Estrogen-related Receptors Stimulate Pyruvate Dehydrogenase Kinase Isoform 4 Gene Expression*

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The pyruvate dehydrogenase complex (PDC) catalyzes the conversion of pyruvate to acetyl-CoA in mitochondria and is a key regulatory enzyme in the oxidation of glucose to acetyl-CoA. Phosphorylation of PDC by the pyruvate dehydrogenase kinases (PDK2 and PDK4) inhibits PDC activity. Expression of the PDK genes is elevated in diabetes, leading to the decreased oxidation of pyruvate to acetyl-CoA. In these studies we have investigated the transcriptional regulation of the PDK4 gene by the estrogen-related receptors (ERRα and ERRγ). The ERRs are orphan nuclear receptors whose physiological roles include the induction of fatty acid oxidation in heart and muscle. Previously, we found that the peroxisome proliferator-activated receptor γ coactivator (PGC-1α) stimulates the expression of PDK4. Here we report that ERRα and ERRγ stimulate the PDK4 gene in hepatoma cells, suggesting a novel role for ERRs in controlling pyruvate metabolism. In addition, both ERR isoforms recruit PGC-1α to the PDK4 promoter. Insulin, which decreases the expression of the PDK4 gene, inhibits the induction of PDK4 by ERRα and ERRγ. The forkhead transcription factor (FoxO1) binds the PDK4 gene and contributes to the induction of PDK4 by ERRs and PGC-1α. Insulin suppresses PDK4 expression in part through the dissociation of FoxO1 and PGC-1α from the PDK4 promoter. Our data demonstrate a key role for the ERRs in the induction of hepatic PDK4 gene expression.

The pyruvate dehydrogenase complex (PDC) catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA (1). Long term changes in PDC activity entail changes in PDC phosphorylation, whereas short term inhibition is mediated by the reaction products acetyl-CoA and NADH (1, 2). The pyruvate dehydrogenase kinases (PDK) decrease PDC activity via phosphorylation, whereas the pyruvate dehydrogenase phosphatases activate the PDC activity by dephosphorylation (3, 4). There are three serine phosphorylation sites on the α-subunit of pyruvate dehydrogenase (E1) that are targeted by PDKs, and phosphorylation of the α-subunit of the E1 element completely inhibits the activity of PDC (4). There is increased phosphorylation of PDC in the heart and skeletal muscle in starvation and diabetes, allowing pyruvate to be conserved while fatty acid oxidation is increased (5–7). In diabetes the decrease in PDC activity is due primarily to the increased PDK activity (5).

Four PDK isoenzymes (PDK1, -2, -3, -4) have been identified and characterized in mammalian tissues (1). The expression patterns of the PDK isoforms are tissue-specific (8). The PDK2 and PDK4 isoforms are highly expressed in liver, heart, and skeletal muscle (9). PDK2 and PDK4 gene expression is elevated with diabetes and starvation, with PDK4 being the most highly regulated isoform (2, 4). Insulin administration and refeeding inhibit the induction of PDK4 gene expression in the skeletal muscles and heart of diabetic and fasted animals, respectively (7, 10). In Morris hepatoma cells, long chain fatty acids, glucocorticoids, and peroxisome proliferator-activated receptor α agonists increase the transcription of the PDK4 gene, whereas insulin blocks PDK4 gene expression (11).

PGC-1α belongs to a family of transcriptional coactivators that includes PGC-1β and the PGC-1-related coactivator (12, 13). PGC-1α was originally cloned from brown adipose tissue through its interactions with the nuclear receptor peroxisome proliferator-activated receptor γ (14). PGC-1α plays a key role in various aspects of energy homeostasis including mitochondrial biogenesis, thermal regulation, and glucose metabolism (15). PGC-1α is highly expressed in the heart, brown fat, and skeletal muscle (16). PGC-1α is expressed at low levels in the liver, but it is induced in fasting animals (17). PGC-1α is also increased in the liver of both type 1 streptozotocin-induced and type 2 db/db diabetic mice (17). PGC-1α stimulates hepatic gluconeogenesis and expression of the phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase genes (17). PGC-1α stimulates PEPCK gene expression through interactions with hepatic nuclear factor 4 (HNF-4), the glucocorticoid receptor, and forkhead transcription factor (FoxO1) (18, 19). Furthermore, PGC-1α regulates pyruvate metabolism in the...
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mitochondria by inducing the PDK4 gene. We have shown that PGC-1α induces PDK2 and PDK4 gene expression in primary rat hepatocytes and cardiomyocytes (20). PGC-1α stimulates expression of the liver isoform of the carnitine palmitoyltransferase-Iα gene, thereby increasing the hepatic mitochondrial fatty acid oxidation (21).

Estrogen-related receptors (ERR) are orphan nuclear receptors. Three ERRs isoforms have been identified, α, β, and γ, which are also called ERR1/NR3B1, ERR2/NR3B2, and ERR3/NR3B3, respectively (22). Like other nuclear receptors, the ERRs contain a DNA binding domain and a ligand binding domain (23). The ligand binding domain is involved in dimerization, transcriptional activation, and interactions with coactivators. The ERRs share a homology with estrogen receptors, but ERRs do not bind to estrogen or other known physiological ligands (23). Several lines of evidence have suggested a role for ERRα and ERRγ in the control of metabolic genes. The ERRα and ERRγ subtypes are expressed in metabolically active tissues including skeletal muscle, heart, kidney, and liver (22, 24). In addition, PGC-1α stimulates ERRα gene expression (25–27). ERRα induces medium chain acyl-CoA dehydrogenase, indicating a role in fatty acid oxidation (28). Several recent reports have shown that the interactions of ERRα and PGC-1α induce oxidative phosphorylation and mitochondrial biogenesis (29, 30).

