Impact of L-FABP and glucose on polyunsaturated fatty acid induction of PPARα-regulated β-oxidative enzymes

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Submitted 23 August 2012; accepted in final form 1 December 2012

The liver is key in maintaining homeostasis of long-chain fatty acid (LCFA) and glucose metabolism (14, 105). Hepatic peroxisome proliferator activated receptor-α (PPARα) is the major transcription factor controlling genes directing LCFA and lipoprotein metabolism (17, 51, 92, 103). n-3 Polysaturated fatty acid [PUFA; e.g., eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)] agonists of PPARα lower triglycerides, are themselves poor substrates for hepatic triglyceride synthesis and have very few known drug interactions or adverse side effects (45–47, 90, 130). Most important, the n-3 PUFA agonists of PPARα were more effective in lowering triglycerides in hypertriglyceridemic diabetic subjects and subjects with more severe hypertriglyceridemia (where fasting blood glucose tends to be higher) than in all diabetic subjects, but much less in normolipidemic nondiabetic subjects (72, 90). However, little is known about the mechanisms whereby PUFAs are more effective in inducing PPARα in the context of high glucose.

Within hepatocyte nuclei, the nuclear receptor PPARα regulates transcription of genes in both glucose (gluconeogenesis) and LCFA (uptake, transport, oxidation) metabolism (14). Our and other laboratories have provided critical evidence demonstrating that unsaturated LCFA, especially PUFA, are endogenous, high-affinity PPARα ligands (37, 39, 53). Our laboratories recently resolved a new pathway of potential PPARα regulation whereby liver fatty acid binding protein (L-FABP) facilitates rapid PUFA uptake into nuclei and directly interacts with PPARα therein (35, 38, 69). Although these studies suggested that L-FABP might be involved in PUFA-mediated activation of PPARα, it is not clear whether 1) L-FABP is required for PUFA-mediated PPARα transcription of LCFA β-oxidative enzymes; 2) L-FABP contributes toward determining the PUFA specificity (i.e., n-3 vs. n-6) for activating PPARα; 3) PUFA redistribute L-FABP into nuclei; and 4) high glucose impacts L-FABP-mediated PPARα transcription of these enzymes in primary hepatocytes. The latter question is especially important in this regard since n-3 PUFA are so beneficial in hepatic PPARα activation of LCFA β-oxidative enzymes to lower triglycerides, especially in hyperlipidemic diabetic populations. The present investigation showed for the first time that not only PPARα, but also L-FABP, was important for facilitating PUFA-mediated PPARα transcription of LCFA β-oxidative enzymes, an effect highly exacerbated by high glucose.

MATERIALS AND METHODS

Materials. Arachidonic acid (AA, C20:4n-6), EPA (C20:5n-3), DHA (C22:6n-3), bovine serum albumin fraction V (fatty acid-free, 10% solution for tissue culture), bovine pancreatic insulin, sodium DL-lactate, D(+) glucose, D(+) maltose, rat tail collagen type I, and dexamethasone were from Sigma-Aldrich (St. Louis, MO). Collage-

THE SEVERAL FORMS OF DIABETES mellitus and associated complications are the sixth leading cause of death in the US, and incidence is rising (22, 101, 123, 132). Nearly 80% of people with diabetes die of cardiovascular disease (CVD), particularly coronary heart disease (20, 102). Thus benefits of any lipid intervention may be greatest in these individuals (97, 102, 114). Although dietary improvements, exercise, and statins have significantly decreased the incidence and severity of CVD, residual CVD risk remains especially high (62–82%) among individuals with diabetes mellitus, even with optimum statin therapy (102, 113). This difference is thought to be due primarily to a residual dyslipidemia in this population, particularly hypertriglyceridemia (113).
nase B was from Roche (Life Technologies, Carlsbad, CA) and MK886 was from Cayman Chemical (Ann Arbor, MI). Standard Williams medium E, custom-made glucose-free Williams medium E, Dulbecco’s modified Eagle’s medium (DMEM)/F12 (1:1), glucose-free DMEM, Hanks’ balanced salt solution free of calcium and magnesium (HBSS), gentamycin, and fetal bovine serum were from Gibco/Life Technologies. RNase-free DNase set and RNasey kit were from Qiagen (Hilden, Germany) and Qiagen Sciences (Germanytown, MD), respectively. TaqMan Gene Expression Assays for acyl coenzyme A oxidase 1 (ACOX1), carnitine palmitoyl transferase 1 A (CPT1A), and carnitine palmitoyl transferase 2 (CPT2) were purchased from Applied Biosystems (by Life Technologies), as were TaqMan and One-Step TR-PCR Master Mix reagents. TO-PRO-3 monomeric cyanine nucleic acid stain and SlowFade reagent were purchased from Life Technologies. Rabbit polyclonal antibody against liver fatty acid binding protein (L-FABP) was produced in our laboratory as described (5). Goat anti-rabbit IgG conjugated to FITC was from Sigma-Aldrich (St. Louis, MO). L-FABP immunogold electron microscopy imaging reagents were as described previously by our laboratory (38). Rabbit anti-CPT1, CPT2, ACOX1, β-actin primary antibodies, and goat horseradish peroxidase-conjugated antibody IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Pierce ECL Western blotting substrates were from Thermo Scientific (Rockford, IL). [9,10-3H]stearic acid (1 mCi/ml in EtOH) was from Amersham/GE Healthcare (Piscataway, NJ).

Animals

All mouse protocols were approved by the Texas A&M University Institutional Animal Care and Use Committee. Wild-type (WT) C57BL/6N mice were purchased from Charles River Laboratories (Wilmington, MA) obtained through the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). L-FABP gene-ablated (−/−, null) mice on a C57BL/10 background were bred in-house to a C57/10 background from N2 generation mice. PPARα-null >N10 backcross generation mice on C57BL/6N background were obtained from Dr. Frank Gonzalez (National Institutes of Health, Bethesda, MD). Cultured primary hepatocytes from WT C57BL/6N mice obtained from Charles River responded similarly as WT littermates from our L-FABP-null and PPARα-null mice that had been backcrossed for 10 generations to the same C57BL/6N background also obtained from Charles River. Mice were kept under constant 12:12 light-dark cycles and had access to control rodent chow and water ad libitum.

METHODS

Culture of primary mouse hepatocytes. Livers were collected from male mice 3–6 mo of age for primary hepatocyte isolation, culture, and viability testing as previously described by our laboratory (5, 117, 119). Mouse primary hepatocytes expressed key proteins, enzymes, and receptors involved in the metabolism of LCFA (L-FABP, SCP-2, FATP5, G0T, FATP2, FATP4) and glucose (GLUT2, GLUT1, glucokinase, insulin receptor) at levels similar to those in liver and maintained these levels constant for 2–3 days and ≥3 days, respectively, in culture as we described earlier (5, 117–119). Likewise, Western blotting showed that expression of nuclear receptors involved in LCFA and glucose metabolism (PPARα, PPARβ, LXR, ChREBP, SREBP) did not differ from that in liver for 2–3 days in culture (not shown), confirming findings of others (75). On the basis of the above findings, all experiments were conducted with mouse primary hepatocytes cultured for ≥2 days as follows.

