Acyl-CoAs Inhibit the Binding of HNF4α to Its Cognate Site—We further studied the effects of fatty acyl-CoA thioesters on the binding of HNF4α to site 3 (i.e., the binding site exhibiting the highest affinity in vitro) by gel shift mobility assay. The binding was markedly inhibited in a dose-dependent manner in the presence of oleoyl-CoA (C18:1(n-9)), linolenoyl-CoA (C18:3(n-3)), and arachidonoyl-CoA (C20:4(n-6)) (Fig. 8). A maximal inhibitory effect of about 60% was observed within a concentration range of 1–10 μM. Half-maximal inhibition was obtained at about 2 μM for arachidonoyl-CoA, 5 μM for linolenoyl-CoA, and 7 μM for oleoyl-CoA (Fig. 8B). In contrast, palmitoyl-CoA (C16:0) and stearoyl-CoA (C18:0) had no inhibitory effects on the binding of HNF4α to C18:0 binding within the same range of concentration, whereas a weak significant activation effect was obtained at 10 μM stearoyl-CoA (Fig. 8B). These data strongly suggested that the mechanism by which PUFA suppress the HNF4α-induced Glc6Pase promoter activity involves an inhibition of the binding of HNF4α to its cognate sites in the presence of polyunsaturated fatty acyl-CoA thioesters.

**DISCUSSION**

The results presented here constitute the first demonstration that LCFA are able to regulate the expression of the Glc6Pase gene at a transcriptional level. We report that PUFA, and to a lesser extent monounsaturated fatty acid (oleic acid), may exert specific suppressive effects on the Glc6Pase promoter activity with regard to saturated fatty acids. In addition, we elucidated the likely molecular mechanism of such an effect, strongly suggesting that it may be mediated via a control of the transactivation effect induced by a liver-specific transactivation factor (i.e., HNF4α). More specifically, we have shown 1) that HNF4α plays a crucial enhancer role in the Glc6Pase promoter activity, through the binding to two specific DNA cognate sites, and 2) that LCFA, via their intracellular metabolites (e.g., fatty acyl-CoA thioesters) are able to modulate the enhancing action of HNF4α, by means of a modulation of its DNA binding activity.

In regard to the original demonstration of the involvement of HNF4α in the regulation of the Glc6Pase promoter, it must be noted that 2 out of 4 putative HNF4α-binding sites predicted from homology with the consensus AGGTCA sequence (33) seem unlikely to have a key role in the HNF4α transactivation of the Glc6Pase promoter. Indeed, an oligonucleotide matching site 2 was unable to compete for the binding of HNF4α to its specific binding sequence of the PEPCK promoter in gel shift assay, and the deletion of site 4 from the −730/+60B construct had no significant effect on the transactivation induced by HNF4α (results of Fig. 6 and 7A). This is in keeping with the
observation that neither site 2 nor site 4 is an integral part of a direct repeat of the DR-1 type (see Fig. 6A). In contrast, both predicted sites 1 and 3 are an integral part of a classical DR-1 repeat, with higher homology with the AGGTCA consensus in the 3’ half-site (see sequence alignments in Table I). In agreement with a crucial role of these sites in the HNF4α transcription of the Glc6Pase promoter, the invalidation by mutation of each or both results in marked suppressions in the HNF4α-induced transcription of the −694/+60B and −80/+60B Glc6Pase promoter constructs. Interestingly, in both cases, the 3’-half sites (1a and 3a) of highest homology with the consensus appear to be the most crucial in the HNF4α transcription. It must be mentioned that HNF4α still transactivates, albeit weakly, the −694/+60B construct having sites 1 and 3 invalidated. This might suggest the presence of another uncovered HNF4α binding site within this promoter region. Another possibility, because HNF4α also transactivates the HNF1α gene, might be an indirect effect of HNF4α mediated by HNF1α on the Glc6Pase transcription. A functional HNF1α binding site has indeed been described in the −220/−210 bp region of the rat Glc6Pase promoter (30, 31).

Regarding to the regulation of Glc6Pase transcription by LCFA, it is of note that each from both sites 1 and 3 is able to confer the suppression of the HNF4α-induced transactivation by PUFA and that the invalidation of both sites results in a total loss of the PUFA-inhibitory effect. However, an intriguing observation has been that site 1 apparently has a prominent role compared with site 3 in transfection experiments, with regard to both the transactivation efficiency (about 50% of total transactivation effect of the −694/+60B construct for site 1 versus 25% for site 3) and to PUFA inhibition (about 20–35% for site 1 versus 10% for site 3; see Fig. 7). In contrast, site 3 exhibits a much better affinity in HNF4α binding in gel shift assays (see e.g. Figs. 6C and 8 for inhibition of the binding in the presence of PUFA-CoA). We have no definitive explanation for the latter. It seems likely that the putative involvement of HNF4α-transcriptional co-activators (e.g. PGC-1) (34) might play a key role in cells, whereas they are absent in in vitro assays.