In these studies we report that ERRα and ERRγ can induce PDK4 and PDK2 gene expression. The ERR isoforms bind to the PDK4 gene promoter, stimulating PDK4 gene expression and suggesting a new role for ERRs in the regulation of hepatic pyruvate metabolism. Finally, we demonstrate that insulin inhibits the induction of the PDK4 gene by both ERR isoforms and PGC-1α.

MATERIALS AND METHODS

Transient Transfection of Luciferase Vectors—PDK4-luciferase (PDK4-luc) constructs were transiently transfected into McA-RH7777 cells by calcium phosphate precipitation (31). Transfections included 2 μg of PDK4-luciferase along with TK-Renilla and mammalian expression vectors for HNF-4, ERRα, ERRγ, or PGC-1α (20). Cells were transfected in Dulbecco’s modified Eagle’s medium containing 5% calf serum, 5% fetal bovine serum and incubated overnight at 37 °C. After two washes with phosphate-buffered saline, the medium was replaced by Dulbecco’s modified Eagle’s medium containing no serum. After 24 h, cells were lysed in passive lysis buffer (Promega). Both luciferase and Renilla activity were measured. Protein content in each lysate was determined by Bio-Rad Protein assay. Luciferase activity was corrected for both protein content and Renilla activity to account for cell density and transfection efficiency respectively. The serial deletions of the rat PDK4 promoter were created by PCR amplification (20). All deletions and site-directed mutants were sequenced and confirmed by DNA sequencing facility at the University of Tennessee Molecular Resource Center.

Adenoviral Infection of McA-RH7777 Cells—The adenoviruses expressing green fluorescent protein (GFP), PGC-1α, ERRα, or ERRγ were purified by cesium chloride purification (17). McA-RH7777 cells were cultured in plain Dulbecco’s modified Eagle’s medium containing 20 mM glucose before infection. The hepatocytes or McA-RH7777 cells were infected at an multiplicity of infection of 50 (20). Sixteen hours after infection the cells were changed to fresh medium. Forty-eight hours after infection RNA was isolated from the McA-RH7777 cells.

Construction of ERRα and ERRγ Adenovirus—The ERR adenovirus was generated using the ViraPower Adenoviral Expression System (Invitrogen) according to the manufacturer’s instructions. The full-length cDNAs for ERRα or ERRγ were cloned into the pENTR1A vector (Invitrogen). The cDNA was transferred into the pAd/CMV/V5-DEST vector (Invitrogen) using the Gateway LR Clonase II enzyme mix according to the manufacturer’s directions (Invitrogen). The ligation reaction was transformed into One Shot TOP10 chemically competent Escherichia coli. Recombinant adenoviral DNA was recovered from the TOP10 E. coli cells and transfected into 293A cells (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s directions. The 293A cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 8% fetal bovine serum (HyClone), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen) in a 37 °C incubator with 5% CO2. Cell lysis was typically apparent in 5–7 days post-infection. Cells and media were collected and subjected to three freeze/thaw cycles. The cell debris was pelleted at 3000 rpm for 15 min. Glycerol was added to the supernatant to a final concentration of 10% and stored at −80 °C.

Western Blot Analysis—Proteins from McA-RH7777 cells were harvested in PD buffer consisting of 40 mM Tris-Cl, pH 8.0, 500 mM NaCl, 0.5% Nonidet P-40, 6 mM EDTA, pH 8.0, 6 mM EGTA, pH 8.0, 1 mM dithiothreitol, and diluted protease inhibitor mixture (Sigma P8340). Cell membranes were removed by centrifugation for 25 min at 4 °C. An equal amount of protein was loaded onto a 12% SDS-PAGE gel and transferred to a 0.45-μm pure nitrocellulose membrane (Bio-Rad). Blots were probed with primary antibodies in phosphate-buffered saline containing 5% nonfat dry milk powder and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Bio-Rad, #170-6515). The primary antibodies were anti-PGC-1α (Santa Cruz, sc-10367), anti-ERRγ (Lifespan Biosciences, LS-A5960), anti-PDK2 (Abgent, #AP7039b) or anti-PDK4 (Abgent, #AP7041b). Protein bands were resolved using Super Signal West Pico or Femto chemiluminescence substrate (Pierce, #34080 or 34094). Bio-Rad Quantity One Software was used to analyze individual bands. Actin (42 KDa) was used as loading control for each lane. The primary antibody was monoclonal mouse anti-actin IgM 1:1000 (Oncogene, #CP01). The goat anti-mouse IgM 1:2000 in the same kit (Oncogene, #CP01) was applied as secondary antibody.

Real-time PCR—RNA was extracted from McA-RH7777 cells using RNA-Stat-60 (Tel-Test) as described previously (21). The RNA was initially treated with DNase I (Ambion) at 37 °C for 1 h. The DNase I was stopped by the addition of DNase inactivation reagent to the sample. Equal amounts of DNA-free RNA were used for the first-strand cDNA synthesis. Up to 1 μg of RNA was mixed with 1 μl of 10 mM dNTP mix and 1 μl of random hexamers (50 ng/μl). Each sample was incubated at
70 °C for 10 min and then placed on ice for at least 1 min. Next, 2 μl of 10× reverse transcriptase buffer, 4.5 μl of 25 mM MgCl2, 2 μl of 0.1 mM dithiothreitol, and 1 μl of RNaseOUT recombinant ribonuclease inhibitor was added to each tube. After incubation at 25 °C for 2 min, each tube was loaded with 1 μl of SuperScript II reverse transcriptase. The tubes were incubated at 25 °C for 10 min, 42 °C for 1 h, and 70 °C for 15 min. RNase H was added to each to each tube and incubated at 37 °C for 20 min.

