Regulation of PGC-1 Promoter Activity by Protein Kinase B and the Forkhead Transcription Factor FKHR

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Peroxisome proliferator–activated receptor-γ coactivator-1 (PGC-1) plays a major role in mediating hepatic gluconeogenesis in response to starvation, during which PGC-1 is induced by the cyclic AMP response element binding protein. Although it is observed that insulin counteracts PGC-1 transcription, the mechanism by which insulin suppresses the transcription of PGC-1 is still unclear. Here, we show that forkhead transcription factor FKHR contributes to mediating the effects of insulin on PGC-1 promoter activity. Reporter assays demonstrate that insulin suppresses the basal PGC-1 promoter activity and that coexpression of protein kinase (PK)-B mimics the effect of insulin in HepG2 cells. Insulin response sequences (IRSs) are addressed in the PGC-1 promoter as the direct target for FKHR in vivo. Coexpression of FKHR stimulates the PGC-1 promoter activity via interaction with the IRSs, while coexpression of FKHR (3A), in which the three putative PKB sites in FKHR are mutated, mainly abolishes the suppressive effect of PKB. Whereas deletion of the IRSs prevents the promoter stimulation by FKHR, that activity is still partially inhibited by insulin. These results indicate that signaling via PKB to FKHR can partly account for the effect of insulin to regulate the PGC-1 promoter activity via the IRSs. *Diabetes* 52:642–649, 2003

It is established that protein kinase B (PKB) plays a critical role in various physiological events by phosphorylating numerous factors in response to insulin, IGF-I, and several growth factors (1–5). PKB-mediated phosphorylation is shown to modulate the function of forkhead transcription family members, such as FKHR AFX, and FKHRL1 (6–10). In the absence of phosphorylation, these forkhead proteins that localize to the nucleus interact with the insulin response sequence (IRS) within the promoters of multiple target genes, e.g., cell cycle regulators, a proapoptotic factor, and glucose metabolic enzymes (8,10–15). Once bound to the target gene promoters via IRS, the forkhead proteins act as a potent activators of transcription. In contrast, when PKB is activated by insulin, the forkhead proteins are phosphorylated, resulting in promotion of their cytoplasmic accumulation, thus inactivating their transcriptional functions (10,16).

Several studies have shown that FKHR controls the expression of glucose metabolic enzymes such as glucose-6-phosphatase catalytic-subunit (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (11–15). They are rate-limiting enzymes in homeostatic regulation of blood glucose concentration by breakdown of glycogen (glycogenolysis) and by de novo synthesis of glucose from noncarbohydrate precursors, such as lactate, pyruvate, glycerol, and alanine (gluconeogenesis). Although previous studies suggest that the PKB-mediated regulation of FKHR accounts for the effect of insulin to inhibit expression of the gluconeogenic genes, they also showed that another molecular mechanism contributes to the inhibitory effects of insulin on these genes expressions (11,13). For example, it is reported that insulin inhibits PEPCK gene transcription independently of the IRS within the PEPCK promoter, indicating that the phosphorylation of forkhead proteins by insulin is not sufficient to explain the suppressive effect of PEPCK gene expression (17). This observation suggests that FKHR induces PEPCK gene expression by an indirect transactivation mechanism through mediating the other transcription factors or coactivators.

Peroxisome proliferator–activated receptor-γ (PPAR-γ) coactivator-1 (PGC-1), first identified as a nuclear receptor coactivator that interacts with PPAR-γ, plays a key role in linking nuclear receptors to the transcriptional program of adaptive thermogenesis and oxidative metabolism (18,19,20). In addition, it is revealed that PGC-1 promotes transcription through the assembly of a complex containing histone acetyltransferase (HAT), such as cAMP response element binding protein (CREB)-p300 (CBP/p300) or steroid receptor coactivator-1 (SRC-1) (21). A recent study by Yoon et al. (22) has demonstrated that although PGC-1 mRNA is not significantly accumulated in the liver in the feeding state, PGC-1 is strongly induced in the liver in fasting mice and in three mouse models deficient for insulin action. Moreover, the overexpression of PGC-1 in hepatic cells increases glucose production and the transcription of genes encoding gluconeogenic enzymes. One of these genes PEPCK, is induced by PGC-1 cooperating with glucocorticoid receptor and the liver-enriched tran-

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ABCD, avidin-biotin conjugate DNA precipitation; CA, constitutively active; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; DN, dominant negative; FBS, fetal bovine serum; G-6-Pase, glucose-6-phosphatase catalytic-subunit; HNF, hepatocyte nuclear factor; IRS, insulin response sequence; luc, luciferase; PEPCK, phosphoenolpyruvate carboxykinase; PK, protein kinase; PPAR-γ, peroxisome proliferator–activated receptor-γ; PGC-1, PPAR-γ coactivator-1; PI, phosphatidylinositol.

scription factor hepatocyte nuclear factor (HNF)-4. On the other hand, Herzig et al. (23) has demonstrated that transgenic mice overexpressing a dominant-negative CREB in the liver exhibit fasting hypoglycemia and reduced expression of gluconeogenic enzymes. Furthermore, it has been shown that CREB binds to the target sequence (cAMP-response element [CRE]) in the PGC-1 promoter, thereby enhancing the expression of PGC-1 in response to cAMP. These findings represent a mechanism by which glucagon, which induces the liver's glucose output in the starved condition, can activate an entire program of gluconeogenesis by increasing in the levels of a coactivator protein, PGC-1. In contrast, they have also shown that the treatment of insulin reduces endogenous PGC-1 mRNA in hepatoma cells (23). Although insulin is usually considered to have the exact opposite effect of hormones like glucagon, adrenaline, and glucocorticoids, which promote glucose output from the liver, the underlying mechanism of how insulin suppresses the PGC-1 expression is still unclear. Hence, we set out to elucidate a negative regulation of PGC-1 expression by insulin.