Taken together, the results presented herein are in keeping with the molecular mechanism previously proposed by Hertz et al. (22) (i.e. that the LCFA regulatory effects are mediated via a modulation of the binding of HNF4α to its DNA cognate sites induced by their intracellular CoA-thioesters derivatives). More specifically, our results are consistent with the previous ones of Hertz et al. (22) in regard to two points: 1) we have found that PUFA inhibit the HNF4α-induced transactivation of the Glc6Pase promoter in cells, and 2) we have found that polyunsaturated fatty acyl-CoA thioesters inhibit the binding of HNF4α to its cognate sites in gel shift mobility assays. Our results are, however, somewhat in disagreement in regard to some other important points: 1) we have not found any enhancement of HNF4α-induced transactivation by palmitate in HeLa cells or of the binding of HNF4α to its DNA binding sites in the presence of C:16-CoA in the gel shift mobility assay; 2) we have not found that stearate and stearoyl-CoA had inhibitory effects similar to those of PUFA and PUFA-CoA, respectively; and 3) we have found that oleate and oleoyl-CoA can induce, at least under some conditions, suppressive effects similar to those of PUFA and PUFA-CoA (with a weaker efficiency, however). We have no definitive explanation for these discrepancies. That the experiments herein have been carried out with a natural gene promoter, while Hertz et al. studied a synthetic promoter could explain at least some of them. With the exception of the slight enhancing effect of stearoyl-CoA on the HNF4α binding in gel shift assay (see Fig. 8), which has been suggested to be possibly due to a detergent-like effect on HNF4α oligomerization (35), we have never found enhancer effects induced by any LCFA. Therefore, we agree with the
opinion of Sladek and co-workers (35) that LCFA-CoA thioesters cannot be considered as a classical ligand for HNF4α. There is no doubt, however, that HNF4α exhibits specific affinity binding sites for LCFA-CoA thioesters (22). Therefore, the consistent suppressive effects induced by PUFA in transfection experiments and PUFA-CoA in binding assays, respectively, led us to propose that PUFA-CoA may be considered as a possible physiological inhibitor of HNF4α-induced transactivation processes. Because some of the most potent inhibitory PUFA (e.g., arachidonic acid) are possible precursors of eicosanoids, it is of note that the experiments in the presence of various inhibitors of the cyclooxygenase and/or lipoxygenase pathways allowed us to definitively rule out the hypothesis that the further metabolism of PUFA via these pathways could be involved in the effects observed. It should also be mentioned that the expression of another liver gene, the 1-pyruvate kinase gene, has been reported to be inhibited by PUFA at the level of transcription (36). The molecular mechanism had not been understood in the latter study, but it is interesting to notice that the promoter region conferring the PUFA response overlaps a HNF4-binding sequence (36).

Hertz et al. (22) argue in their paper that dietary stearate (C18:0) and PUFA have been reported having effects 1) similar between them, on the one hand, and 2) opposed to those of dietary palmitate (C16:0), on the other hand, in some physiological processes such as blood coagulability or the level of blood lipids. Our findings that palmitate and stearate and their respective CoA-counterparts did not inhibit the HNF4α to its DNA cognate sites in vitro. 1) Saturated LCFA (C16:0 and C18:0) did not suppress HNF4α-induced transactivation, and their respective CoA-counterparts did not inhibit the HNF4α-binding.

2) The monounsaturated LCFA (C18:1(9-)) exhibited a weak suppressing effect on the HNF4α transactivation in HeLa cells, and C18:1-CoA had a significant inhibitory effect on the HNF4α-DNA binding only at the highest concentrations studied in gel shift mobility assays (see Fig. 8B). 3) PUFA markedly suppressed the HNF4α-transactivating effect on the Glc6Pase promoter, and their respective CoA-thioester derivatives inhibited the binding of HNF4α to its cognate DNA binding sites from the lowest concentrations studied. This suggests that the inhibitory action of LCFA-CoA could be at least in part dependent on either the number of double bonds in the carbon chain or on the conformational constraints dictated by these double bonds.