Freshly isolated hepatocytes were plated on type I collagen-coated six-well (or 100-mm) tissue culture dishes (for RNA and protein) or two-well chamber glasses and slides (confocal imaging). The hepatocytes were then incubated overnight at 37°C, 5% CO2 with DMEM/F12 (1:1) medium supplemented with 10 mM HEPES pH 7.4, 0.1 mg/ml gentamycin sulfate and 5% fetal bovine serum. The next morning, the medium was removed and hepatocytes incubated for 1 h under conditions as previously described (81), except that the Williams’ medium E used was commercially custom prepared to be glucose free (Life Technologies). Following addition of glucose (6, 11, 20 or 30 mM) and either 40 μM fatty acid-free albumin alone (Alb) or in complex with 200 μM AA (C20:4n-6), EPA (C20:5n-3), or DHA (C22:6n-3) prepared as described earlier (112), the hepatocytes were incubated for an additional 5 h, medium was removed, and RNA was isolated (see Quantitative real-time PCR). The choice of glucose concentrations was based on those in mouse serum under a variety of physiological and pathological conditions: 5–6 mM as in normal mice after overnight fast; 9–11 mM as in transient normal (after a meal) or diabetic; 14–25 mM as in overnight-fasted high-fat diet-fed, ob/ob, or diabetic mice; and 35 mM postprandial as in severe uncontrolled diabetic (Jackson Laboratory mouse genome database, http://phenome.jax.org/). Culturing mouse primary hepatocytes with 200 μM PUFAs/40 μM albumin complexes for 5 h results in maximal PUFAs induction of PPARα-regulated gene transcription (81).

Quantitative real-time PCR. RNA was used to isolate total RNA from hepatocytes as per the manufacturer’s instructions followed by spectrophotometric quantitation. Relative level of mRNA expression for CPT1A, CPT2, and ACOX1 was determined by quantitative real-time PCR using TaqMan One-Step Master Mix and Gene Expression Assays for mouse CPT1A (mM 00550438_m1), CPT2 (Mm00487202_m1), ACOX1 (Mm00443579_m1), L-FABP (Mm00443440_m1), acyl-CoA binding protein (ACBP; Mm03048192_g1), and PPARα (Mm00440939_m1), HNF4α (Mm0433959_m1), and HNF1α (Mm04943434_m1), and 18S housekeeping gene. All experiments were performed in triplicate with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a thermal protocol of 48°C for 30 min, 95°C for 10 min before the first cycle, 95°C for 15 s, and 60°C for 60 s, repeated 40 times. Data were analyzed with ABI PRISM 7000 SDS software (Applied Biosystems) to determine ΔCt for each well of a 96-well plate, relative to a positive control 18S housekeeping gene. Unless otherwise indicated, relative abundance of each transcript was calculated as described earlier (5, 70).

Western blotting. Hepatocytes were cultured similarly as above for mRNA quantitation except that hepatocytes were incubated for longer time (24 h) to allow changes in mRNAs to be translated into altered protein levels. Hepatocyte protein was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) followed by gel electrophoresis, transfer to nitrocellulose, and Western blotting with antibodies to CPT2, ACOX1, or β-actin (internal control) as described earlier (5, 70).

Determination of L-FABP distribution to hepatocyte nuclei by confocal microscopy. Redistribution of L-FABP from cytoplasm to nucleus in response to PUFA was determined by confocal imaging of cultured primary hepatocytes from WT mice labeled with anti-L-FABP and a vital nucleic acid. Briefly, hepatocytes were incubated with 6 or 20 mM glucose plus albumin/PUFA complexes as above for mRNA determination except that incubation times were from 0.5 to 24 h. At each time point, the medium was removed, hepatocytes were washed three times with HBSS, and hepatocytes were fixed with 3.3% paraformaldehyde in HBSS (Electron Microscopy Sciences, Hatfield, PA) for 1 h at room temperature (RT). Excess paraformaldehyde was then removed by washing with HBSS and neutralization with ammonium chloride (50 mM NH4Cl for 15 min at RT). Nonspecific protein binding was blocked by incubating the fixed hepatocytes with 5% fetal bovine serum in HBSS for 30 min at room temperature. Hepa-
Hepatocytes were then immunolabeled for 1 h with primary rabbit anti-L-FABP polyclonal serum, followed by an additional 1 h with secondary goat anti-rabbit IgG conjugated to FITC. For fixed hepatocytes immunolabeled only with anti-L-FABP, the hepatocytes were then stained for 30 min with 1 μM of TO-PRO-3 monomeric cyanine nucleic acid stain (Life Technologies), extensively washed with HBSS, air dried, and stabilized with SlowFade reagent from Molecular Probes. A MRC 1024 Bio-Rad laser scanning confocal microscopy (LSCM) system (Carl Zeiss MicroImaging, Thornwood, NY) equipped with krypton/argon laser was then used to simultaneously excite FITC-labeled L-FABP and the nuclear stain TO-PRO-3 at 488 and 647 nm, respectively. Emission was then detected through two separate photomultipliers. These photomultipliers were equipped with 540/30 (for FITC) and 680/32 (for TO-PRO-3) emission filters. ImageJ (NCBI website) was then used to analyze the simultaneously acquired confocal images to obtain fluorescence intensities of nuclei and cytoplasmic areas of hepatocytes (average of fluorescence intensity per surface unit) and therefrom the nuclear vs. cytoplasmic ratios.

Intracellular glucose concentration. Hepatocytes isolated and cultured as described above were washed three times with PBS, followed by incubation as indicated previously (81) for 1 h at 37°C, 5% CO₂ in glucose-free DMEM [instead of the Williams medium E] to which was added 6, 20, or 30 mM glucose. Medium was then removed and hepatocytes were washed with ice-cold 100 mM MgCl₂ containing 0.1 mM phloretin as an inhibitor of glucose transport (131). Hepatocytes were then scraped from the dishes with PBS and protease inhibitor at 4°C followed by disruption with a probe sonicator (Sonic Dismembrator 550, Fisher Scientific, Waltham, MA) at 4°C. After centrifugation at 10,000 g, 4°C, for 20 min, the supernatant and a standard were added 6, 20, or 30 mM, Medium was then removed and hepatocytes were washed with ice-cold 100 mM MgCl₂ containing 0.1 mM phloretin as an inhibitor of glucose transport (131). Hepatocytes were then scraped from the dishes with PBS and protease inhibitor at 4°C followed by disruption with a probe sonicator (Sonic Dismembrator 550, Fisher Scientific, Waltham, MA) at 4°C. After centrifugation at 10,000 g, 4°C, for 20 min, the supernatant and a standard curve of glucose were used for quantitating glucose analysis with an Amplitite Glucose Quantitation Kit (AAT Bioquest Sunnyvale, CA) as described earlier (5).

De novo fatty acid synthesis from [¹⁴C]glucose. Glucose-free DMEM/F12 containing fatty acid-free albumin (40 μM), 6 or 20 mM glucose, and tracer amounts of [¹⁴C]glucose at the same specific activity, was prepared for culturing mouse primary hepatocytes for 0 h in serum-free medium containing glucose (6, 11, 14 mM glucose concentration within each treatment group.

