Dietary Polyunsaturated Fatty Acids Interfere with the Insulin/Glucose Activation of L-Type Pyruvate Kinase Gene Transcription

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L-type pyruvate kinase (L-PK) is a key glycolytic enzyme regulating the flux of metabolites through the pyruvate-phosphoenolpyruvate cycle (1). The regulation of L-PK is complex involving both hormones and nutrients. We have found that feeding rats diets containing polyunsaturated fatty acids (PUFA) significantly inhibits hepatic pyruvate kinase enzyme activity (~60%) and suppresses mRNAPK abundance (~70%). Studies with primary hepatocytes indicate that PUFA act directly on hepatocytes. Specifically, arachidonic (20:4, ω6) and eicosapentaenoic (20:5, ω3) acid suppressed both mRNAPK levels and the activity of a transfected PKCAT (-4300/+12) fusion gene by ~70%. This is due to an inhibition of the insulin/glucose-mediated transactivation of L-PKCAT. Deletion analysis localized PUFA-regulated cis-acting elements to a region within the L-PK proximal promoter, i.e. between -197 and -96 base pairs. This region binds two transcription factors involved in the hormone/nutrient regulation of L-PK gene transcription, i.e. a major late transcription factor-like factor and HNF-4. Linker scanning mutation analysis localized the PUFA-regulated cis-acting elements to the vicinity of the HNF-4 binding site. Thus, PUFA-regulated factors abrogate the insulin/glucose activation of L-PK gene transcription by targeting the HNF-4 elements. These studies suggest that PUFA may have significant effects on hepatic carbohydrate metabolism by inhibiting the L-PK side of the pyruvate-phosphoenolpyruvate cycle. (Molecular Endocrinology 8: 1147–1153, 1994)

INTRODUCTION

L-type pyruvate kinase (L-PK) is a key glycolytic enzyme regulating the flux of metabolites through the pyruvate-phosphoenolpyruvate cycle (1). The activity of the enzyme is subject to acute control by covalent modification and allosteric effectors. Chronic control is achieved by regulating PK gene transcription and mRNAPK abundance. The regulation of PK gene expression is complex and involves both hormones and dietary factors (2–4). While hormones elevating hepatic cAMP levels inhibit PK gene expression, insulin and dietary carbohydrate augment PK gene transcription. The cis-linked targets for both the negative cAMP and positive insulin/glucose control of L-PK gene transcription have been localized to a 87-base pair (bp) region upstream from the PK gene, i.e. between -163 and -96 bp (5–13). We recently reported that dietary polyunsaturated fatty acids (PUFA) rapidly suppress mRNAPK along with other hepatic mRNAs encoding both glycolytic and lipogenic enzymes (proteins), including fatty acid synthase (FAS), the S14 protein, malic enzyme, and glucokinase (14). The transcription of both hepatic FAS and S14 genes was rapidly inhibited by dietary PUFA (14–17). Transfection analysis of primary hepatocytes using S14CAT fusion genes revealed the presence of PUFA-regulated cis-acting elements within the proximal promoter region (-220 to -80 bp) of the S14 gene (16).

Because mRNAPK was subject to the same temporal suppression by PUFA as FAS and S14, we were interested in determining whether PK was also transcriptionally regulated by PUFA. In this report we have examined the effects of PUFA on hepatic L-PK enzyme activity and mRNAPK in vivo and have used transfection of primary hepatocytes with PKCAT fusion genes to...
locate PUFA-regulated cis-linked elements within the PK promoter. Our results suggest that PUFA target components are involved in the insulin/glucose regulation of L-PK gene transcription.

RESULTS

Effects of Dietary Fatty Acids on L-PK Activity and mRNAPK Abundance

Rats were meal fed high glucose diets supplemented with 10% dietary fat (Fig. 1). The level of PK enzymatic activity in olive oil-fed rats (960.5 ± 108.4 IU/liver) compared favorably with L-PK levels reported for rats fed a high carbohydrate-low protein diet (18). Substituting fish (menhaden) oil or its derivatives for olive oil led to a greater than 60% decline in PK activity. Both 20:5,ω3 and 22:6,ω3 were as effective as menhaden oil at suppressing L-PK activity indicating that the ω3-fatty acids in menhaden oil probably account for the inhibitory effect on PK activity. The decline in PK activity paralleled the fall in mRNAPK indicating that fatty acids suppressed L-PK activity at the pretranslational level.