We have also taken in consideration the hypotheses that either PPARα or SREBP1c (see Introduction) could be involved in the PUFA modulation of Glc6Pase gene transcription. However, in none of our transfection experiments did clofibrate (0.5–500 μM) alter the Glc6Pase promoter activity (data not shown). In addition, there was no difference in the abundance of the Glc6Pase mRNA in the liver in PPARα null (−/−) mice (37) as compared with wild-type mice, whether the animals were fed with fenofibrate or not (data not shown). These data strongly suggested that PPARα might not be involved in the PUFA modulation of the Glc6Pase gene transcription. Furthermore, our preliminary results have shown that the overexpression of SREBP1c in HepG2 cells or HeLa cells did not alter the activity of the Glc6Pase promoter (data not shown). This allowed us to rule out the hypothesis that PUFA-induced suppression of the Glc6Pase promoter activity might be dependent on a decrease in the abundance of SREBP1c, as has been suggested, accounting for the PUFA-suppressive effects on the transcription of genes involved in fatty acid synthesis (19, 20).

In conclusion, we report here that PUFA are able to moderate the Glc6Pase gene transcription by means of a specific PUFA-CoA thioester-mediated inhibition of the binding of HNF4α to specific enhancer sites on the Glc6Pase promoter. These results further extend the previous ones of Hertz et al. (22). Because of the crucial importance of the Glc6Pase gene on the one hand and of the nutritional facts on the other hand in the context of hepatic insulin resistance and type 2 diabetes, the data presented herein may likely explain at least in part the beneficial effects of PUFA on insulin resistance at the level of endogenous glucose production.

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Polyunsaturated Fatty Acyl Coenzyme A Suppress the Glucose-6-phosphatase Promoter Activity by Modulating the DNA Binding of Hepatocyte Nuclear Factor 4 α

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Polyunsaturated fatty acyl coenzyme A suppress the glucose-6-phosphatase promoter activity by modulating the DNA binding of hepatocyte nuclear factor 4a.

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There is one error repeated several times in the text and in Fig. 7 on pages 15740–15742. In Fig. 7 in columns A and B, 3a should be 3b, 3b should be 3a, 1a+3a should be 1a+3b, and 1a+3b should be 1a+3a. There is no error in the inset of Fig. 7. In the text on pages 15740–15742, 3b should be substituted for 3a, and 3a should be changed to 3b. Hence, it appears that it is site 3b, such as it has been defined in Fig. 6A, which is more crucial than site 3a for the binding of HNF4 and the sensitivity to PUFA (whereas the contrary is published).

This might be of some importance because when the sequence of site 3 is analyzed, it is possible to find direct repeat 1 (DR1) sequences on both sense and antisense strands. Thus, in agreement with our discussion (page 15742) that the 3'-half sites generally appear of highest homology with the AGGTCA consensus (and also the most crucial in the HNF4 transactivation in this case), the DR1 sequence on the sense strand should constitute a better fit with the consensus for the binding of HNF4 dimers (see alternative Table I below).

### Table I

**Comparison of HNF4a-binding sequences**

In the consensus site, the consensus nucleotide(s) found in HNF4a-binding sequences is represented in capital letters; the lowercase letters point out divergences from the consensus that are represented at least three times in the analyzed sequences. The numbers in parentheses in the right column indicate references. AS, antisense strand.

| Sequence                  | Consensus Site | References |
|---------------------------|----------------|------------|
| L-PK                      | TCT TGGACT C TGGCCC CCA | (39)       |
| PEPCK (AS)                | CCA CGGCCA A AGGTCA TGA | (40)       |
| ApoCIII                   | CCG TGGSCA A AGGTCA CCT | (41)       |
| a1-AT (AS)                | ACA GGGGCT A AGGTCA CTG | (42)       |
| HGFIL                     | AGCC AGGTCT C AGGTCA GGG | (43)       |
| ANG region C (AS)         | CAG AGGGCA G AGGGCA GGG | (44)       |
| ANG region J (AS)         | GTC GGGGCA A AGGTTC CCA | (44)       |
| ApoAI                     | GCA GGGGTC A AGGTCT CAS | (45)       |
| ApoB                      | AAA GGGCCA A AGGGG CCT | (46)       |
| Bikunin box 7             | AGC AGTCAA A AGTCAA GTG | (47)       |
| Factor X                  | GCT GAGGCA A AGTCAA CCG | (48)       |
| Transhyretin TTR          | CCC TAGGCA A GGTCAA TAT | (49)       |
| HNF1                      | TGA AGTCAA A AGGTCA GTC | (50)       |
| WHV                       | AGG AGTCAA A AGGTCA TTA | (28)       |
| Glc6Pase site 1 (AS)      | CCG TGGCCC T GGTCC AGA |           |
| Glc6Pase site 3           | AAC TGACCC C AGTCC TCT |           |
| Consensus HNF4a-binding site | NNN GGGCCA A AGGTCA NNN |     |

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