The parameters for real time PCR were as follows: 95 °C for 11 min followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min and for the melt curve, 100 cycles of 10 s from 60 to 100 °C in 0.4 °C increments. The final concentration of primers in each well in the PCR plates was 0.1 μM. The oligonucleotide standards were diluted to 10−6, 10−5, 10−7, 10−6, 10−5 μM and used as a template for standard curve. The sequences of the target gene primers are provided in Ma et al. (20). A 1:500 dilution of each cDNA as template was used to measure 18 S ribosomal RNA (rRNA), and 1 μl of 1:10 or 1:20 dilution of each cDNA was used as a template to assess target genes. The 18 S rRNA normalized all PDK mRNA determinations.

**Gel Shift Mobility Assays**—The probes for gel shift assays were created by labeling double-stranded oligonucleotides using Klenow enzyme and [α-32P]dCTP (32, 33). The sequence of the top strand oligomers for ERRα and ERRγ binding is shown in Fig. 6. The labeled probe (30,000 cpm) was combined with in vitro translated ERRα and ERRγ (TNT T7) quick coupled transcription-translation system, Promega) in binding buffer containing 80 mM KCl, 25 mM Tris HCl, pH 7.4, 0.1 mM EDTA, 1 mM diithiothreitol, and 10% glycerol (33). For the identification of FoxO1 binding sites, the oligomers were incubated with GST-FoxO1 (Upstate #14-343). The nonspecific competitor added to each binding reaction was 1 μg of a 1:1 ratio of poly(dI:C):poly(dA:dT). Binding reactions were incubated at room temperature for 20 min and resolved on a 5% non-denaturing acrylamide gel (80:1, acrylamide/bisacrylamide) in Tris borate running buffer (0.5 × Tris borate-EDTA) at 4 °C (33).

**Chromatin Immunoprecipitation (ChIP) Assays**—Rat primary hepatocytes (3 × 106 in a 60-mm dish) were maintained for 48 h in RPMI 1640 media. These cells were treated with 100 nM insulin for 24 h before the harvest. McA-RH7777 cells (3 × 107 in a 150-mm dish) were maintained in plain Dulbecco’s modified Eagle’s medium (DMEM). The McA-RH7777 cells were infected with adenovirus expressing ERRα or GFP for 3 days before cross-linking. Cross-linking was performed with 1% formaldehyde for 15 min at room temperature and stopped by the addition of 125 mM glycine for 5 min (21). Cross-linked hepatocytes were washed with phosphate-buffered saline. Cells were scraped from the plate and collected by centrifuging for 5 min at 2000 × rpm. The DNA was sonicated as we have described previously (21). The samples were precleared by the addition of 80 μl of Salmon Sperm DNA, protein A-agarose, 50% slurry (Upstate Biotechnology, #16-157) for 30 min at 4 °C with rotation. The antibodies used included anti-PGC-1α (Santa Cruz, sc-13067), anti-HNF-4 (Santa Cruz, sc-8987), anti-FoxO1 (Santa Cruz, sc-11350), or anti-ERRγ (Lifespan Biosciences, LS-A5960) at 4 °C overnight using rabbit IgG as the control. To collect the antibody-protein-DNA complex, 60 μl of protein A-agarose slurry was added for 2 h at 4 °C.

The protein A-agarose was washed multiple times as described previously (21).

The genomic DNA was precipitated with the addition of 30 μl of 5 M NaCl followed by centrifugation at 13,000 × g for 3 min. The supernatant was removed, and the pellet was resuspended in 100 μl of water, 4 μl of Tris, pH 6.8, 2 μl of 0.5 M EDTA, pH 8.0, and 1 μl of 20 mg/ml proteinase K. After incubation at 45 °C for 1 h, genomic DNA fragments were purified using Qiagquick spin columns (Qiagen, #28104). The final eluted volume was 50 μl. A total of 2–5 μl of purified sample was used in 30–35 cycles of PCR. Primers for the rat PDK4 gene are listed in Ma et al. (20). The PCR products were analyzed on 2% Nusieve 3:1 agarose (Cambrex, #50094) and visualized with Multimage Light Cabinet with Quantity One software.

**RESULTS**

**PGC-1α Stimulates the PDK4 Gene**—Previously, we reported that PGC-1α increases PDK4 mRNA levels in rat hepatocytes (20). Our first experiments examined whether PGC-1α altered the protein abundance of PDK4 in rat hepatocytes. PDK4 mRNA abundance was increased 6.0-fold after infection of hepatocytes with an adenoviral vector expressing PGC-1α (Ad-PGC-1α) (Fig. 1A). PDK4 protein abundance was assessed by Western blot analysis. As shown in Figs. 1, B and C, PGC-1α increased PDK4 protein levels 2.1 ± 0.2-fold.