Effect of Oleic (18:1,ω9), Arachidonic (20:4,ω6), and Eicosapentaenoic Acid (20:5,ω3) on mRNAPK Abundance and PKCAT Activity in Primary Hepatocytes

In vivo studies showed that rats fed diets enriched in ω3 fatty acids led to a decline in both L-PK enzyme activity and mRNAPK. We previously reported that both ω3- and ω6-fatty acids acted directly on primary hepatocytes to suppress mRNAPK activity (14). To determine whether the mechanism of PUFA action was at the level of L-PK gene transcription, primary hepatocytes were transfected with a PKCAT fusion gene (Fig. 2). The PKCAT fusion gene contained sequences extending from −4300 to +12 bp relative to the 5’-end of the L-PK gene (9).

Primary hepatocytes were treated with insulin and 25 mM glucose to maximally stimulate L-PK gene transcription (6, 9, 11). To evaluate the effects of fatty acids, albumin-bound fatty acids were added at 300 μM. One group of cells was assayed for mRNAPK while a second group was transfected with PKCAT and assayed for chloramphenicol acetyltransferase (CAT) activity. When compared to albumin-treated cells, the level of mRNAPK in hepatocytes treated with 18:1, 20:4, and 20:5 fell by 35, 77, and 85%, respectively. This pattern of inhibition of PK gene expression is comparable to our previous findings (14). PKCAT activity showed a corresponding 36%, 68%, and 85% decline after treatment with 18:1, 20:4, and 20:5, respectively (Fig. 2). Thus the decline in mRNAPK by 20:4 and 20:5 is due to an inhibition of L-PK gene transcription.

While the dose of fatty acid used in these studies was 300 μM, dose-response analysis indicated that the ED50 for both 20:4 and 20:5 was ≤ 80 μM (not shown), a value similar to the effect of these fatty acids on S14 gene transcription (16). The effect of 20:4 and 20:5 on hepatocyte mRNAPK or PKCAT activity was essentially identical (not shown). Moreover, the effects of PUFA

Fig. 1. Effect of Dietary Fats on Hepatic L-PK Enzyme Activity and Gene Expression

Five groups of rats were meal fed a HICO diet containing 10% olive oil for 10 days. One group was maintained on olive oil for an additional 5 days while the other groups were switched to a HICO plus 10% ω3 fatty acid diet for 5 days: olive oil (OO), fish oil (FO), fish oil esters (FOE), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA). Rats received food from 0900 h until 1200 h. The rats were killed at 1700 h on the 8th day of treatment. Livers were removed for analysis of L-PK mRNA abundance (solid bars), n = 4 and total L-PK enzyme activity (open bars), n = 3. Results are expressed as percent of the olive oil treatment, mean ± SE.

Fig. 2. Effect of Oleic (18:1,ω9), Arachidonic (20:4,ω6), and Eicosapentaenoic (20:5,ω3) Acid on mRNAPK Abundance and PKCAT Activity in Primary Hepatocytes

Primary hepatocytes were treated with 50 μM albumin (ALB) and either oleic acid (18:1) or arachidonic acid (20:4) or eicosapentaenoic acid (20:5) at 300 μM. Cells were harvested after 48 h of treatment for analysis of mRNAPK levels (open bars). Cells transfected with PKCAT-4.3 (~−4300)/+12 were harvested after a similar 48 h treatment and assayed for CAT activity (solid bars). Results are expressed as percent of the albumin-treated group, Mean ± SE; n = 3.
were not due to generalized detergent or toxic effects because mRNAs encoding β-actin, thyroid hormone receptor β1, tyrosine aminotransferase, and c/EBPα were not consistently affected by these treatments. Moreover, RSV CAT activity was insensitive to fatty acid treatment (14, 16).

**PUFA Block the Insulin/Glucose Transactivation of L-PK CAT**

We next examined the effect of fatty acids on the insulin/glucose activation of L-PK gene transcription (Fig. 3). Hepatocytes transfected with PK CAT and maintained in media containing 1 μM insulin and 10 mM lactate expressed low CAT activity (208 ± 42 U). However, replacing lactate with glucose (25 mM) promoted a 6-fold increase in PK CAT activity, a level of induction comparable to previous reports (9). Addition of 18:1 to the glucose-treated cells did not significantly impair the glucose-mediated activation of PK CAT. However, addition of 20:4 fully abrogated the glucose-mediated activation of PK CAT. Similar results were obtained with 20:5 (not shown). Thus, PUFA interfered with the insulin/glucose-mediated induction of L-PK CAT gene transcription.