RESULTS

PUFAs Induced PPARα Transcription of Key Mitochondrial and Peroxisomal Genes Rate Limiting in Fatty Acid β-Oxidation in Mouse Primary Hepatocytes Cultured with Normal Physiological Glucose

Most prior studies of PPARα activation in cultured primary hepatocytes were performed with media containing high (11–30 mM) glucose levels, much higher than normal (6 mM) physiological glucose (5, 50, 58, 70, 75, 81, 93). Therefore, it was important to first determine the extent to which PUFAs regulate PPARα transcriptional activity in the context of normal (6 mM) physiological glucose. Transcription of CPT1A, CPT2, and ACOX1 genes was examined because they are key indicators of fatty acid β-oxidation that are regulated by PPARα, but not by SREBP1 or ChREBP (reviewed in Ref. 47).

When mouse primary hepatocytes were cultured with 6 mM glucose plus PUFA, transcription of CPT1A, CPT2, and ACOX1 was only modestly upregulated compared with fatty acid-free albumin without ligand. As shown by the black bars in Fig. 1A, both n-6 (AA) and n-3 (EPA, DHA) PUFAs increased CPT1A transcription by 2.6-, 1.9-, and 1.5-fold, respectively, in WT cultured primary mouse hepatocytes. These PUFAs also induced, but less so, transcription of CPT2 (black bars Fig. 1B), 2.1-, 1.3-, and 1.3-fold, respectively, as well as transcription of ACOX1 (black bars, Fig. 1C), 2.3-, 2.0-,
Impact of Very High (30 mM) Glucose on PPARα level in the culture medium. were not due to increased osmolality due to elevated sugar being more effective (at 11–20 mM glucose). These effects were dependent on the presence of PUFA. Incubating hepatocytes with (6 mM glucose not taken (71), as an osmotic control. Culturing hepatocytes in media containing 11–20 mM glucose at a constant osmolality independent of sugar uptake. This was accomplished by using maltose, a disaccharide sugar impact of a change in osmolality independent of sugar uptake. Since high (20 mM) glucose not only increases intracellular glucose level in hepatocytes but also increases the osmolality of the culture medium, it was also important to determine the impact of a change in osmolality independent of sugar uptake. This was accomplished by using maltose, a disaccharide sugar not taken (71), as an osmotic control. Culturing hepatocytes with (6 mM glucose + 14 mM maltose) did not potentiate PUFA induction of CPT1A (Fig. 1A, gray bar), CPT2 (Fig. 1B, gray bar), or ACOX1 (Fig. 1C, gray bar). In the case of ACOX1, the osmotic control maltose (6 mM glucose + 14 mM maltose) actually decreased PUFA induction (Fig. 1C, gray bars). The basis for this decrease is not known. Thus high glucose not only potentiated PUFA-mediated transcription of LCFA β-oxidative enzymes but also shifted the order of PUFA-mediated transcription of CPT1A, CPT2, and ACOX1 from the n-6 PUFA (AA) being slightly more effective (at 6 mM glucose) toward the n-3 PUFA (DHA, EPA) being more effective (at 11–20 mM glucose). These effects were not due to increased osmolality due to elevated sugar level in the culture medium.

Impact of Very High (30 mM) Glucose on PPARα Transcription of Genes in LCFA β-Oxidations (CPT1A, CPT2, ACOX1) in the Absence of PUFA

The above effects of high glucose in the 11–20 mM range on PUFA-mediated transcription of CPT1A, CPT2, and ACOX1 were dependent on the presence of PUFA. Incubating hepatocytes in media containing 11–20 mM glucose at a constant level of lipid-free albumin (i.e., no exogenous lipidic ligand) did not significantly alter CPT1A (Fig. 1A), CPT2 (Fig. 1B), or ACOX1 (Fig. 1C) mRNA levels. In contrast, at the highest glucose concentration tested (Fig. 1, 30 mM glucose, finely cross-hatched bars), potentiation of PUFA-mediated transcription of CPT1A, CPT2, and ACOX1 was dependent not only on the presence of PUFA but also on the 30 mM glucose directly enhancing transcription even in the absence of PUFA. Incubating WT hepatocytes with 30 mM glucose plus AA, EPA, or DHA stimulated CPT1A expression up to 9.1-, 16.3-, and 17.4-fold, respectively, i.e., a 3.5-, 8.6-, and 11.6-fold potentiation compared with 6 mM glucose. However, even in the absence of PUFA the very high 30 mM glucose directly stimulated CPT1A, CPT2, and ACOX1 by 6.1-, 3.6-, and 4.5-fold, respectively (Fig. 1, Alb; finely cross-hatched bar).

In summary, high (11–20 mM) glucose itself did not activate transcription of LCFA β-oxidative enzymes, but it nevertheless potentiated PUFA-mediated transcription of LCFA β-oxidative enzymes. In contrast, very high (30 mM) glucose in the absence of PUFA itself activated transcription of LCFA β-oxidative enzymes as well as potentiated PUFA-mediated transcription of LCFA β-oxidative enzymes. The direct effect of 30 mM glucose alone may be due to the limited capacity of hepatocytes to store excess glucose as glycogen such that excess glucose is metabolized to acetyl-CoAs that are utilized for de novo LCFA synthesis, ~7% of which are mono- and disaturated (C18:1n-9 and C18:2n-6) that much more weakly activate hepatic PPARα than the PUFA (37, 39, 48, 53, 81, 95).

PUFA-Mediated PPARα Transcription of Proteins Involved in LCFA, LCFA-CoA Binding/Transport (L-FABP and ACBP), and PPARα Itself, in the Context of Normal Physiological and High Glucose

Transcription of both L-FABP and ACBP is regulated by PPARα (27, 58). High glucose (20 mM) enhanced n-6 AA (Fig. 2A) and n-3 EPA (Fig. 2A) mediated transcription of L-FABP. Similarly, PUFA-mediated transcription of ACBP was enhanced in the presence of high glucose (Fig. 2B).

The promoter regions of PPARα as well as two other nuclear receptors in LCFA metabolism, HNF4α and HNF1α, contain DRI response elements (91, 121). Both PPARα (37, 39, 53, 78, 126) and HNF4α (31–33, 84, 85, 121) have high affinity for LCFA/LCFA-CoA. Studies with gene-ablated mice indicate that both PPARα and HNF4α induce hepatic transcription of L-FABP (2, 29, 30). Furthermore, HNF4α in turn is induced by HNF1α (59). HNF1α may also induce L-FABP through a different response element (1, 34, 67). Therefore, it was important to determine the impact of PUFA and high glucose on transcription of PPARα itself as well as transcription of HNF4α and HNF1α in WT cultured primary mouse hepatocytes. At normal physiological 6 mM glucose, transcription of PPARα itself was modestly induced by both n-6 AA and n-3 EPA (Fig. 2C). In contrast, neither n-6 AA nor n-3 PUFA (EPA and DHA) stimulated transcription of HNF4α (Fig. 2D) or HNF1α (Fig. 2E). In fact, the n-6 AA decreased transcription of HNF4α (Fig. 2D) and both n-3 EPA and n-3 DHA decreased transcription of HNF1α (Fig. 2E) at 6 mM glucose. High (20 mM) glucose potentiated both AA- and EPA-mediated transcription of PPARα itself (Fig. 3A). In contrast, neither the n-6 AA, nor the n-3 fatty acids (EPA and DHA) stimulated transcription of HNF4α (Fig. 2D) or HNF1α (Fig. 2E). In fact, n-3 EPA and n-3 DHA inhibited transcription of HNF4α at high glucose (Fig. 2D).