**ERR and PGC-1 Stimulate PDK4 Gene Expression**—ERRα has been implicated in the regulation of fatty acid oxidation in skeletal and cardiac muscle (28, 30). We tested whether the ERRα or ERRγ isoforms would induce the expression of the PDK2 and PDK4 genes in hepatoma cells. We infected the McA-RH7777 rat hepatoma cells with adenoviruses expressing ERRα (Ad-ERRα), ERRγ (Ad-ERRγ), or PGC-1α (Ad-PGC-1α). We measured the mRNA level by real-time PCR. Both ERRs induced the expression of PDK4 mRNA. ERRγ strongly stimulated expression of the PDK4 gene with a 7.7 ± 1.2-fold increase in PDK4 mRNA levels (Fig. 2A), whereas ERRα increased the PDK4 gene expression 2.8 ± 0.4-fold. ERRγ consistently induced PDK4 more strongly than ERRα. ERRα increased PDK2 mRNA levels 2.0-fold, whereas ERRγ had no effect on PDK2 mRNA abundance. PGC-1α induced PDK4 and PDK2 mRNA levels 7.9 ± 0.9- and 2.2 ± 0.2-fold, respectively.

We measured the induction of PDK4 and PDK2 protein abundance by ERRγ. We infected the McA-RH7777 cells with Ad-ERRγ and harvested cell lysates. ERRγ increased the PDK4 protein abundance 1.9 ± 0.2-fold and had no effect on PDK2 protein levels (Fig. 2B). These observations are consistent with our RNA measurements. PGC-1α increased PDK4 levels 2.7 ± 0.45-fold, but PDK2 levels were not changed (Fig. 2C). The induction by PGC-1α is very similar to the induction of PDK4 by PGC-1α in primary rat hepatocytes (20). Overall, these results show that the ERRs and PGC-1α induce PDK4 expression but have little effect on PDK2. The remainder of our studies focused on the regulation of the PDK4 gene.

To determine whether ERRα or ERRγ directly stimulated the PDK4 promoter, we cotransfected the −2083/+87 rat PDK4-luciferase (PDK4-luc) vector with ERRα or ERRγ expression vectors into McA-RH7777 cells. ERRα and ERRγ increased the

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FIGURE 1. PGC-1α increases PDK4 enzyme abundance in hepatocytes. A, primary rat hepatocytes were infected with adenoviruses expressing either green fluorescent protein (Ad-GFP) or PGC-1α (Ad-PGC-1α). The infection conditions are described under “Materials and Methods.” The RNA was isolated, and PDK4 mRNA abundance was measured by real time PCR. The data are expressed as the average induction ± S.E. in three independent preparations of hepatocytes. B, the abundance of PGC-1α and PDK4 was determined by Western analysis. After infection for 48 h, whole cell lysates were prepared. Lanes 1 and 2 contain proteins isolated from independent plates of GFP-infected hepatocytes, and lanes 3 and 4 contain proteins isolated from independent plates of PGC-1α-infected cells. Actin was used as the loading control. C, The fold induction of PDK4 protein by PGC-1α was expressed as the mean ± S.E. The data are the average of three determinations done in duplicate from different preparations of hepatocytes.

expression of PDK4-luciferase 3.9- and 6.6-fold, respectively (Fig. 3). PGC-1α induced the PDK4 luciferase reporter gene 2.0-fold. Cotransfection of PGC-1α and ERRs further enhanced the induction of PDK4-luciferase. As with the adenoviral infections, ERRα stimulated PDK4 less robustly than ERRγ.

We created serial deletions from the 5′ end of the PDK4 promoter that were ligated to the luciferase reporter gene and transfected these reporter genes into McA-RH7777 cells. The ERR induction was lost with deletions through the −578 to −325 region of the promoter (Fig. 3). The −375/+87 PDK4-luc vector still responded to ERRγ or the combination of ERRα/PGC-1α, whereas the −325/+87 vector was not induced by ERR/PGC-1. It is possible that there is an accessory factor in the −423/−375 region that is required for ERRα action. These results suggest that the −578/−325 region mediates the ERR/PGC-1 response, and our experiments focused on this region of the promoter.

Insulin Inhibits ERR-mediated Induction of the PDK4 Gene—Insulin inhibits the expression of the PDK4 and PDK2 genes (34). To investigate whether insulin suppressed the induction of the PDK genes by ERRs and PGC-1α, we infected McA-RH7777 cells with adenoviruses expressing ERRs and ERRγ and treated the cells with insulin. As shown in Fig. 4A, ERRα and ERRγ strongly stimulated PDK4 gene expression 3.1- and 6.6-fold, respectively. The addition of insulin reduced the ERRα and ERRγ induction of PDK4 mRNA by 48 and 44%, respectively. Insulin also decreased the PDK4 mRNA abundance in GFP-infected cells 36%. The induction by PGC-1α alone was blocked 30% by insulin. We co-infected McA cells with Ad-ERRs and Ad-PGC-1α. We observed that the ERRα/PGC-1α and ERRγ/PGC-1α increased PDK4 mRNA levels 23.8- and 18.2-fold, respectively, but that they did not induce PDK2. The synergy between ERRα/PGC-1α was stronger than ERRγ/PGC-1α on the PDK4 gene (Fig. 4A). PDK2 mRNA was increased 2-fold by PGC-1α, but the ERRs had little effect. These results indicate that ERRs and PGC-1α will activate PDK4 but not PDK2 expression. The induction by ERRα/PGC-1α and ERRγ/PGC-1α was reduced 37 and 43%, respectively, by insulin administration. In contrast, insulin did not repress the PDK2 mRNA levels after ERR infection (Fig. 4A).