**Localization of PUFA Response Elements (PUFA-REs) by Deletion Analysis**

The foregoing studies indicated that PUFA-regulated cis-acting elements (PUFA-RE) were present within 4.3 kilobases (kb) of the L-PK gene. The PUFA-RE were localized by deletion analysis (Fig. 4). When compared to 18:1 treated hepatocytes, all deletion constructs extending from -4300 to -197 bp were inhibited by more than 60% after 20:4 treatment. Similar effects were seen when 20:5 was substituted for 20:4 (not shown). In contrast, CAT activity in cells transfected with PK CAT-0.096 was not consistently affected by either 20:4 or 20:5 treatment. This pattern of control suggests that the region between -197 to -96 bp relative to the 5'-end of the PK gene contains PUFA-sensitive cis-acting elements.

**Linker Scanning Mutagenesis of the PK Proximal Promoter**

The region between -197 and -96 bp upstream from the L-PK gene has been reported to bind several transcription factors including a major late transcription factor (MLTF)-like factor, HNF-4 (LF-A1), and NF-1 (5, 8, 10, 11, 13). The transcription factors, MLTF-like and HNF-4, have been implicated in the insulin/glucose activation of L-PK gene transcription (10, 11). To determine which factors might be involved in the PUFA suppression of PK gene transcription we employed linker scanning mutants of the L-PK promoter region from -197 to -96 bp. Each mutation contained a cluster of six to eight bases changed to an unrelated sequence (Fig. 5). Hepatocytes that did not receive fatty acid treatment (the albumin control) displayed a loss of CAT activity.
Fig. 5. Linker Scanning Mutagenesis of the PUFA-Responsive Region of the L-PK Gene

Linker scanning mutations in the −183 to −97 bp region of the L-PK gene proximal promoter were described previously (11). The location of the mutations is shown at the bottom of the figure. Transfected primary hepatocytes were maintained in 25 mM glucose media containing only albumin or supplemented with either 18:1ω9 or 20:4ω6 at 300 μM for 48 h. Top panel, PKCAT activity (units) for hepatocytes treated with 50 μM albumin only. These results are representative of three separate studies with three samples per group. Mean ± SD; n = 3. Bottom panel, Results are expressed as the percent inhibition of PKCAT activity by arachidonic acid (20:4ω6). These results represent the mean of three separate studies with three samples per group. Mean ± SD; n = 9.

with mutation in regions designated as LS-2, LS-3, and LS-4 (Fig. 5, upper panel). This pattern is identical to previous reports and spans the elements recognized by MLTF-like and HNF-4 transcription factors (11). Treatment of hepatocytes with 20:4 led to ≥ 60% inhibition of PKCAT activity in cells transfected with LS-1, LS-2, LS-3, LS-6, and LS-7. In contrast, PKCAT activity in hepatocytes transfected with LS-4 and LS-5 was only marginally affected by 20:4 treatment, i.e. 20 and 15% inhibition, respectively. This pattern of control suggests that the PUFA-RE do not map to the MLTF-like element, but to the vicinity of elements recognized by HNF-4.

DISCUSSION

L-Type pyruvate kinase (L-PK) is a target for dietary PUFA inhibition in vivo and in cultured primary hepatocytes. The in vivo studies reported here extend our previous observations (14) by showing that PUFA suppress both the activity of the enzyme as well as the corresponding mRNA, i.e. mRNAPK. Moreover, PUFA override the stimulatory effects of insulin and glucose to inhibit L-PK gene transcription. Interestingly, the PUFA-regulated factors target one of two elements involved in the insulin/glucose transactivation of the L-PK gene (Fig. 6).

The insulin/glucose regulated cis-acting elements upstream from the L-PK gene binds two factors, an MLTF-like factor and HNF-4 (2, 10, 11). While the precise identity of the MLTF-like factor remains to be determined, the current view is that transcription factors binding at the −168 to −145 bp region are closely related to members of the myc-family of nuclear transacting factors (10, 11, 20). HNF-4 is structurally related to nuclear receptors in the steroid/thyroid supergene
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Fig. 6. Organization of the Carbohydrate and PUFA-Regulated cis-Acting Elements within the L-PK Promoter