Thus, whereas high glucose potentiated PUFA-mediated transcription of L-FABP, ACBP, and PPARα, high glucose and/or EPA or DHA inhibited transcription of HNF4α or HNF1α. Since both HNF4α and HNF1α as well as PPARα induce transcription of L-FABP, these findings suggest that the reduced expression of HNF4α and HNF1α may actually counteract in part the upregulation of PPARα by PUFA at high glucose.
Impact of L-FABP Gene Ablation on PUFA-Mediated PPARα Transcription of LCFA β-Oxidative Genes in Mouse Primary Hepatocytes Cultured with Normal (6 mM) Physiological Glucose

To determine whether the glucose potentiation of PUFA-mediated transcriptional activity was specifically dependent on expression of L-FABP, the above experiments were repeated in primary mouse hepatocytes from L-FABP-null mice. Hepatocytes were cultured in medium containing increasing glucose and either albumin only (basal) or albumin complexed with AA (C20:4 n-6), EPA (C20:5 n-3), or DHA (C22:6 n-3) as described in MATERIALS AND METHODS.

At 6 mM glucose, L-FABP gene ablation significantly impacted PUFA-mediated transcriptional activity. Loss of L-FABP abolished AA and EPA induction of CPT1 mRNA (Fig. 3A), DHA induction of CPT2 mRNA (Fig. 3B), as well as EPA and DHA induction of ACOX1 mRNA (Fig. 3C). L-FABP ablation abolished or slightly inhibited the ability of AA and EPA to induce transcription of ACBP (Fig. 3D) and PPARα itself (Fig. 3E). Most significantly, L-FABP gene ablation almost completely abolished the ability of high (11–30 mM) glucose to potentiate PUFA-mediated transcription of CPT1A, CPT2, ACOX1, ACBP, and PPARα. However, high (11–30 mM) glucose slightly increased the ability of only EPA to induce transcription of PPARα back to basal levels at albumin only (Fig. 3E).

Taken together these findings indicated that L-FABP gene ablation reduced the ability of PUFA to induce transcription of multiple PPARα-regulated genes in LCFA metabolism and completely abolished the potentiation by high (11–30 mM) glucose. Finally, L-FABP gene ablation also abolished the ability of very high (30 mM) glucose to induce transcription of PPARα-regulated genes in LCFA β-oxidation in the absence of PUFA.
Second, the impact of the PPARα inhibitor MK886 (129) on the ability of high glucose to potentiate PUFA-mediated PPARα transcription of CPT1A, CPT2, and ACOX1 was examined in cultured primary hepatocytes from WT mice. Again, to facilitate comparisons, the basal (albumin plus MK886) levels of CPT1A, CPT2, and ACOX1 mRNA at physiological (6 mM) glucose were set as 1 (Fig. 5, A–C). Treatment with MK886 abolished the ability of 1) AA and EPA to induce PPARα transcription of CPT1A, CPT2, and ACOX1 at 6 mM glucose (Fig. 5, A, C, and E; AA, EPA) and 2) high glucose to potentiate AA- and EPA-mediated PPARα transcription of CPT1A, CPT2, and ACOX1 (Fig. 5, A, C, and E; AA, EPA).

Taken together, these data indicate that high glucose potentiation of PUFA (especially EPA and DHA) mediated transcription of LCFA β-oxidative enzymes in cultured primary mouse hepatocytes is primarily PPARα dependent.

Impact of High Glucose on Protein Levels of LCFA β-Oxidative Enzymes Induced by PUFA and High Glucose in Cultured Primary Mouse Hepatocytes

Western blot analysis of CPT2 and ACOX1 protein levels was performed to determine the effects of albumin (40 μM) complexed with PUFA (200 μM) compared with albumin only as negative control, in the presence of physiologically normal (6 mM) and high (20 mM) glucose (Fig. 6). Western blot bands for CPT2, ACOX1, and β-actin, as loading control, are shown side by side (Fig. 6A), demonstrating an increase in the expression level of these proteins upon treatment with PUFA, especially with EPA and DHA at both 6 and 20 mM glucose. Quantitative analysis of the Western blots and plotting of CPT2/β-actin optical density for multiple bands for each treatment (Fig. 6B) indicated that AA, EPA, and DHA increased CPT2 protein level by 1.4-, 2.3-, and 5.6-fold, respectively, at 6 mM glucose. High glucose-potentiated AA, EPA, and DHA increased CPT2 protein even more to 1.9-, 3.8-, and 9.1-fold, respectively, at 20 mM glucose.

Whereas AA only mildly increased ACOX1 protein by 1.4-fold at 6 mM glucose, EPA and DHA increased ACOX1 protein by 2.2- and 7.8-fold, respectively, at 6 mM glucose (Fig. 6C). High glucose did not potentiate AA induction of ACOX1 protein level but potentiated that mediated by both EPA and DHA to 2.4- and 12-fold, respectively, at 20 mM glucose.

Attempts to detect and measure CPT1A by Western blot analysis of cultured primary hepatocytes incubated with high glucose and PUFA were unsuccessful. This was consistent with CPT1A mRNA being much lower than those of CPT2 and ACOX1 as well as with earlier reports that CPT1A protein could only be detected by Western blotting of isolated mitochondria (16).

Thus the n-3 PUFA (EPA, DHA) had greater enhancing effect than n-6 PUFA (AA) at 6 mM and even more so at 20 mM glucose with regard to CPT2 and ACOX1 protein expression in WT mouse hepatocytes.

Impact of High Glucose and PUFAs on LCFA β-Oxidation in Cultured Primary Mouse Hepatocytes

The ability of high glucose together with n-3 PUFA to stimulate LCFA β-oxidation was determined in WT hepatocytes incubated with media containing 6 or 20 mM glucose.

Fig. 3. L-FABP gene ablation markedly reduced high glucose potentiation of PUFA-mediated PPARα transcription of genes in LCFA β-oxidation, transport (ACBP), and PPARα. As indicated by the vertical bars from left to right, L-FABP-null hepatocytes were cultured for 6 h in serum-free medium containing glucose (6, 11, 20 or 30 mM) supplemented with either fatty acid-free Alb (40 μM) or Alb complexed with 200 μM AA, EPA, or DHA as described in MATERIALS AND METHODS. Total RNA was isolated and relative fold change in the expression level of PPARα-regulated genes was determined by quantitative PCR: CPT1A mRNA (A), CPT2 mRNA (B), ACOX1 mRNA (C), ACBP mRNA (D), and PPARα mRNA (E). Values for each genotype were expressed relative to Alb + 6 mM glucose within that genotype. Values are means ± SE, n = 3–4. *P < 0.05 vs. Alb alone at the same glucose concentration; #P < 0.05 vs. 6 mM glucose concentration within each treatment group.
EPA/BSA for 24 h. This assured induction not only of mRNA but also of protein expression of LCFA-oxidative enzymes as described in the preceding section. The cells were then washed and further incubated for additional 24 h with medium containing 6 or 20 mM glucose, 200 μM stearic acid/BSA, and a trace amount of [3H]stearic acid as described in MATERIALS AND METHODS.