Here, we show that FKHR participates in stimulation and insulin inhibition of the gene for PGC-1. FKHR-stimulated PGC-1 promoter activity or/and PGC-1 basal promoter activity is negatively modulated in PKB-dependent phosphorylation in HepG2 cells. We also identify the functional IRSs in the PGC-1 promoter as *cis*-acting elements for FKHR binding in vivo and in vitro. Furthermore, we find that activated PKB counteracts the induced PGC-1 promoter activity by FKHR wild-type but not by FKHR (3A) mutant deficient for PKB phosphorylation. These results suggest that PKB-mediated FKHR transinactivation in the PGC-1 gene confers the suppressive effect of insulin on PGC-1 expression.

RESEARCH DESIGN AND METHODS

Cell culture, transfections, and reporter gene assays. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were performed by the calcium phosphate method. pRSV-β-galactosidase plasmid was included in each transfection experiment to control for the efficiency of transfection. To ensure equal DNA amounts, empty plasmids were added in each transfection. After transfection, cells were incubated in DMEM supplemented with 10% FBS for 24 h. Thereafter, the medium was replaced with serum-free DMEM containing 0.1% BSA and incubation was continued for 18 h. When necessary, insulin or wortmannin were added. The luciferase activity was measured with Auto-Lumat (Berthold) and normalized for β-galactocidase activity in the same sample.

Cloning and plasmids. pcDNA3-FLAG-FKHR was generated by RT-PCR–based cloning of mouse FKHR cDNA into pcDNA3-FLAG vector as described previously (24). The putative PKB phosphorylation sites at Thr-24, Ser-253, and Ser-316 were mutated to Ala by PCR mutagenesis. A genomic clone encompassing human PGC-1 promoter extended from –992 to +90 bp relative to the transcription initiation site was subcloned into the *Bgl*II site of the promoterless pRL-basic luciferase reporter gene vector to generate PGC-1–promoter luc. The deletion mutants, various PGC-1 promoter (ΔIRS)-luc and (ΔCRE)-luc reporter plasmids, were generated by PCR mutagenesis. Constitutively active or dominant-negative PKB expression plasmids were obtained from Upstate Biotechnology.

Generation of anti-FKHR antibody. A rabbit polyclonal antibody specific for mouse FKHR was raised against the bacterially expressed GST-FKHR (541–652 a.a.) fusion protein. The purified protein was injected into a rabbit and boosted five times. Serum from immunized rabbits was affinity purified by use of the antigen (Tanpaku Seisei Kougyou, Gunma, Japan).

Chromatin immunoprecipitation assay. Cells were grown to 95% confluence in DMEM supplemented with 10% FBS. Thereafter, medium was replaced with serum-free DMEM containing 0.1% BSA and incubation was continued for 18 h. Following the treatment with 100 nmol/l insulin for 30 min or not, cells were washed twice with PBS and cross-linked with 1% formaldehyde at 37°C for 10 min. Cells then were rinsed with ice-cold PBS twice and collected into PBS containing protease inhibitors and centrifuged for 4 min. Cells were resuspended in SDS lysis buffer (1% SDS, 10 mmol/l EDTA, 50 mmol/l Tris-HCl, pH 8.1 and protease inhibitors) and incubated for 10 min on ice. The lysate was sonicated four times for 10 s each (MISONIX, microson) followed by centrifugation for 10 min. Supernatants were collected and diluted in chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/l EDTA, 167 mmol/l NaCl, 16.7 mmol/l Tris-HCl, pH 8.1, and protease inhibitors) followed by preclean with 30 μg sheared salmon sperm DNA, 5 μg normal rabbit IgG, and 60 μl protein G-Sepharose-50% slurry for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with anti-FKHR-C3 antibody. After immunoprecipitation, 60 μl protein G-Sepharose-50% slurry and 20 μg salmon sperm DNA were added and the incubation was continued for another 1 h. Precipitates were washed sequentially for 5 min each in low salt (0.1% SDS, 1% Triton X-100, 2 mmol/l EDTA, 20 mmol/l Tris-HCl, pH 8.1, and 150 mmol/l NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mmol/l EDTA, 20 mmol/l Tris-HCl, pH 8.1, and 500 mmol/l NaCl), and LiCl wash buffer (0.25 mol/l LiCl, 1% NP-40, 1% deoxycholate, 1 mmol/l EDTA, and 10 mmol/l Tris-HCl, pH 8.1). Precipitates were then washed twice with TE buffer and eluted twice with 1% SDS and 0.1 mol/l $NaHCO_3$. Eluates were pooled and heated at 65°C for at least 4 h to reverse the formaldehyde cross-linking. Following the addition of Protease K and incubated for 1 h at 45°C, DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation. Precipitated DNA fragments were analyzed by PCR amplification using primers detected against the human PGC-1 promoter region or the β-actin coding region as negative control. The PCR products were analyzed by electrophoresis using 0.7% agarose gel and visualized with ethidium bromide staining.

