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Peroxisome Proliferator-Activated Receptor-α Regulates the Expression of Pancreatic/Duodenal Homeobox-1 in Rat Insulinoma (INS-1) Cells and Ameliorates Glucose-Induced Insulin Secretion Impaired by Palmitate

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Both peroxisome proliferator-activated receptor-α (PPARα) and pancreatic/duodenal homeobox-1 (PDX-1) have been reported to be associated with glucose-stimulated insulin secretion (GSIS), but the relationship between PPARα and PDX-1 is not yet fully understood. In the present study, we tested the hypothesis that PPARα regulates the expression of PDX-1 in β-cells. Isolated pancreatic islets from Wistar rats and rat pancreatic insulinoma (INS-1) β-cells were cultured in media supplemented with and without 0.2 or 0.4 mM palmitate, and treated with and without a PPARα agonist (fenofibrate) or PPARα antagonist (MK886). Results indicated that treatment with fenofibrate significantly enhanced PPARα mRNA and protein expression in cells cultured with elevated palmitate concentrations compared with cells that did not receive fenofibrate treatment. In turn, this enhanced expression led to an increase in PDX-1 mRNA and nuclear protein, as well as DNA binding activity of PDX-1 with the insulin promoter. Accordingly, the expression of the PDX-1 downstream targets, insulin and glucose transporter-2, increased, resulting in increased intracellular insulin content and GSIS. Treatment with MK886 inhibited expression of PPARα, blocking PPARα-regulated PDX-1 expression, and the downstream transcription events of PDX-1. EMSA revealed that nuclear protein might bind with the peroxisome proliferator response element sequence located in the PDX-1 promoter. Collectively, these results demonstrate a regulatory relationship between PPARα and PDX-1 in INS-1 cells. Furthermore, PPARα activation potentiates GSIS under elevated palmitate conditions possibly via up-regulation of PDX-1. Our findings have potential clinical implications for the use of PPARα agonists in the treatment of type 2 diabetes. (*Endocrinology* 149: 662–671, 2008)

CHRONIC EXPOSURE of pancreatic β-cells to elevated levels of circulating free fatty acids (FFAs) is implicated in β-cell dysfunction, with the inhibition of glucose-stimulated insulin secretion (GSIS) both *in vivo* and *in vitro* (1, 2). Exposure to FFAs has influenced the expression of the transcription factor, pancreatic/duodenal homeobox-1 (PDX-1), which in turn affects GSIS by altering the expression of the insulin gene (3). Peroxisome proliferator-activated receptor-α (PPARα) is also influenced by FFAs (3) and may prevent fatty acid-induced impairment of GSIS in pancreatic islets (4). To date, the relationship between PPARα and PDX-1 has not yet been fully elucidated.

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Abbreviations: BIS, Basal insulin secretion; DAPI, 4,6-diamidino-2-phenylindole; FFA, free fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT2, glucose transporter-2; GSIS, glucose-stimulated insulin secretion; INS-1, rat insulinoma; IP, immunoprecipitation; ISI, insulin secretion index; PDX-1, pancreatic/duodenal homeobox-1; PPARα, peroxisome proliferator-activated receptor-α; PPRE, peroxisome proliferator response element.

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PPARα is a ligand-activated transcription factor that is expressed in a broad range of tissues that include the liver, heart, kidney, skeletal muscle, and pancreas (5–7). Upon activation and binding by their ligands, PPARα regulates the expression of its target gene by binding to a specific peroxisome proliferator response element (PPRE) in the enhancer site of its targeted gene(s) (8, 9). These genes include those involved in cellular uptake, cellular transport, and β-oxidation of fatty acids (9–12). Clinically, PPARα ligands are used to treat patients with dyslipidemia. There is also evidence to indicate that the activation of PPARα may have effects on insulin secretion (13–17). Yoshikawa *et al*. (3) reported that isolated pancreatic islets exposed to FFAs for 48 h exhibited both a decrease in the expression of PPARα, and a decrease in GSIS. In another study, Wang *et al*. (18) reported that augmented PPARα mRNA expression in pancreatic islets of Zucker diabetic fatty rats was associated with the restoration of normal levels of insulin and GSIS.