The ability of n-3 EPA to enhance oxidation of stearic acid was highly dependent on glucose concentration in the medium. At physiological glucose, n-3 EPA only weakly enhanced oxidation of stearic acid by 9 ± 1% (Table 1). In contrast, high glucose the ability of n-3 EPA to stimulate oxidation of stearic acid was increased greater than threefold to 28 ± 1% (Table 1). In contrast, high glucose did not potentiate n-3 EPA induction of stearic acid oxidation in L-FABP-null hepatocytes (not shown).

Thus these data correlated with n-3 EPA potentiation of mRNA and protein levels of LCFA-oxidative enzymes in WT, but not L-FABP-null, hepatocytes as shown in the preceding sections.

**Distribution of L-FABP in nucleus and cytoplasm of cultured primary mouse hepatocytes.** Subcellular fractionation, immunofluorescence confocal, and immunogold electron microscopy established that L-FABP was distributed to both the nucleus and slightly more so to cytoplasm of rat and mouse hepatocytes (9, 38). To show whether L-FABP’s distribution was stable over time, hepatocytes were fixed, dual labeled with fluorescent antibody to L-FABP (Fig. 7, green pixels) and nuclear dye (Fig. 7, red pixels), and imaged through separate photomultipliers as described in MATERIALS AND METHODS. L-FABP in nuclei was shown by superposition of these images with only pixels of L-FABP colocalized with nuclei (Fig. 7, yellow pixels).

L-FABP was distributed in both cytoplasm and nucleus of mouse hepatocytes cultured at physiological 6 mM glucose.

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**Fig. 4.** PPARα gene ablation markedly reduced both PUFA-mediated PPARα transcription of fatty acid β-oxidative genes as well as potentiation by high glucose. Primary hepatocytes from WT (A, C, and E) and PPARα-knockout (PPARα-KO; B, D, and F) mice were isolated and cultured with serum-free medium containing 11 or 30 mM glucose plus 40 μM fatty acid-free Alb or Alb complexed with 200 μM of AA, EPA, or DHA as described in MATERIALS AND METHODS. CPT1A mRNA (A and B), CPT2 mRNA (C and D), and ACOX1 mRNA (E and F) levels were then measured relative to an internal housekeeping gene control as described in MATERIALS AND METHODS. Values for each genotype were then expressed relative to Alb + 6 mM glucose treatment within that genotype. Values are means ± SE, n = 3–4. *P < 0.05 vs. Alb at the same glucose concentration; #P < 0.05 for 30 mM vs. 11 mM glucose within each treatment group (i.e., Alb, AA, EPA, or DHA).

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**Fig. 5.** PPARα inhibitor MK886 prevented stimulation of fatty acid β-oxidation gene transcription by high glucose in WT mouse hepatocytes. CPT1A (A), CPT2 (B), and ACOX1 (C) mRNA levels were measured in hepatocytes cultured with serum-free medium containing glucose (6 or 20 mM) and Alb (40 μM) or Alb complexed with 200 μM of AA or EPA. Values are means ± SE, n = 3–4. *P < 0.05 vs. Alb at the same glucose concentration. #P < 0.05 for 20 mM vs. 6 mM glucose within each lipid treatment group (i.e., Alb, AA, EPA).
L-FABP AND GLUCOSE POTENTIATE PUFA-MEDIATED PPARα TRANSCRIPTION

Fig. 6. Western blots for CPT2 and ACOX1: effect of PUFA and high glucose. Hepatocytes from WT L-FABP (+/+) mice were treated with Alb only (Alb, 40 μM) as negative control and Alb in complex with PUFA (AA, EPA, DHA, 200 μM) in medium containing either 6 or 20 mM glucose for 24 h followed by Western blotting (see MATERIALS AND METHODS). A: Adobe Photoshop (Adobe Systems, San Jose, CA) and CorelDraw X5 (Corel, Ottawa, ON, Canada) were used to crop and compile the cropped images from Western blots for CPT2, ACOX1, and β-actin (as loading control). B: relative quantitative determination of CPT2 protein level vs. treatments as determined by use of Scion Image. C: quantitative analysis of ACOX1 protein level as a function of treatments; values are means ± SE, n = 4. *P < 0.05 vs. Alb alone at the same glucose concentration; †P < 0.05 vs. Alb at 6 mM glucose. ‡P < 0.05 vs. Alb/EPA at 6 mM glucose.

Table 1. Impact of high glucose on ability of n-3 PUFA to induce LCFA β-oxidation in cultured primary hepatocytes

<table>
<thead>
<tr>
<th>BSA/Complex</th>
<th>Glucose, mM</th>
<th>BSA/Complex</th>
<th>Glucose</th>
<th>Stearic Acid Oxidation, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>6</td>
<td>BSA</td>
<td>6</td>
<td>680 ± 13</td>
</tr>
<tr>
<td>BSA/n-3 EPA</td>
<td>6</td>
<td>BSA/steearic acid</td>
<td>6</td>
<td>744 ± 7*</td>
</tr>
<tr>
<td>BSA</td>
<td>20</td>
<td>BSA/steearic acid</td>
<td>20</td>
<td>1,015 ± 9†</td>
</tr>
<tr>
<td>BSA/n-3 EPA</td>
<td>20</td>
<td>BSA/steearic acid</td>
<td>20</td>
<td>1,299 ± 11‡</td>
</tr>
</tbody>
</table>

Primary hepatocytes from wild-type mice were incubated for 24 h with 6 or 20 mM glucose in medium containing either BSA or BSA/200 μM n-3 eicosapentaenoic acid (EPA) as described in legend to Fig. 8. To determine the ability these hepatocytes to β-oxidize stearic acid, the hepatocytes were then further incubated for 24 h with 6 or 20 mM glucose in medium containing BSA/200 μM stearic acid plus trace quantity of [1-14C]stearic acid as in MATERIALS AND METHODS. PUFA, polyunsaturated fatty acids; LCFA, long-chain fatty acid. Values are means ± SE, n = 6. *P < 0.05 vs. BSA at same glucose concentration; †P < 0.05 vs. BSA at 6 mM glucose. ‡P < 0.05 vs. BSA/EPA at 6 mM glucose.
redistribution of L-FABP quantitatively represents a significant shift in L-FABP protein mass appearing in hepatocyte nuclei. However, PUFA induced redistribution of L-FABP to nuclei did not appear potentiated by high glucose.