Avidin-biotin conjugate DNA precipitation assay. The following double stranded oligonucleotides were used, all biotinylated at the 3′-end of the sense strand, IRS1: AGTGTGTTGG*TATTTTT*CCCTCAGTTC, IRS2: ACATACAGGC*TATTTTG*-TTGATTAAAC, and IRS3: GCCACTTGCT*TGTTTTG*GAAGGAAAAT. The consensus insulin response sequences are italicized. 3×IRS consisted of three copies of the IRS derived from the IGFBP-1 promoter, while 3×IRS mut carried substitutions in IRS core residues of 3×IRS. An avidin-biotin conjugate DNA precipitation (ABCD) assay was performed by incubating cell extracts derived from HepG2 cells with double-stranded DNA immobilized on streptavidin agarose in binding buffer (20 mmol/l HEPES [pH 7.5], 150 mmol/l KCl, 0.5% Triton X-100, 0.5 mmol/l EDTA, 20 g/ml poly [dI-dC], and protease inhibitors). After 20 min of incubation at 4°C, the beads were washed four times with the same buffer and proteins were resolved by SDS-PAGE, followed by electrotransfer onto polyvinylidine fluoride membrane and probed with the anti-FKHR antibody. Chemiluminescent detection relied on horseradish peroxidase–conjugated secondary antibodies.

RESULTS

Insulin inhibits basal PGC-1 promoter activity via PKB-dependent pathway. A recent study has shown that insulin reduces endogenous PGC-1 mRNA in FAO hepatoma cells (23). This observation raises the question of how insulin modulates PGC-1 expression. To reveal the molecular mechanism whereby insulin suppresses PGC-1 expression, a promoter sequence of the human PGC-1 gene was isolated and inserted into a promoterless luciferase reporter gene plasmid. Because we found that a fragment of the human PGC-1 promoter from −992 to +90, relative to the transcription start site, contains three consensus IRSs, we hypothesized that a trans-factor(s) modulated by insulin signaling binds to IRSs and regulates the PGC-1 promoter activity.

To investigate the effect of insulin on PGC-1 promoter, we conducted a series of transient transfection assays in HepG2 cells. First, the reporter plasmid containing the human PGC-1 promoter (PGC-1 promoter luc) was transfected into HepG2 cells, following treatment with or without 10% FBS or 100 nmol/l insulin. Serum stimulation substantially suppressed PGC-1 promoter activity, and particularly, insulin treatment was far more effective in reducing it by 50% than incubation with serum-free me-