Transient transfection experiments were conducted to test the ability of insulin to inhibit the expression of the PDK4-luc gene in the McA-RH7777 cells (Fig. 4B). Previously, we had found that HNF-4 increased expression of the PDK4 gene and that this induction occurred through an element located at −1115/−1092 (20). To test whether insulin might block this induction, we cotransfected HNF-4 with PDK4-luc. HNF-4 induced PDK-luc expression 5.2-fold, but this stimulation was not inhibited by insulin. On the other hand, a similar induction of PDK4-luc by ERRα (3.8-fold) or ERRγ (6.6-fold) was blocked 56 and 64%, respectively, by insulin administration. The induction by ERRs and PGC-1α was also inhibited by insulin. These results suggest that insulin may repress PDK4 expression in part through inhibition of ERR transactivation.

Our next experiments were designed to investigate the regulation of the −578/−325 region of the PDK4 promoter by ERRs and insulin. We ligated the −578/−325 region of the PDK4 promoter in front of the enhancerless SV40-luciferase vector. As determined in Fig. 3, this portion of the promoter responds to ERR/PGC-1α. We transfected this vector into McA-RH7777 cells with PGC-1α and ERR vectors. As shown in Fig. 5, ERRα and PGC-1α increased the expression of the reporter 1.4 and 1.8-fold, respectively, whereas the cotransfection of ERRα/PGC-1α synergistically induced −578/−325 SV40-luciferase vector 6.4-fold. ERRγ induced the −578/−325 SV40-luciferase vector 3.3-fold. Deletion of the −375/−325 region of the promoter reduced the induction by ERRs, ERRγ, and PGC-1α to 1.4-, 1.0-, and 0.8-fold, respectively. These data indicate that the −375/−325 portion of the promoter is crucial for the induction by the ERRs and PGC-1α.
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ERRs Bind to the −375/−330 Region of PDK4 Promoter—The next experiments were designed to identify binding sites for ERRs in the −375/−325 region of the PDK4 promoter. We generated two double-stranded oligonucleotides that contained all the sequences in the region between nucleotides −375 and −330 of PDK4 promoter. On the bottom strand nucleotides −371/−363 contained an ERR binding site (TCAATGTCA) that had a sequence that varied from a typical TCAAGGTCA type motif by one nucleotide (35). As shown in Fig. 6A, gel shift mobility assays demonstrated that ERRα and ERRγ bound to the −375/−350 region of PDK4 promoter. The ERRs were obtained by in vitro translation in a reticulocyte lysate system. Mutagenesis of nucleotides −370/−365 (Mut-370) eliminated the ERR binding, whereas mutation of nucleotides of −359/−352 (Mut-359) did not disrupt the binding of ERRα or ERRγ (Fig. 6A). There is a second ERR binding site motif between nucleotides −343 and −334 (ACAAGGACA) of the rat PDK4 promoter. Using gel shift mobility assays, we found that the ERRs bound to oligomers containing nucleotides −359/−331 (Fig. 6B). The mutation of the ACA nucleotides at the 5′ end of this ERR binding site (Mut-338) blocked the binding of ERRs to this element. In addition, there is a highly conserved FoxO1 site (−347/−340) (GTAAAGTTCA) in this region that is conserved between the mouse, human, and rat promoters (36). Our gel shifts indicate that FoxO1 can bind this site (Fig. 6B).

To test whether ERRγ can associate with PDK4 promoter in vivo, we conducted ChIP assays. After infection of the McA-RH7777 hepatoma cells with Ad-ERRγ or Ad-GFP, we cross-linked the cells with 1% formaldehyde and sheared the chromosomal DNA by sonication. The DNA–protein complexes were precipitated by a specific antibody against ERRγ or control rabbit IgG. Using primers for the PDK4 promoter, we observed a PCR product with ERRγ antibody pulldown in the cells overexpressing ERRγ but not by GFP (Fig. 6C) (20).

To examine the regulation through the ERR sites, we ligated two copies of the wild type −375/−325 region of the PDK4 promoter in front of SV40-luciferase vector (Fig. 7A). This
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Fold Induction of PDK4-luciferase

|    | ERRα | PGC-1α | PGC-1α | ERRγ | PGC-1α |
|----|------|--------|--------|------|--------|
| -2083 | 3.9 ± 0.8 | 5.8 ± 1.0 | 2.0 ± 0.2 | 6.6 ± 0.9 | 11.4 ± 0.8 |
| -578 | 3.6 ± 0.1 | 5.7 ± 1.5 | 1.9 ± 0.1 | 5.5 ± 0.2 | 8.7 ± 0.2 |
| -504 | 2.8 ± 0.5 | 4.0 ± 1.0 | 1.7 ± 0.1 | 5.4 ± 0.2 | 7.7 ± 0.1 |
| -461 | 2.2 ± 0.1 | 2.8 ± 0.4 | 1.5 ± 0.1 | 3.4 ± 0.3 | 5.0 ± 0.1 |
| -433 | 2.3 ± 0.2 | 3.0 ± 0.1 | 1.5 ± 0.1 | 3.6 ± 0.4 | 5.1 ± 0.4 |
| -375 | 1.0 ± 0.1 | 2.3 ± 0.2 | 1.1 ± 0.1 | 2.1 ± 0.1 | 2.6 ± 0.2 |
| -325 | 0.8 ± 0.1 | 1.0 ± 0.1 | 0.9 ± 0.1 | 1.1 ± 0.1 | 1.2 ± 0.1 |
| -100 | 0.2 ± 0.1 | 0.7 ± 0.1 | 1.2 ± 0.3 | 0.8 ± 0.1 | 1.2 ± 0.2 |
| -18  | 0.9 ± 0.3 | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 |