### Increasing Glucose Concentration in the Culture Medium

**Increased Cytosolic Glucose Levels in Cultured Primary Mouse Hepatocytes**

Cytosolic glucose concentration was near 3 mM in mouse primary hepatocytes cultured with medium containing normal physiological glucose, i.e., 6 mM (Table 2). Increasing glucose level in the culture medium to 20 or 30 mM resulted in cytosolic glucose increasing in parallel to 12 and 17 mM, respectively (Table 2). Taken together, these data indicated that cytosolic glucose concentration in cultured primary hepatocytes was 40–50% of that in the culture medium and increased proportionally with increasing extracellular glucose. A similar pattern of increasing intracellular glucose at higher extracellular glucose was observed by use of noninvasive glucose nanosensors for real-time imaging of intracellular glucose levels in cultured hepatic cells (19). Since noninvasive imaging of glucose nanosensors in cultured cells showed that nucleoplasmic and cytoplasmic glucose concentrations are similar in cultured cells (18), this suggested that glucose levels in cultured mouse primary hepatocyte nuclei would also be in a mM range similar to cytoplasmic glucose level therein.

### Impact of High Glucose on De Novo LCFA Synthesis from Cultured Primary Mouse Hepatocytes

Cultured primary mouse hepatocytes were incubated for 6 h with medium containing 6 or 20 mM glucose plus tracer amounts of \[^3H\]glucose (at same specific activity), followed by lipid analysis and isotope incorporation as described in MATERIALS AND METHODS. The total mass of LCFA as well as triglyceride remained constant regardless of whether the hepatocytes were incubated for 6 h with normal physiological (6 mM) or high (20 mM) glucose (Table 3, top section). This was despite the fact that \[^3H\]glucose-derived radiolabel rapidly (within 3 min) appeared not only in the nonesterified fatty acid fraction, but also in equal or greater amount in the triglycerides (Table 3). After 6 h incubation with 6 mM glucose, \[^3H\]glucose-derived radiolabel remained constant in the nonesterified fatty acid fraction while significantly increasing in the triglycerides (Table 3). Incubation with high glucose for 6 h increased the appearance of
Thus, although incubation of cultured primary hepatocytes for 6 h with high glucose increased de novo synthesis of [3H]LCFA from [3H]glucose, the amount was small, did not increase as [3H]triglyceride, and did not alter the total mass of LCFA or triglycerides.

**DISCUSSION**

In humans, the adverse effects of chronic hyperglycemia are exacerbated by high dietary fat, especially by saturated LCFA (79, 123). Conversely, dietary n-3 PUFAs (C20:5n-3; C22:6n-3) as in fish oil are positively linked to decreased serum triglycerides and cholesterol concomitant with decreased incidence of heart disease, diabetes, and obesity (116). Dietary n-3 PUFAs, but much less saturated LCFA, impact hepatic gene expression and lipid metabolism by binding and activating PPARα transcription of LCFA oxidative enzymes (reviewed in Refs. 37, 39, 109). Conversely, by controlling the nuclear abundance of ChREBP and SREBP1, the dietary n-3 PUFAs inhibit transcription of ChREBP- and SREBP1-regulated genes involved in glucose metabolism and de novo LCFA synthesis (reviewed in Ref. 47). Dietary n-3 PUFA agonists of PPARα lower glucose-induced hypertriglyceridemia and improve lipoprotein patterns in normoglycemic or well-controlled diabetic individuals (45–47, 90, 130). Paradoxically, some reports in rodents (57, 106) as well as human subjects (49, 72, 90, 111)

![Fig. 8. PUFA enhanced nuclear distribution of L-FABP in cultured mouse hepatocytes, as demonstrated by quantitative analysis of confocal images. WT mouse primary hepatocytes were isolated, cultured, and processed for confocal microscopy as described in MATERIALS AND METHODS. Graphs represent analysis of images of hepatocytes cultured in 6 mM (A) and 20 mM (B) glucose. The L-FABP fluorescence intensity per unit surface area was measured within as well as outside the nucleus area, as determined by the nuclear stain, and the ratio of nuclear L-FABP to cytoplasmic L-FABP was determined. Values are means ± SE, n = 40 cells. *P < 0.05 vs. Alb control at the same time point; #P < 0.05 vs. the identical lipid treatment at 0.5 h.](http://ajpgi.physiology.org/)

Table 2. **Intracellular glucose level is proportional to extracellular concentration**

<table>
<thead>
<tr>
<th>Extracellular Glucose, mM</th>
<th>Cytosolic Glucose, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2.56 ± 0.17</td>
</tr>
<tr>
<td>20</td>
<td>12.45 ± 1.34</td>
</tr>
<tr>
<td>30</td>
<td>16.83 ± 0.68</td>
</tr>
</tbody>
</table>

Wild-type mouse primary hepatocytes were cultured with medium containing 40 μM fatty acid-free albumin and either 6 or 20 mM glucose for 1 h as described in MATERIALS AND METHODS. Culture medium and hepatocytes were then collected and cytosolic glucose level was determined as described in MATERIALS AND METHODS.

![Table 3. **High glucose concentration effect on de novo synthesis of lipids from [3H]glucose**](http://ajpgi.physiology.org/)

<table>
<thead>
<tr>
<th>Lipid Class, nmol/mg protein</th>
<th>Glucose (mM)</th>
<th>3 min</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonesterified fatty acid</td>
<td>6</td>
<td>54 ± 2</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>Nonesterified fatty acid</td>
<td>20</td>
<td>44 ± 3</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>6</td>
<td>103 ± 4</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>20</td>
<td>100 ± 4</td>
<td>96 ± 4</td>
</tr>
</tbody>
</table>

[3H]Glucose incorporation into fatty acid and triglyceride, nmol/mg protein

<table>
<thead>
<tr>
<th>Lipid Class, nmol/mg protein</th>
<th>Glucose (mM)</th>
<th>3 min</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonesterified Fatty Acid</td>
<td>6</td>
<td>1.90 ± 0.27</td>
<td>2.59 ± 0.06</td>
</tr>
<tr>
<td>Nonesterified Fatty Acid</td>
<td>20</td>
<td>1.94 ± 0.07</td>
<td>5.96 ± 0.46**</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>6</td>
<td>1.47 ± 0.02</td>
<td>6.22 ± 0.37**</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>20</td>
<td>1.37 ± 0.50</td>
<td>5.02 ± 0.04*</td>
</tr>
</tbody>
</table>

Cultured primary hepatocytes from wild-type mice were incubated with medium containing 40 μM fatty acid-free albumin, either 6 or 20 mM glucose, and tracer amounts of [1-3H]-glucose at the same specific activity as described in MATERIALS AND METHODS. Culture medium and hepatocytes were then collected and processed as described in MATERIALS AND METHODS. Culture medium and hepatocytes were then collected and processed as described in MATERIALS AND METHODS. Values are means ± SE, n = 4, P < 0.02. *Significant difference at 6 h vs. 3-min time point. **Significance for 20 mM vs. 6 mM glucose.
suggest that n-3 PUFAs may be more effective in lowering serum triglycerides in the presence of high glucose. Nearly 30% of diabetic subjects are poorly compliant and have significantly elevated glucose (13, 100, 107). Despite these studies, however, almost nothing is known regarding the molecular basis for this conundrum whereby high glucose both induces hypertriglyceridemia but at the same time makes n-3 PUFAs more effective in lowering hypertriglyceridemia when glucose remains elevated. Our laboratories recently elucidated a new L-FABP lipophilic ligand signaling to PPARα in the nucleus (109). L-FABP has a high affinity for PPARα ligands such as n-3 PUFA (69, 78, 126); L-FABP enters nuclei and directly binds PPARα; and L-FABP/PPARα interaction is enhanced by high glucose (35, 38, 69). Taken together, these findings suggest a potential mechanism(s) whereby high glucose may impact L-FABP-mediated n-3 PUFA induction of PPARα in the nucleus. The results presented herein with cultured primary mouse hepatocytes from WT and L-FABP-null hepatocytes provide the following new insights into the roles of L-FABP and high glucose in regulating n-3 PUFA-induced PPARα transcription of LCFA β-oxidative genes.