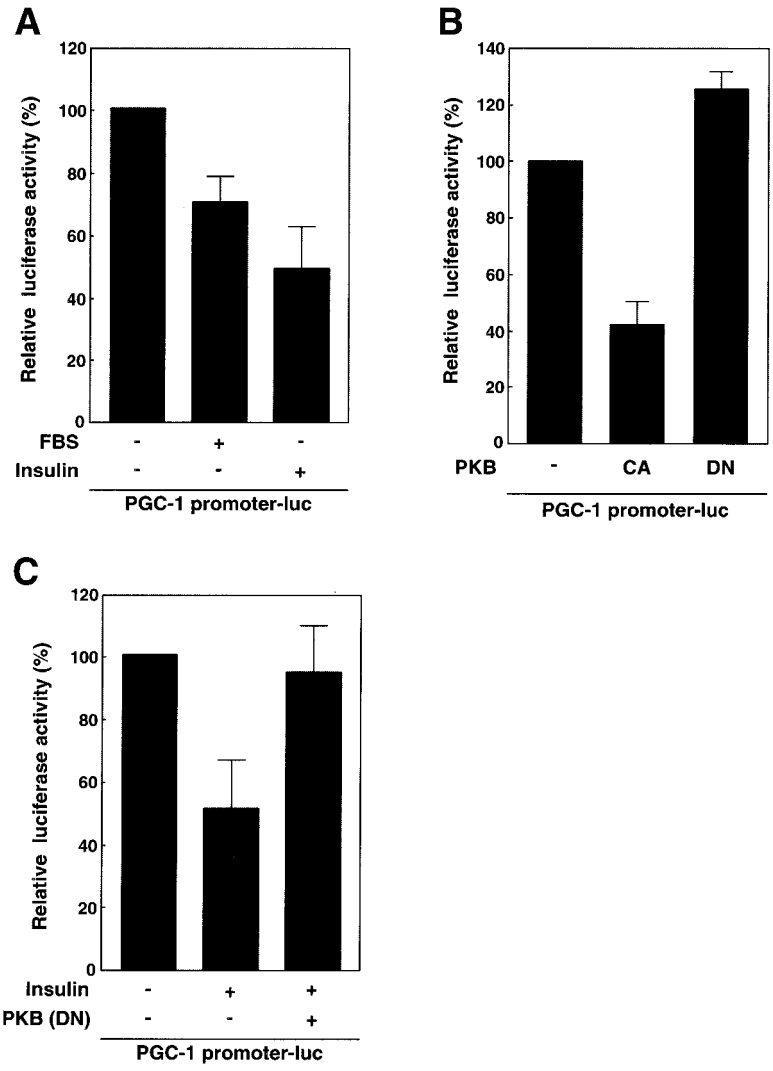


FIG. 1. Insulin inhibits basal PGC-1 promoter activity via PKB pathway. *A*: HepG2 cells were transfected with 100 ng human PGC-1 promoter luc plasmid containing promoter sequence from –992 to +90 (PGC-1 promoter luc). Following transfection, cells were incubated in the absence or presence of 10% FBS for 18 h and treated with or without 100 nmol/l insulin. *B*: HepG2 cells were transfected with 100 ng PGC-1 promoter luc together with 50 ng empty vector, constitutively active mutant PKB (CA), or catalytically inactive mutant PKB (DN) expression plasmids. Following transfection, cells were serum-starved for 18 h. *C*: HepG2 cells were transfected with 20 ng PGC-1 promoter luc together with 20 ng empty vector or PKB (DN) expression plasmid. Following transfection, cells were treated with 100 nmol/l insulin for 18 h. The results are presented as relative luc activities compared with the activity in serum-starved condition (*A* and *C*) or the activity after the coexpression of the empty vector (*B*), which was set as 100%.

dium (Fig. 1*A*). This is in agreement with previous data in which insulin suppresses PGC-1 gene transcription in intact hepatoma cells (23), and thus we considered that the PGC-1 promoter luciferase (luc) reporter plasmid included the abilities to mimic the inhibitory effect of insulin on PGC-1 expression. Next, because expression of active PKB is sufficient to mimic insulin's inhibitory effects on transcription through IRS-containing promoters (25–27), we tested whether the basal PGC-1 promoter activity is regulated by coexpression of a constitutively active (CA) or a dominant negative (DN) mutants of PKB. Figure 1*B* shows that transfected PKB (CA) mutant represses basal PGC-1 promoter activity to the same extent as insulin treatment (Fig. 1*A*), whereas no inhibition was detected with PKB (DN) mutant, suggesting that the inhibitory effect of insulin on the PGC-1 promoter are mediated by PKB. Furthermore, to confirm that the effect of insulin actually involves the PKB pathway, we investigated whether the insulin-inhibited PGC-1 promoter activity is recovered by coexpression of a PKB (DN). As expected, overexpressed PKB (DN) counteracted the inhibitory effect of insulin on the PGC-1 promoter (Fig. 1*C*). These results indicated that the PKB pathway acts downstream of insulin receptor signaling to modulate PGC-1 gene transcription in HepG2 cells.

Insulin inhibits FKHR-stimulated PGC-1 promoter activity. It has been shown that PKB phosphorylates the Forkhead transcription factor FKHR, and consequently represses its ability of transactivaton, probably by disturbing the nuclear import of FKHR (28–30). To study a potential role of FKHR in regulating PGC-1 promoter activity, HepG2 cells were cotransfected with the PGC-1 promoter luc and an FKHR expression plasmid. Under a condition of serum-starvation, transfected FKHR in HepG2 cells stimulated PGC-1 promoter activity in a dose-dependent manner, indicating that the transactivation of PGC-1 gene is indeed mediated by FKHR (Fig. 2*A*). Furthermore, to examine the effects of insulin on FKHR-induced PGC-1 promoter activity, HepG2 cells were cotransfected with the PGC-1 promoter luc and FKHR expression plasmids and then incubated in serum-free medium with or without insulin treatment (Fig. 2*B*). Compared with the control insulin-free condition, insulin was able to suppress FKHR-stimulated PGC-1 promoter activity by ~50% and to a similar extent for inhibiting basal activity (Fig. 2*B*). These results suggest that insulin inhibits induction of PGC-1 gene by specifically interfering with FKHR activity.

Insulin inhibits the binding of FKHR to PGC-1 promoter in vivo. In response to insulin, PKB-mediated phosphorylation is shown to modulate the function of

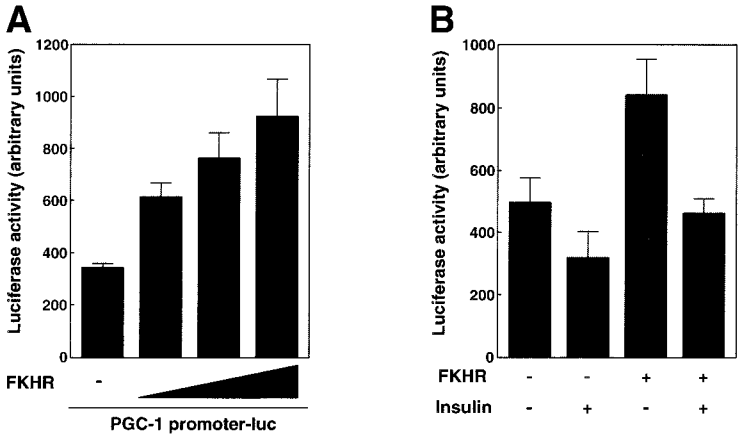


FIG. 2. FKHR activates PGC-1 promoter activity (*A*), and the FKHR-stimulated PGC-1 promoter activity is inhibited by insulin treatment (*B*). *A*: HepG2 cells were transfected with 100 ng PGC-1 promoter luc together with 10, 20, and 50 ng FKHR expression plasmids. Following transfection, cells were serum-starved for 18 h. The results are presented as arbitrary units. *B*: HepG2 cells were transfected with 100 ng PGC-1 promoter luc together with or without 20 ng FKHR expression plasmids. Following transfection, cells were serum-starved for 18 h in the presence or absence of 100 nmol/l insulin. The results are presented as arbitrary units.