FIGURE 3. ERRs and PGC-1α stimulate rat PDK4 gene expression. McA-RH7777 hepatoma cells were transfected with 2 μg of rat PDK4-luciferase reporter gene and expression vectors for ERRα (50 ng), ERRγ (50 ng), and PGC-1α (0.5 μg). TK-Renilla (1.0 μg) was included as a transfection control. Serial deletions were created from the 5′ end of the rat PDK4 promoter. A model of the truncated promoters is shown on the left. Cells were harvested 36 h after transfection. The luciferase activity was corrected for protein content and Renilla activity. The data are expressed as the mean of the fold induction ± S.E. by the particular transcription factor. All transfections were done in duplicate and repeated three or four times.

region contains both ERR binding sites and the FoxO1 binding site. ERRα and PGC-1α induced expression of the −375/−325 SV40-luciferase gene 2.5- and 2.4-fold, respectively. ERRγ induced this vector 5.4-fold (Fig. 7A). Mutation of the more 5′ ERR binding site (Mut-370) strongly decreased the ability of both ERR and PGC-1α to induce the −375/−325 luciferase gene expression. Mutation of the 3′ ERR binding site (Mut-338) also decreased the induction by ERRs and PGC-1α. The results indicate that both of the ERR sites are involved in the recruitment of PGC-1α to the −375/−325 region of the PDK4 promoter. In addition, we disrupted the FoxO1 site (Mut-347). Interestingly both the ERRs and PGC-1α were able to increase expression of the reporter gene as strongly as the wild type promoter. However, cotransfection of ERRα or ERRγ and PGC-1α resulted in a less than additive induction. These results suggest that FoxO1 plays a role in the interaction of ERR and PGC-1α.

We investigated the insulin regulation of the ERR and PGC-1α induction of PDK4 gene. As seen in Fig. 7, insulin reduced the expression of two copies of the wild type −375/−325 region of PDK4 promoter driving SV40 luciferase by ERRα, ERRγ, or PGC-1α. Mutation of the FoxO1 binding site (Mut-347) eliminated the inhibition by insulin. However, insulin still inhibited luciferase expression by ERRγ/PGC-1α induction with either ERR binding site mutation. Insulin did not inhibit the PGC-1α induction of −375/−325 luciferase gene with the FoxO1 binding site (Mut-347) disrupted, suggesting that FoxO1 is important for the insulin suppression of the PDK4 gene. It was found that FoxO1 would stimulate the mouse PDK4 promoter, although the induction was very weak (36). In our experiments, the mutation of the FoxO1 site in the context of the −375/−325 × 2 SV40-luciferase vector did not decrease the basal expression of the PDK4 gene (data not shown). Finally, we tested whether ERRγ and PGC-1α could interact physically. Interestingly, ERRα and ERRγ interact with

FIGURE 4. Insulin inhibits the ERR and PGC-1α induction of rat PDK4 gene expression. A, McA-RH7777 cells were infected with ERRα (Ad-ERRα), ERRγ (Ad-ERRγ), PGC-1α (Ad-PGC-1α), or GFP (Ad-GFP) as described in the legend to Fig. 2. Insulin (100 nM) was added for 24 h (striped bars), and cells were harvested. PDK4 (top panel) and PDK2 (bottom panel) mRNA were measured by real time PCR. The p < 0.01 for the insulin inhibition is indicated by the asterisk, and p < 0.05 is indicated by number symbol (#). B, McA-RH7777 cells were transfected with 2 μg of −2083/+87 rat PDK4-luciferase reporter gene and expression vectors for HNF-4 (250 ng), PGC-1α (500 ng), ERRα (50 ng), and ERRγ (50 ng). TK-Renilla (1.0 μg) was included as a transfection control. Cells were exposed to insulin for 24 h. The induction of luciferase activity was corrected for Renilla and protein content. All transfections were done in duplicate and repeated three times. The p < 0.01 for the insulin inhibition is indicated by the asterisk, and p < 0.05 is indicated by number symbol (#).
a leucine-rich motif in PGC-1α at amino acids 209–213 as well as the LXXL motif used by other nuclear receptors (28). In Fig. 7B, we show that the GST-PGC-1α containing the first 400 amino acids of PGC-1α will interact more strongly with ERRα than the first 170 amino acids of PGC-1α. Similar results were observed with ERRγ.

Insulin Decreases PGC-1α Association with the PDK4 Promoter—Previously, we demonstrated by ChIP assay that PGC-1α is associated with the PDK4 promoter in vivo on rat primary hepatocytes (20). One mechanism by which insulin may regulate the PDK4 gene is by inducing dissociation of PGC-1α from the PDK4 promoter. We added insulin to the rat hepatocytes and examined the interaction of HNF-4 and PGC-1α with the PDK4 promoter using the ChIP assay. We did not observe any change in the binding of HNF-4, but we did see a decrease in the association of PGC-1α with the PDK4 promoter (Fig. 8). These results suggest that the dissociation of PGC-1α is a mechanism by which insulin suppresses PDK4 gene expression. In addition, we examined whether FoxO1 is associated with the PDK4 promoter and whether insulin might affect this interaction. Our ChIP assays demonstrated that FoxO1 is associated with the PDK4 gene and that insulin will reduce the binding of FoxO1 to the PDK4 gene.

DISCUSSION

In this study we have investigated the contribution of the ERRα and ERRγ orphan receptors to PDK4 gene expression in hepatoma cells. Previous studies have shown that ERRα and PGC-1α promote fatty acid oxidation in skeletal muscle and the heart (25, 30). We have identified a new action for the ERRs involving the regulation of hepatic PDK4 gene expression. Our
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Overall, our results suggest that ERRα and ERRγ have crucial roles in the regulation PDK4 gene expression.