First, in hepatocytes cultured with normal physiological glucose (i.e., 6 mM) the n-3 PUFA (EPA, DHA), only modestly induced PPARα transcription of LCFA β-oxidative enzymes (CPT1, CPT2, ACOX1), LCFA/LCFA-CoA binding/transport proteins (L-FABP, ACBP), or PPARα itself. PUFA induction of PPARα transcription correlated with significant L-FABP redistribution to hepatocyte nuclei (shown herein) and with uptake/redistribution of fluorescent L-FABP analogs to nuclei of cultured cells (69). Furthermore, at normal physiological glucose (6 mM) the n-3 PUFA were slightly weaker than n-6 PUFA in inducing PPARα transcription despite the fact that n-3 PUFAs exert more beneficial triglyceride-lowering actions mediated through PPARα (8, 43, 44, 55, 73, 74, 80, 88, 110, 116). Thus, although the n-3 PUFA enhance L-FABP distribution to the nucleus at physiological normal 6 mM glucose, under these conditions the n-3 PUFA only weakly induce transcription of PPARα-regulated genes.

Second, high glucose (11–30 mM) dramatically potentiated the n-3 PUFA (EPA, DHA)-induced PPARα transcription of LCFA β-oxidative enzymes (CPT1, CPT2, ACOX1), LCFA/LCFA-CoA binding/transport proteins (L-FABP, ACBP), or PPARα itself. In addition, high glucose altered the order of effectiveness to n-3 PUFA (EPA, DHA) > n-6 PUFA (AA). Increased mRNA levels of PPARα-regulated genes such as CPT2 and ACOX1 correlated with increased levels of CPT2 and ACOX1 proteins as well as with increased LCFA β-oxidative activity. Increased transcription of CPT1A, CPT2, and ACOX1 mRNAs is known to correlate with protein translation and fatty acid oxidative activity in rat and mouse cultured primary hepatocytes (27, 58). It is important to note that in the context of high glucose the DHA was more effective than the other PUFAs in inducing PPARα transcription of ACOX1 in mouse primary hepatocytes, a finding consistent with an earlier study of primary rat hepatocytes cultured with high glucose (58). Finally, high glucose did not potentiate PUFA-mediated PPARα transcription by further increasing L-FABP distribution to nuclei beyond that elicited by PUFA at physiological glucose. Previously the hypolipidemic effect of PUFAs was attributed primarily to enhanced transcription of LCFA β-oxidative enzymes, inhibition of fatty acid and TG synthesis via SREBP1 and ChREBP, suppression of the glycolytic enzyme L-pyruvate kinase through ChREBP/MLX transcription factors, and decreased use of glucose for lipid synthesis by enhancing its storage into glycogen (reviewed in Ref. 47). The data presented herein for the first time showed that PUFA are much more effective at stimulating PPARα transcription in the context of high glucose. This finding may contribute to our understanding of why the n-3 PUFA agonists of PPARα are more effective in hypertriglyceridemic diabetic subjects and subjects with more severe hypertriglyceridemia (where fasting blood glucose tends to be higher) than in all diabetic subjects (72, 90).

Third, high glucose potentiation of n-3 PUFA induced transcription of PPARα-regulated genes (e.g., CPT1, CPT2, ACOX1, ACBP, PPARα) required an intact L-FABP lipophilic ligand signaling pathway to PPARα. L-FABP gene ablation almost completely abolished potentiation of PUFA-mediated PPARα transcriptional activity by high glucose. L-FABP gene ablation also abolished the ability of incubation with the very highest concentration (30 mM) to activate PPARα transcription even in the absence of PUFA. Likewise, PPARα ablation or inhibition (MK886) impaired the ability of high glucose to potentiate n-3 PUFA-mediated PPARα transcription of LCFA β-oxidative enzymes. High glucose potentiation of PUFA-induced PPARα transcriptional activity was not due to increased de novo fatty acid synthesis from glucose, but instead correlated with increased cytosolic glucose concentration. Taken together these findings suggested that high glucose potentiation of PUFA-induced PPARα transcriptional activity was also not due to indirect effects of glucose on other signaling pathways.

Fourth, very high glucose (30 mM) alone activated PPARα transcription of LCFA β-oxidative enzymes, but less so than in the presence of exogenous PUFA. Although the mechanism(s) whereby high glucose alone activated PPARα transcriptional activity is not completely clear, ablation of either L-FABP or PPARα clearly abolished the effect. Thus this effect of high glucose alone was also mediated through the L-FABP/PPARα pathway rather than indirectly through other signaling pathways impacted by glucose. The direct effect of very high (30 mM) glucose alone on PPARα transcriptional activity may be attributed at least in part to de novo synthesis of fatty acids from glucose. Since liver has limited capacity to store excess glucose as glycogen, the excess is metabolized to acetyl-CoAs that are utilized for de novo LCFA synthesis. Such de novo synthesized LCFA are >93% saturated (C14:0, C16:0, C18:0) with the remainder <7% primarily mono- and disaturated (C18:1n-9 and C18:2n-6) (95). Whereas the saturated LCFA are not bound by and do not activate PPARα, the mono- and diunsaturated are bound by and do not activate PPARα than the PUFA (37, 39, 48, 53, 81). Although high glucose did not increase the total mass of LCFA or triglyceride, incorporation of [3H]glucose into the LCFA (but not triglyceride) pool was significantly increased. Although at 20 mM glucose this pool of [3H]glucose-derived LCFA (i.e., mono- or diunsaturated LCFA) did not appear large enough to induce PPARα in the absence of PUFA, at 30 mM glucose this pool may have been sufficiently large to induce PPARα in the absence of PUFA. Consistent with this possibility, inhibition of fatty acid synthase by C75 prevented high glucose induction of PPARα (not shown), as did fatty acid synthase gene ablation.
Similarly, sumoylation transcriptionally represses PPAR activity (25, 26) suggest that very high 30 mM glucose potentiation of PUFA-mediated PPARα transcriptional activity occurred through the L-FABP/PPARα pathway rather than through indirect effects of glucose on other signaling pathways. Increasing data from our and other laboratories favor a fourfold process. First, L-FABP enhances PUFA uptake (69), consistent with L-FABP's high cytoplasmic concentration (2–5% of cytosolic protein) and high affinity for PUFAs (78, 94, 126). Second, L-FABP cotransports bound PUFA into nuclei. L-FABP targets PUFA and LCFA to the nucleus as shown in vitro with purified nuclei and in living cells overexpressing L-FABP or hepatocytes null in L-FABP (41, 42, 52, 69, 70). Concomitantly, the PUFA enhance L-FABP distribution to nuclei (Fig. 7). Third, L-FABP directly interacts with PPARα for PUFA transfer by direct channeling of L-FABP-bound PUFA to PPARα for induction of transcriptional activity (35, 38, 109, 122, 127). Finally, high glucose potentiates this process by interacting with both L-FABP and PPARα (35, 36, 40, 115) but especially by increasing L-FABP/PPARα binding affinity (35). Cytoplasmic glucose levels in hepatocytes (Table 2) and liver (p. 59, Ref. 28; Refs. 24 and 125) are normally in the 3–4 mM range but increase proportionally to extracellular glucose (Table 2). Since nucleoplasmic glucose levels are similar to those in cytoplasm (18), high extracellular glucose increases nucleoplasmic glucose levels to a level sufficient for enhancing L-FABP/PPARα binding (35). This increased L-FABP/PPARα binding affinity in turn would facilitate molecular channeling of bound PUFA from L-FABP to PPARα for induction of transcriptional activity (35, 38, 109, 122, 127).