FKHR, which directly binds to the IRS in several gene promoters in vitro (7,10–13,24). We have identified the three consensus IRSs in the PGC-1 promoter: IRS1, IRS2, and IRS3 (Fig. 3*A*). To investigate whether the PGC-1 promoter is a direct target for FKHR in vivo, we performed ChIP assays in the presence or absence of insulin treatment. HepG2 cells were serum-starved for 18 h followed by treatment with or without insulin, and then soluble chromatin was prepared from cultured cells after cross-linking. For immunoprecipitation of FKHR-bound genomic DNA fragments, a specific antibody against FKHR was used and the immunoprecipitated DNAs were analyzed by PCR using the specific pairs of primers spanning the IRSs (Fig. 3*A*). Figure 3*B* shows that the PGC-1 promoter was efficiently recovered from immunoprecipitates of FKHR but not of control normal rabbit IgG. No PCR product was obtained from normal rabbit IgG or FKHR immunoprecipitates using control β-actin primers. In addition, FKHR recruitment to the PGC-1 promoter was significantly decreased after insulin treatment. These data show that endogenous FKHR can bind to the chromatinized PGC-1 promoter in the serum-starved condition in HepG2 cells.

FKHR binds to PGC-1 promoter IRSs in vitro. Because the PGC-1 promoter is identified as an in vivo target for FKHR, we next determined whether FKHR could interact with three putative IRSs within the PGC-1 promoter in vitro. To this end, we performed an ABCD assay using biotin conjugate, double-stranded oligonucleotides that contains consensus insulin response sequences termed IRS1, IRS2, or IRS3 (Fig. 4*A*). Whole cell lysates extracted from intact HepG2 cells were mixed with each oligonucleotide immobilized on streptavidin agarose. As shown in Fig. 4*B*, endogenous FKHR strongly binds to IRS2 and IRS3, but weakly to IRS1 probe. In the control experiment, a biotinylated 3×IRS, which consists of the three copies of IRS derived from the IGFBP-1 promoter sequence, precipitated FKHR efficiently, whereas, a mutated IRS (3×IRS mut) did not. These findings indicate that FKHR binds to all putative IRSs in the PGC-1 promoter in vitro.

IRS elements are required for both the FKHR-induced and the basal PGC-1 promoter activity. Furthermore, to confirm the FKHR response region in the PGC-1 promoter, we performed the reporter assay using a series of putative IRS- and CRE-deleted promoter constructs. Respective IRSs located from –976 to –970 (IRS1), −586 to –580 (IRS2), and –354 to –348 (IRS3) are depicted in Fig. 3*A*, and the transcriptional activities of these constructs were tested in the presence or absence of the FKHR expression plasmid. Compared with intact PGC-1 promoter luc, the deletion of each IRS1, IRS2, or IRS3 reduced the FKHR-mediated activation by 30%, whereas no substantial activation was observed using the ΔIRS1/2/3 promoter, which lacks all the IRSs (Fig. 4*C*). Remarkably, this construct reduced basal promoter activity by 40% (data

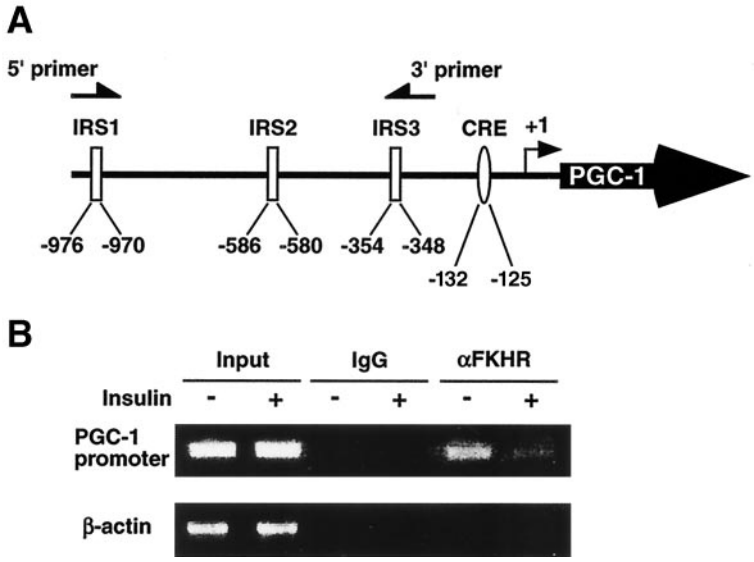


FIG. 3. Endogenous FKHR binds the PGC-1 gene regulatory region. *A*: A schematic representation of the human PGC-1 gene regulatory region. The presence of putative IRSs and a consensus CRE are shown by solid or oval boxes, respectively. Arrows indicate a primer pair used for amplifying corresponding the DNA fragment. The numbers are the positions upstream to the PGC-1 gene transcription start site (+1). *B*: Chromatin immunoprecipitation assays of FKHR occupancy on the human PGC-1 promoter region. HepG2 cells were serum-starved and treated with or without 100 nmol/l insulin. Soluble chromatin was immunoprecipitated with antibodies against FKHR or normal rabbit IgG as a negative control. Precipitated DNA fragments were analyzed by PCR amplification using primers against the human PGC-1 promoter region described in *A* or the β-actin coding region as a negative control.