Pyruvate oxidation to acetyl-CoA is a key step in glucose oxidation in the liver and skeletal muscle (1). PDC catalyzes the oxidation of pyruvate to acetyl-CoA thereby controlling the fate of pyruvate. The phosphorylation state of PDC, which is determined by the relative activity of PDKs and pyruvate dehydrogenase phosphatases, is the primary determinant of PDC activity (1). There is increased expression of the PDK4 gene in the liver of fasted animals (7). Recent studies have indicated that inhibition of PDKs with dichloroacetate and other inhibitors will reduce hyperglycemia in type II diabetic rats (37). In PDK4 knock-out mice the blood glucose levels are further reduced in starvation due to the increased utilization of glucose (38). These observations suggest that modulation of PDC activity by the PDKs is an important component of glycemic control in the whole animal.

PGC-1α is induced in the liver of the fasting and diabetic animals, and it stimulates the expression of genes involved in hepatic gluconeogenesis and mitochondrial fatty acid oxidation (16). PGC-1α elevates gluconeogenesis by inducing the expression of the PEPCK and glucose-6-phosphatase genes through interactions with the glucocorticoid receptor, HNF-4, and FOXO1 (17, 19). In contrast to the PEPCK gene, our studies have not found that interactions of PGC-1α and HNF-4 are involved in the PGC-1α induction of the PDK4 gene (20). The central role of PGC-1α in regulating gluconeogenic genes has been shown through knock-down experiments. Koo et al. (39), using adenovirus to deliver PGC-1α RNA-mediated interference to mice by tail vein injection, reported decreased PEPCK and glucose-6-phosphatase mRNA. Isolated hepatocytes from PGC-1α knock-out mice have reduced PEPCK and glucose-6-phosphatase mRNA compared with hepatocytes from wild-type mice (40, 41). PGC-1α may inhibit glycolysis by inhibiting PDC activity. Previously, we found that PGC-1α induces PDK4

| A.             | Fold induction of SV40-luciferase | Fold induction in the presence of insulin |
|----------------|---------------------------------|-----------------------------------------|
| -375/-325 X 2 WT |                                 |                                         |
| SV40-luc        | 1                               | 0.9 ± 0.2                              |
| SV40-luc + PGC-1α | 2.4 ± 0.3                      | 1.0 ± 0.1*                             |
| SV40-luc + ERRα | 2.5 ± 0.4                       | 1.4 ± 0.4*                             |
| SV40-luc + ERRα/PGC-1α | 6.5 ± 1.0             | 3.1 ± 0.9*                             |
| SV40-luc + ERRγ | 5.4 ± 0.9                       | 2.5 ± 0.4*                             |
| SV40-luc + ERRγ/PGC-1α | 11.7 ± 1.8          | 5.6 ± 1.0*                             |
| -375/-325 X 2 Mut-370 |                            |                                         |
| SV40-luc        | 1                               | 0.6 ± 0.1                              |
| SV40-luc + PGC-1α | 0.6 ± 0.2                      | 0.4 ± 0.1                              |
| SV40-luc + ERRα | 1.9 ± 1.2                       | 0.7 ± 0.1*                             |
| SV40-luc + ERRα/PGC-1α | 1.0 ± 1.8             | 0.6 ± 0.1                              |
| SV40-luc + ERRγ | 1.9 ± 1.2                       | 0.7 ± 0.1*                             |
| SV40-luc + ERRγ/PGC-1α | 2.3 ± 0.5             | 0.7 ± 0.1*                             |
| -375/-325 X 2 Mut-338 |                            |                                         |
| SV40-luc        | 1                               | 0.9 ± 0.1                              |
| SV40-luc + PGC-1α | 1.0 ± 0.1                       | 0.6 ± 0.1*                             |
| SV40-luc + ERRα | 1.2 ± 0.2                       | 0.7 ± 0.1*                             |
| SV40-luc + ERRα/PGC-1α | 2.0 ± 0.6             | 0.9 ± 0.2*                             |
| SV40-luc + ERRγ | 2.7 ± 0.7                       | 1.3 ± 0.3*                             |
| SV40-luc + ERRγ/PGC-1α | 2.7 ± 1.1             | 0.9 ± 0.2*                             |

| B.           | 10% input | GST-PGC-1α |
|--------------|-----------|------------|
| ERRγ         |           | I-400 I-170 |
| ERRα         |           | I-400 I-170 |

FIGURE 7. Insulin inhibits induction by ERRγ through the -375/-325 region of the PDK4 promoter. A, McA-RH7777 hepatoma cells were transfected with 2 μg of SV40-luciferase reporter gene and expression vectors for ERRα, ERRγ, or PGC-1α as described in the legend to Fig. 3. TK-Renilla was included as a transfection control. Insulin at a concentration of 100 nM was added for 24 h. Cells were harvested 36 h after transfection. The luciferase activity was corrected for protein content and Renilla activity. The data are expressed as the mean of the fold induction ± S.E. by the particular transcription factor. The p < 0.01 for the insulin inhibition is indicated by the asterisk. All transfections were done in duplicate and repeated three to six times. B, interactions between PGC-1α and ERRγ or ERRα were demonstrated with GST pulldown assays. 35S-Labeled ERRγ or 35S-labeled ERRα were prepared in a reticulocyte lysate system. The interactions were conducted as described in the under “Materials and Methods.” rPDK, rat PDK.

data demonstrate that both ERRα and ERRγ bind to the PDK4 promoter and stimulate PDK4 expression. The ERRs further promote PDK4 gene expression by recruiting PGC-1α to the PDK4 promoter. Insulin, which is a key regulator of PGC-4, inhibits the PDK4 induction by both ERRs and ERR/PGC-1α.