The L-PK gene is flanked by several response elements binding specific transcription factors, i.e. TATA, HNF-4, NFI, HNF-1, and MLTF-like (2, 10, 11). Both the MLTF-like factors and HNF-4 bind as dimers (20, 21). The insulin/glucose response elements (CHO-response region) include the MLTF-like factors (at -168 to -145 bp) and HNF-4 (at -145 to -126 bp), while the PUFA-regulated factors target elements within the vicinity of the HNF-4 binding site, i.e. the PUFA-response region.

family and binds at -145 to -126 bp (2, 10, 11). HNF-4 functions in hepatic-specific gene transcription (21). When these elements were tested separately for hormonal/nutrient control, only the MLTF-like elements conferred insulin/glucose control to the PK promoter (10, 11). The MLTF-like element also represents the target for negative control by cAMP/A-kinase regulated pathway (10). HNF-4 alone confers neither positive control by insulin/glucose or negative control by cAMP/A-kinase. Thus HNF-4 plays an ancillary role in the hormonal/nutrient regulation of L-PK gene transcription. In the context of the L-PK promoter, the MLTF-like factor cooperates with the contiguous HNF-4 to promote both the transcriptional activation by insulin/glucose and repression of transcription by cAMP/A-kinase (10, 22). By targeting the HNF-4 side of this complex, PUFA-regulated factors presumably interfere with the role HNF-4 plays in the insulin/glucose-mediated activation of the L-PK gene.

If PUFA-mediated inhibition of L-PK gene transcription simply represented an abrogation of insulin or glucose action or an activation of a cAMP/A-kinase regulatory pathway, we might expect the PUFA-RE to map to the MLTF-like elements of the L-PK gene. By finding the HNF-4 side of the complex targeted by PUFA-regulated factors suggest that PUFA do not have generalized effects on specific signaling pathways, e.g. insulin receptor tyrosine kinase activity, but rather target specific genes through effects on tissue-specific elements associated with those genes.

HNF-4 has been implicated in the metabolic control of both the tyrosine aminotransferase (23) and phosphoenolpyruvate carboxykinase genes (24). However, we have found neither gene to be consistently affected by PUFA (14, 16). It is not clear from our studies whether HNF-4, per se, is regulated by PUFA (or metabolites) or whether the HNF-4 motif is targeted by distinct PUFA-regulated transcription factors. The HNF-4 motif may also bind related transcription factors, such as chicken ovalbumin upstream promoter-transcription factor (COUP-TF), Apo AI regulatory protein (ARP-1), peroxisome proliferator activated receptor (PPAR), and retinoid X receptor (25-27). Such factors might be regulated by PUFA and compete for HNF-4 binding leading to inhibition of PK gene transcription. Gel shift analysis has not revealed quantitative or qualitative differences in specific DNA-protein interaction within the -145 to -126 bp (HNF-4) region (not shown). This is not unexpected if the PUFA-regulated transacting factor is a low abundance protein [e.g. a ligand-activated receptor like PPAR, peroxisome proliferator-activated receptor (27)]. We are currently evaluating the role factors such as COUP-TF, PPAR, ARP-1, and retinoid X receptor play in the PUFA-mediated control of L-PK gene transcription.

The S14 gene is also activated by insulin/glucose through an MLTF-like element located at -1440 bp (28). While PUFA also inhibit S14 gene transcription, the PUFA-RE were not found in the vicinity of this MLTF element, but within the S14 proximal promoter region between -220 and -80 bp (16). PUFA were found to attenuate the T3-mediated transactivation of the S14 gene. There are two regions upstream from the S14 gene that are important for T3-mediated transactivation: 1) the S14 thyroid hormone response elements (TRE) are located between -2.9 and -2.5 (29, 30); and 2) elements within the promoter between -120 and -80 bp (B-Region). This region binds tissue-specific factors that function to potentiate T3 activation of the S14 gene transcription (16). PUFA did not have generalized effects on either the S14 TREs or a canonical TRE (DR-4: AGGTCAAnnnnAGGTCA) when linked to a heterologous promoter. Because the PUFA-REs were located just upstream from the B-region, we speculated that PUFA-regulated factors interfered with the role the B-region factors played in T3-mediated transactivation of the S14 gene (16).

The analysis of PUFA control of S14 and PK gene transcription indicates that PUFA-regulated factors do not have generalized effects on endocrine regulatory pathways, but rather are promoter (gene) specific. Examination of the PUFA-responsive regions of these genes will be presented in future communications.
genes shows a region with 80% sequence identity, i.e. S14: -128 GGACACTGGCGACCA -114; L-PK: -142 GGACTCTGCGCCCA -128. For the S14 gene this region is located immediately upstream from the element that potentiates T3 transactivation of the S14 gene, i.e. B-region at -80 to -120 bp. For the L-PK gene this region is located within the putative HNF-4 binding site (-145 to -126 bp). The role this element plays in PUFAMediated control of the S14 and PK gene is currently under investigation.