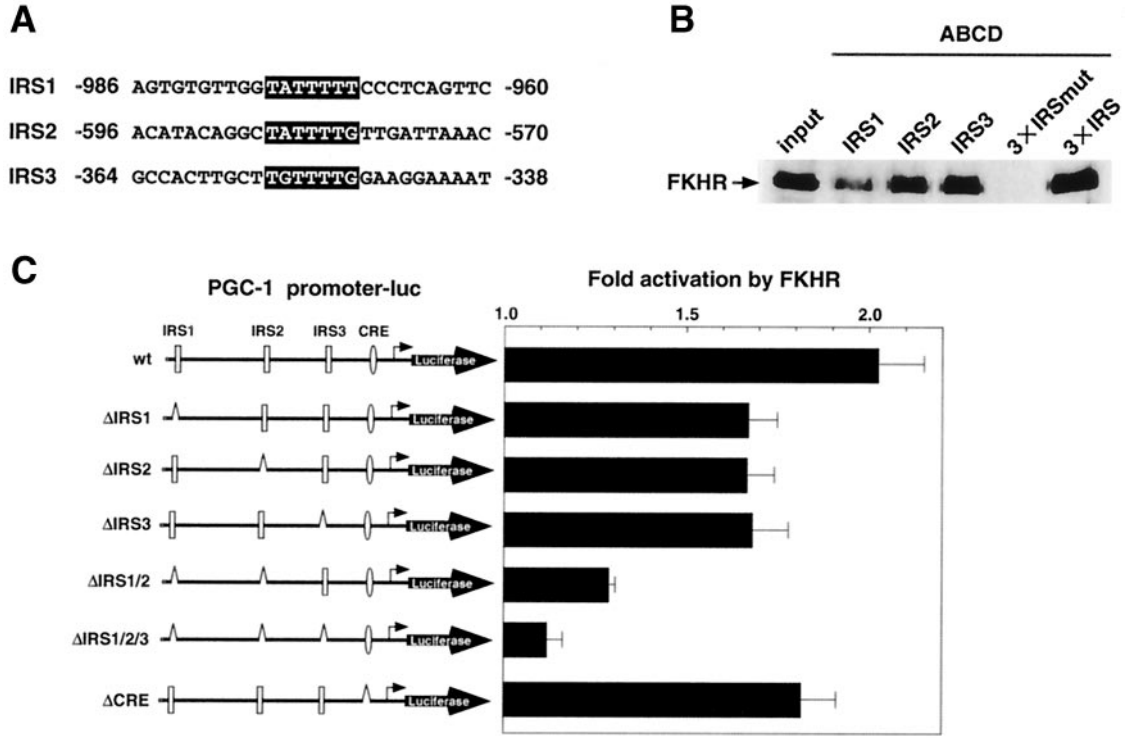


FIG. 4. FKHR binds all putative IRSs and potentiates PGC-1 promoter activity. *A*: The biotinylated oligonucleotides used in an ABCD assay are shown. Black boxes depict putative IRSs, termed IRS1, IRS2, and IRS3, respectively. *B*: The ABCD assay was performed using a double-stranded IRS1, IRS2, IRS3, 3×IRS mut, and 3×IRS with whole cell extract from nontransfected HepG2 cells. The 3×IRS and 3×IRS mut were three copies of the wild-type or mutated IRS derived from the IGFBP-1 promoter sequence, respectively. After washing the beads, proteins were detected by Western blot analysis. The input lane represents 5% of the total volume of whole cell extracts used for the binding assay. *C*: Effects of deletion of the IRSs on FKHR-induced PGC-1 promoter activity. HepG2 cells were transfected with 100 ng of PGC-1 promoter luc, various IRS-, or CRE-deleted mutants described in left panel, together with or without 50 ng FKHR expression plasmid. Following transfection, cells were serum-starved for 18 h. The results are presented as fold activation compared with the relative luc activities after the coexpression of the empty vector control, which was set as 1.

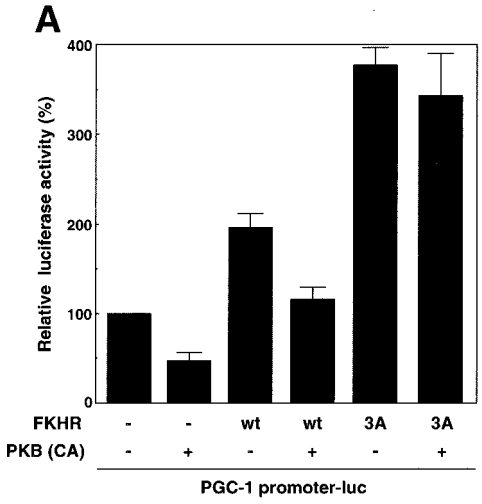
not shown), indicating that the three IRSs are required for maintaining the basal promoter activity in HepG2 cells. In contrast, the deletion of CRE (from –132 to –125) in the PGC-1 promoter had no influence on the extent of fold activation by FKHR compared with the intact PGC-1 promoter activity. Taken together, these results demonstrate that the three consensus IRSs, characterized in the PGC-1 promoter (−992 to +90), are required for both the basal and FKHR-stimulated reporter activity.