FIGURE 8. Insulin decreases PGC-1α association with the PDK4 promoter in vivo. Chromatin immunoprecipitation assays were conducted to analyze the binding of proteins to the PDK4 promoter. A model of the PDK4 promoter and the location of the PCR primers are shown at the top of the figure. Rat hepatocytes were cross-linked with 1% formaldehyde as described under "Materials and Methods." Cross-linked DNA was immunoprecipitated with either rabbit IgG or an antibody to HNF4 (anti-HNF4) PGC-1α (anti-PGC-1α), or FoxO1 (anti-FoxO1). The bands in the gel are ethidium bromide-stained PCR products. PCR conditions are described under "Materials and Methods." The ChIP assays were repeated three times on independent hepatocytes preparations.

Overall, our results suggest that ERRα and ERRγ have crucial roles in the regulation PDK4 gene expression.

Pyruvate oxidation to acetyl-CoA is a key step in glucose oxidation in the liver and skeletal muscle (1). PDC catalyzes the oxidation of pyruvate to acetyl-CoA thereby controlling the fate of pyruvate. The phosphorylation state of PDC, which is determined by the relative activity of PDKs and pyruvate dehydrogenase phosphatases, is the primary determinant of PDC activity (1). There is increased expression of the PDK4 gene in the liver of fasted animals (7). Recent studies have indicated that inhibition of PDKs with dichloroacetate and other inhibitors will reduce hyperglycemia in type II diabetic rats (37). In PDK4 knock-out mice the blood glucose levels are further reduced in starvation due to the increased utilization of glucose (38). These observations suggest that modulation of PDC activity by the PDKs is an important component of glycemic control in the whole animal.

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The ERRs stimulate fatty acid oxidation in part through the recruitment of PGC-1α (28, 42, 43). Several recent reports have indicated that ERRα and PGC-1α interactions induce oxidative phosphorylation and mitochondrial biogenesis (29, 30). ERRα stimulates medium chain acyl-CoA dehydrogenase, indicating a role in fatty acid oxidation (28). ERRα activated peroxisomal proliferation-activated receptor α gene expression in cardiac myocytes, providing an additional mechanism by which ERRα might promote fatty acid oxidation (25). While this manuscript was in preparation, it was reported that ERRα induces mouse PDK4 gene expression in skeletal muscle (44, 45). These studies confirmed our previous observation that PGC-1α increases PDK4 gene expression (20). These groups identified two different ERR sites as the ERRα response element in the muscle promoter (44, 45). These elements correspond to the −371/−363 and −342/−334 sites found in this study. Our results demonstrate that in hepatic cells both the ERR elements contribute significantly to the ERR induction and the recruitment of PGC-1α. These differences may reflect species differences in the promoters or the fact that our studies focused on hepatic cells, whereas other groups used C2C12 cells. However, both ERR sites are perfectly conserved in rat, mouse, and human PDK4 genes. Wende et al. (44) reported that FoxO1 was not involved in the regulation of mouse PDK4 gene by ERRα (44). Our results partially agreed with this observation in that mutation of the FoxO1 site in the rat PDK4 promoter did not decrease the ERRα induction. However, FoxO1 does appear to be needed for the synergistic effects of ERRα and PGC-1α.

It was reported that ERRα inhibited the expression of the gluconeogenic gene PEPCK by PGC-1α, indicating that ERRα does not support gluconeogenesis (46). We have also observed that ERRα will block the PGC-1α induction of PEPCK in McA-RH7777 cells (data not shown). It may be that ERRα and ERRγ have a role in specifically inhibiting glycolytic flux but not in inducing gluconeogenic genes. In the same report it was shown that ERRα induced the expression of several mitochondrial genes including ATP synthase subunit β and cytochrome c-1 (46). However, we found that overexpression of ERRα and ERRγ does not induce the expression of carnitine palmitoyltransferase-1α gene (data not shown). Carnitine palmitoyltransferase-1α is a rate controlling step in the oxidation of long chain fatty acids in mitochondria (47). Therefore, ERR isoforms are likely to regulate hepatic gene expression in a complex manner.

Multiple factors have been implicated in the insulin regulation of gene expression (48, 49). One mechanism that has been defined for the insulin inhibition of gene expression entails the insulin-induced phosphorylation and subsequent nuclear exclusion of FoxO1 (48, 50–52). PGC-1α can coactivate FoxO1 to induce PEPCK gene expression (19). Insulin may inhibit PEPCK in part through the nuclear exclusion of FoxO1 and disruption of the regulatory complex of PGC-1α and FoxO1 (50). ChIP assays were utilized to demonstrate that FoxO1 and PGC-1α association with the PEPCK promoter was decreased by insulin (19). Our studies indicated that insulin decreases the binding of FoxO1 and PGC-1α to the PDK4 promoter. Studies of the human PDK4 gene have shown that insulin will inhibit the glucocorticoid induction of PDK4 gene expression through FoxO1 (34). It is not known if this mechanism functions in the rat gene, as the glucocorticoid response element is located at a different position in the promoter (data not shown). Dissociation of FoxO1 from the promoter may disrupt the ERR-PGC-1α complex and lead to the 50% decrease in gene expression that we observed in our studies. In addition, insulin may repress PDK4 gene expression by directly inhibiting the transactivation ability of ERR, but further studies will be needed to examine this possibility.

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