Finally, L-PK plays a key role in hepatic carbohydrate metabolism. Elevation of L-PK activity is associated with enhanced hepatic glucose utilization, while inhibition is associated with gluconeogenesis (1). These studies suggest that PUFA may have significant effects on hepatic carbohydrate metabolism by inhibiting the L-PK side of the pyruvate-phosphoenolpyruvate cycle. Dietary fat has been reported to have a significant impact on hepatic glucose output and extrahepatic glucose utilization leading to a decline in L-PK enzymatic activity might explain, at least in part, the increased hepatic glucose output seen in rodents and humans fed high fat diets (32, 33).

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats (125-150 g) were obtained from Charles River Breeding Laboratories (Kalamazoo, MI). Animals were housed in a University facility, and all experimental protocols were approved by the All University Animal Use Committee at Michigan State University. A high carbohydrate, fat-free diet (HiCHO, 38% carbohydrates) was obtained from ICN (Cleveland, OH). Rats (one per cage) were trained to a meal-feeding regimen, i.e. access to food from 0900 to 1200 h (14, 16). Body weights and food intakes were measured daily. Rats were first trained to a meal-feeding protocol by feeding a high glucose diet supplemented with 10% olive oil plus 0.1% butylated hydroxytoluene. After 10 days of adaptation, the rats were then switched to a diet containing 10% fish oil or its fatty acid constituents. The fish oils tested included: 1) menhaden oil; 2) concentrated ethyl esters of menhaden oil containing 59% docosahexaenoic acid (22:6,ω3); 3) unesterified ethyl esters of menhaden oil containing 34% ddocosahexaenoic acid (22:6,ω3). All fish oils were obtained from Nu-Chek Prep (Elysian, MN) and were more than 95% pure by gas chromatography.

Preparation of Hepatocytes

Hepatocytes were prepared and maintained in Williams E medium containing 25 mM glucose, 10 mM dexamethasone, 1 μM insulin, and albumin (50 μg) plus specific fatty acids (14, 16). Hepatocytes used for RNA analysis were seeded onto 100-mm Primaria culture dishes at 107 cells per dish. Media also contained 1 μM vitamin E and 2.7 μM butylated hydroxytoluene to prevent oxidation of the fatty acids (14, 16). All fatty acids were obtained from Nu-Chek Prep (Elysian, MN) and were more than 95% pure by gas chromatography.

RNA Extraction and Measurement of PK mRNA

Liver and hepatocyte total RNA was isolated by the guanidinium thiocyanate procedure (34) and used for measurement of PK mRNA (mRNApk) (14). mRNApk was examined by Northern analysis and quantified by dot blot analysis. The corresponding cDNApk (pLPK) was kindly provided by A. Kahn (Institute of National Medical Research, Paris, France) and labeled with [32P]dCTP by random priming (14).

Transfection of Primary Hepatocytes

Primary hepatocytes used for transfection were seeded onto either Primaria 30 mm (six-well, at 106 cells per well) or 60-mm dish (at 3 × 106 cells per dish). Hepatocytes were transfected with PKCAT fusion genes using Lipofectin (Life Technologies, Inc, Gaithersburg, MD) as described previously (16).

Plasmid Constructions

Construction of the PKCAT chimeric genes has been described elsewhere (11). Linker scanning mutants were prepared essentially as described elsewhere (11). Briefly, clustered point mutants were made by site-directed mutagenesis (35). L-PK sequences from -197 to +12 were cloned into pBluescript SK+ vector (Stratagene, La Jolla, CA) and single-stranded uracil-containing DNA was created using Escherichia coli strain CJ236. In vitro synthesis of second strand was performed using mutant oligonucleotides as primers. Each oligonucleotide (32-35 nucleotides) contained a six- to eight-nucleotide mutation creating a unique NsiI site. Mutant constructs were isolated after transformation of E. coli strain MV1190. The PK promoter sequences were cloned into pCAT(AN) so that the promoter drives expression of the CAT gene (36). The sequence of the mutant PK promoter sequence in the PKCAT constructs was verified by dideoxynucleotide sequence analysis (11).

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