Phosphorylation of FKHR accounts for inhibitory effect of insulin on PGC-1 promoter activity. To determine whether PKB-dependent phosphorylation of FKHR is critical for the PGC-1 gene suppression, we used a FKHR (3A) mutant, which is mutated at the three PKB phosphorylation sites (T24, S253, and S316) to alanines. This mutant is known to be resistant to the inhibitory effect by phosphorylation, resulting in the promotion of nuclear retention and of access to the target sequences (7–10,28,29). Cotransfection assays showed that FKHR (3A) possessed the activity to induce the PGC-1 gene transcription about twofold more than wild-type FKHR, whereas that activity was not suppressed by coexpression of PKB (CA) compared with the suppression of basal or FKHR wild-type–stimulated activity (Fig. 5*A*). Consistent with the cotransfection assays, insulin was not able to inhibit FKHR (3A)-stimulated PGC-1 promoter activity (data not shown). These results indicate that phosphorylation of putative PKB sites in FKHR is required for PKB-dependent suppression of FKHR-stimulated PGC-1 promoter activity.

Furthermore, to examine whether the three IRSs in the PGC-1 promoter are sufficient for the inhibitory effect of insulin, HepG2 cells were transfected with intact or ΔIRS1/2/3 PGC-1 promoter luc and then incubated with insulin and the phosphatidylinositol (PI)-3 kinase inhibitor, wortmannin, as indicated in Fig. 5*B*. In the intact promoter, treatment with wortmannin abolished the inhibitory effect of insulin, revealing the involvement of PI3 kinase in the insulin-mediated PGC-1 inhibition. In contrast, although the inhibitory effect of insulin on the PGC-1 promoter was not completely diminished by eliminating all three IRSs, no substantial inhibition was observed by insulin and wortmannin treatments in the ΔIRS1/2/3 PGC-1 promoter (Fig. 5*B*). Remarkably, this construct reduced basal promoter activity by 50%, indicating that the three IRSs are required for maintaining the basal promoter activity in HepG2 cells. The attenuated inhibition in ΔIRS1/2/3 PGC-1 promoter implies that FKHR partially confers the inhibitory effect of insulin via the IRSs in the PGC-1 promoter.

DISCUSSION

Based on our results, we propose a model for the transcriptional regulation of the PGC-1 promoter in which both FKHR and CREB stimulate the promoter activity via each response element (Fig. 6). Glucagon is known to



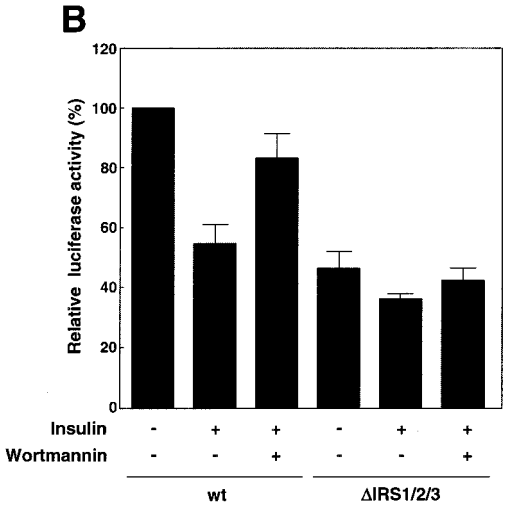


FIG. 5. Insulin inhibits PGC-1 promoter activity via IRSs. *A*: HepG2 cells were transfected with 100 ng PGC-1 promoter luc together with 20 ng empty vector, FKHR wild-type, or FKHR (3A) expression plasmids with or without 10 ng PKB (CA) expression plasmid. Following transfection, cells were serum-starved for 18 h. The results are presented as relative activities compared with that in cotransfection of empty vector (*lane 1*), which was set as 100%. *B*: HepG2 cells were transfected with 20 ng PGC-1 promoter luc, or all IRS-deleted mutant (PGC-1 ΔIRS1/2/3 promoter luc) plasmids. Following transfection, cells were serum-starved for 18 h in the presence or absence of 100 nmol/l insulin and 200 nmol/l wortmannin, which was added 30 min before insulin. The results are presented as relative activities compared with that in transfection of PGC-1 promoter luc in the absence of insulin or wortmannin (*lane 1*), which was set as 100%.

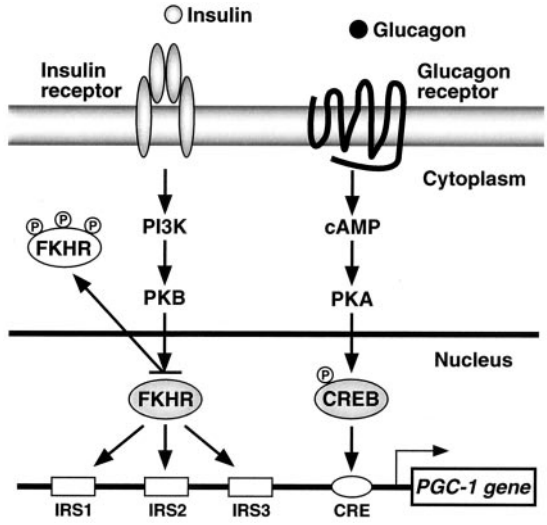


FIG. 6. Transcriptional regulation of the PGC-1 promoter by FKHR and CREB. A schematic model for PGC-1 gene transcription mediated by FKHR and CREB through each responsive element via different hormone signals. *Left*: insulin activates PI3 kinase signaling pathway, which stimulates phosphorylation of FKHR by PKB, followed by nuclear exclusion and repression of PGC-1 gene transcription. *Right*: Glucagon activates cAMP signaling pathway, which stimulates phosphorylation of CREB by PKA, and thereby induces the expression of PGC-1.