Potential roles for other mechanism(s) such as posttranslational modification of L-FABP by phosphorylation, ubiquitinylation, and sumoylation in glucose potentiation of PPARα transcriptional activity are unlikely since L-FABP is not known to undergo posttranslational modification by these mechanisms (76, 108). Glucose potentiation of PPARα transcriptional activity by such posttranslational modification of PPARα are also unlikely. Although increased serum insulin enhances PPARα phosphorylation and transcriptional activity (15), in the present studies the level of insulin was not increased in the culture medium. Furthermore, ubiquitinylation targets PPARα for degradation and high dietary glucose increased rather than decreased PPARα mRNA and protein in mice (not shown). Similarly, sumoylation transcriptionally represses PPARα (89), but high glucose potentiated rather than inhibited PPARα transcriptional activity in the present studies. Other than at the highest level studied (30 mM), the high glucose alone without exogenous PUFA ligand did not induce PPARα transcription, suggesting that potential posttranslational modifications of L-FABP or PPARα alone in response to high glucose were not likely to account for the potentiation of PUFA-mediated PPARα transcriptional activity.

With regards to the physiological implications of these results, the human L-FABP T94A polymorphism is the most prevalent mutation in the FABP protein family, occurring at high frequency of 26–38% (8+2% homozygous) in multiple populations (minor allele frequency for 1,000 genomes in NCBI dbSNP database; ALFRED database; Refs. 11, 21, 60, 82, 99, 124, 128). The phenotype of L-FABP-null mice exhibits significant similarities to that of humans carrying the L-FABP T94A variant: 1) ablation of L-FABP reduced VLCn-3 PUFA and LCFA uptake (5, 69, 70, 118), a phenotype shared by “Chang liver” cells overexpressing human L-FABP T94A variant vs. WT L-FABP overexpressors (23); 2) serum triglyceride was increased in L-FABP-null mice (3, 4, 61, 64, 65, 77), a phenotype shared by humans with the L-FABP T94A variant (10, 21); 3) synthetic (fibrate) and natural (branched-chain LCFA) peroxisome proliferators were less effective in lowering serum and hepatic triglycerides in L-FABP-null mice (6, 7). Low levels of clofibrate and/or dietary phytol decreased serum and hepatic triglyceride in WT but not L-FABP-null mice (6, 7). This phenotype of L-FABP-null mice was similar to that of fenofibrate-treated subjects expressing the L-FABP T94A variant. To date, however, there have been no reports examining the impact of high glucose on the ability of dietary n-3 PUFA to mitigate these phenotypes in either L-FABP-null mice or human subjects expressing the L-FABP T94A variant.

In summary, the present work studied the effect of glucose and L-FABP on the expression of PPARα target genes, when PPARα was activated by PUFA agonists. n-3 PUFA-mediated PPARα transcription of genes in LCFA β-oxidation (CPT1A, CPT2, ACOX1), LCFA/LCFA-CoA binding/transport (L-FABP, ACBP), and PPARα itself was potentiated when the concentration of glucose in the culture medium exceeded 6 mM in WT hepatocytes. These effects were abrogated by L-FABP gene ablation, PPARα gene ablation, or treatment of WT hepatocytes with MK886 (PPARα antagonist). Thus PUFAs may be more effective in inducing transcription of PPARα-regulated genes, especially in the fatty acid β-oxidation pathway, in the context of high glucose. The potential role of L-FABP in lowering serum triglyceride levels is underscored by studies in humans reporting a L-FABP T94A polymorphism that occurs with high frequency, 32–37% of (10–13% homozygous) in all populations studied to date (11, 21, 99, 124, 128). The T94A substitution raises serum LDL cholesterol and triglyceride, traits associated with increased risk of cardiovascular disease and Type 2 diabetes (10, 21), and decreases LCFA uptake in cultured cells (23). Likewise, L-FABP-null mice exhibited increased age-dependent obesity, elevated serum triglyceride, and decreased hepatic L-FABP uptake, exacerbated by high-fat diet (3, 4, 62, 63, 66). Finally, the findings presented herein may help to explain why dietary n-3 PUFAs (C20:5n-3; C22:6n-3) are even more positively linked to decreased serum triglycerides, serum cholesterol, and incidence of heart disease in hypertriglyceridemic diabetic subjects and subjects with more severe hypertriglyceridemia (where fasting blood glucose tends to be higher) than in hypertriglyceridemic, normoglycemic individuals, but with little effect on serum triglycerides in normolipidemic, normoglycemic subjects (72, 90, 116). Nearly 30% of diabetic subjects are poorly compliant and have significantly elevated glucose (13, 100, 107). It is noteworthy that PPARα activation by natural/endogenous fatty acid ligands is...
not thought to be as likely to lead to toxicity compared with synthetic ligands (e.g., bezafibrate) used for the treatment of conditions such as dyslipidemias and diabetes (83). In general, PPARs exhibits weaker affinities for these synthetic ligands, and thus pharmaceutical levels of such drugs may more likely produce toxicity via off-target and/or receptor-mediated mechanisms that are not always well understood (83). Taken together, these findings show for the first time that the n-3 PUFA-mediated PPARs activation of hepatic fatty acid β-oxidative genes is significantly impacted by an intact L-FABP lipidic ligand signaling pathway to PPARs, an effect even more prominent in the context of high glucose. Potentiation of these effects at high glucose may contribute significantly to the hypolipidemic effects of n-3 PUFA-rich fish oil in dyslipidemic insulin-resistant or the nearly 30% of poorly compliant diabetic subjects in whom glucose remains elevated (13, 45, 90, 96–98, 100, 107, 113, 114, 120).

REFERENCES


30. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepato- 

toxic nuclear factor 4α (nuclear receptor 2A1) is essential for mainte- 

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L-FABP and Glucose Potentiate PUFA-Mediated PPARα Transcription


78. Norris AW, Spector AA.

79. Parks EJ, Skokan LE, Timlin MT, Dingfelder CS.

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83. Peng XE, Wu YL, Lu QQ, Ju ZJ, Lin X.

84. Parks EJ, Skokan LE, Timlin MT, Dingfelder CS.

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