potentiate the cAMP signaling pathway, resulting in phosphorylation of CREB by PKA (protein kinase A), and consequently the phosphorylated CREB appears to recruit a coactivator CBP to the PGC-1 promoter through the CRE, and induce PGC-1 expression (23). In this study, we elucidated that FKHR activates the PGC-1 promoter through the three consensus IRSs identified, while insulin inhibits both basal and FKHR-stimulated PGC-1 promoter activities by PKB-dependent phosphorylation of FKHR (Figs. 1–5). These findings are reminiscent of a recent study analyzing transgenic mice with the cardiac-specific expression of activated PKB (31). The significant down-regulation of PGC-1 is observed by overexpression of activated PKB, indicating that PKB signaling suppresses the basal PGC-1 expression in vivo.

Alternatively, we found that the PGC-1 promoter was partially suppressed by insulin (Fig. 5*B*) or transfected PKB (CA) (data not shown), even after deletion of the three IRSs (Fig. 5*B*). A possible explanation for this inhibition is that a ΔIRS1/2/3 PGC-1 promoter construct still contains the functional CRE. A previous study demonstrated that insulin-dependent activation of the Ras pathway represses CREB activity by inducing recruitment of the S6 kinase $pp90_{RSK}$ to CBP (32). Therefore, binding of $pp90_{RSK}$ to CBP might counteract basal cAMP signal transduction, and thereby suppresses the ΔIRS1/2/3 PGC-1 promoter activity. In addition to the PGC-1 promoter, similar observations are reported in the G-6-Pase and PEPCK genes, where disruption of IRS does not completely impair the inhibitory effect of insulin on their promoters (11,17). These findings suggest that other *cis*-activating elements or *trans*-acting factors also play roles in mediating the effect of insulin signaling.

The genes encoding limiting enzymes for gluconeogenesis are powerfully controlled at the transcriptional level by key hormones, particularly insulin, glucagon, and glucocorticoid. Although recent data have shown a critical role for PKB in insulin-dependent inhibition of hepatic gluconeogenesis, it has not been elucidated whether that component is involved in the regulation of PGC-1 expression (33). In this paper, we show that insulin can suppress PGC-1 promoter activity by mediating the function of FKHR via PKB signaling pathway. This finding provides an idea that FKHR can regulate PEPCK gene expression, not only through direct binding to that promoter, but also through indirect activation, in which the induced PGC-1 protein works as a coactivator with HNF-4 and glucocorticoid receptors in PEPCK gene transcription (22). This two-dimension model may explain a paradox that insulin inhibits PEPCK gene transcription independently of the IRS on that promoter (17).

The liver-specific insulin-receptor knockout (LIRKO) mice are unique models exhibiting dramatic insulin resistance in the liver, severe glucose intolerance, and the failure of insulin to suppress hepatic glucose production (34). This line of the mice also displays the transcriptional induction of G-6-Pase and PEPCK genes in the liver. Interestingly, recent data have shown that the expression of PGC-1 distinctively increases in the livers of the LIRKO mice (22). Although an indirect effect of insulin cannot be excluded, this in vivo observation implies that insulin is the dominant regulator of PGC-1 expression, possibly through FKHR-regulated transcription. Here, we propose a model in which FKHR is essential for maintaining basal PGC-1 expression, while CREB is necessary for stimulating further induction of PGC-1 by glucagon in the starved condition.

Our results argue for a three-stage model in the activation of PGC-1 gene by FKHR and CREB from feeding to fasting. First, in the feeding stage, glucagon secretion drops to the basal level while insulin increases to the maximal level, resulting in phosphorylation of sole FKHR by PKB. Because phosphorylated FKHR is excluded from the nucleus and unphosphorylated CREB cannot recruit CBP as a coactivator, neither of them can stimulate PGC-1 transcription. Hence, hepatic gluconeogenesis through the induced expression of PGC-1 would be almost eliminated in the feeding stage. Second, during early fasting stage (interphase), glucagon secretion gradually goes up while insulin level moderately drops, consequently increasing in the proportions of phosphorylated CREB and unphosphorylated FKHR, and then both activated transcription factors can induce PGC-1 expression. Third, under prolonged fasting stage (so called starved), glucagon secretion further increases to the maximal level while insulin decreases to the basal level, leading to CREB phosphorylation by PKA and preventing FKHR from PKB-dependent phosphorylation. Consequently, both phosphorylated CREB and unphosphorylated FKHR may synergistically induce PGC-1 expression, and hence the hepatic gluconeogenesis occurs rapidly to maintain basal blood glucose level for protecting the body against hypoglycemia.

In addition to the previous reports in which FKHR was shown to play an important role in mediating the expression of gluconeogenic genes including G-6-Pase and PEPCK, we have shown here that FKHR also regulates PGC-1 expression in the transcriptional level. This result suggests that the molecular mechanism of FKHR transcription is principally linked to regulating gluconeogenesis. Because excessive hepatic glucose production by the gluconeogenesis is known to be a major contributor to both the fasting hyperglycemia and the exaggerated postprandial hyperglycemia in diabetes (35), controlling FKHR functions in the liver could be a remarkable target for antidiabetic therapy. To conduct that strategy, however, further studies would be required to elucidate the tissue-specific physiological significance of FKHR functions.

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