PDX-1 plays a central role in pancreatic β-cell differentiation and insulin secretion (19–21). It controls the expression of insulin and other β-cell-specific genes, such as glucose transporter-2 (GLUT2), by transactivation at the transcriptional level (22–24). Gremlich *et al*. (25) reported that palmi-

tate treatment of isolated pancreatic islets induced a marked decrease in PDX-1 mRNA and protein expression, as well as in PDX-1 binding activity with insulin and GLUT2 genes, which was associated with a decline in insulin secretion.

Collectively, these findings suggest that a link between PPARα and PDX-1 may exist in pancreatic β-cells under elevated palmitate conditions. Currently, details of this relationship and knowledge of its existence under normal palmitate concentrations are unknown.

In the present study, we hypothesized that a regulatory interaction exists between PPARα and PDX-1 in pancreatic β-cells exposed to FFAs. To test this, we exposed isolated pancreatic islets and rat insulinoma (INS-1) β-cells to normal and elevated concentrations of palmitate, and measured the effects on insulin content and secretion, and PPARα and PDX-1 expression. Data from this study may provide evidence that PPARα is a potent regulator of PDX-1, and improves β-cell insulin secretion under normal or elevated palmitate concentrations.

Materials and Methods

Animals

Male Wistar rats (weighing ~230–275 g) were used. All experiments were approved by the animal ethics committee of Shandong University, China.

Isolation of pancreatic islets

Pancreatic islets were isolated via collagenase digestion, as previously described (26). Islets were maintained in complete RPMI 1640 medium (Invitrogen, Grand Island, NY) containing 11.1 mM glucose and 20% (vol/vol) fetal bovine serum (Invitrogen) for 24 h in a humidified atmosphere (5% CO_2, 95% air) at 37 C.

Cell and islet culture and treatment

The INS-1 cell line was donated by Nanjing Medical University, China. The INS-1 cells (passages < 40) and isolated pancreatic islets were grown in monolayer culture, as described previously (27) in RPMI 1640 medium containing 11.1 mM glucose supplemented with 10 mM HEPES, 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere (5% CO_2, 95% air) at 37 C.

When the INS-1 cells reached 80–90% confluence, or the islets had been cultured for 1 d, the cells were washed, and fresh media were added. The replacement media were supplemented with either 0.2 or 0.4 mM palmitate (Sigma-Aldrich, St. Louis, MO), and either 5 μM fenofibrate, a PPARα agonist (kindly donated by Laboratories Fournier SA, Rue de Pres Potets; Fontaine-les-Dijon, France), or 1 μM MK886, a PPARα antagonist (BIOMOL Internatiional, Plymouth Meeting, PA), for 24 or 48 h. Palmitate was dissolved in 95% (vol/vol) ethanol (3). Fenofibrate and MK886 were dissolved in dimethylsulfoxide. As controls, the islets and INS-1 cells were also cultured in media supplemented with an equivalent volume of the vehicle used in each experiment.

In addition, another INS-1 stable cell line, Pdx-1 no. 6 (donated by Professor Haiyan Wang, University Medical Center, Geneva, Switzerland) was used. Under normal conditions, the PDX-1 expression is very weak in this cell line relative to INS-1 cells. However, when the media are supplemented with doxycycline (Sigma-Aldrich), PDX-1 expression can be induced. The Pdx-1 no. 6 cells were cultured in RPMI 1640 medium supplemented with 100 μg/ml hygromycin and 100 μg/ml G418 (Sigma-Aldrich) (28). Increasing concentrations of doxycycline (0, 75, 150, and 500 ng/ml) were added to the culture medium for 24 or 48 h.

Insulin secretion and insulin content assays

Pancreatic islets (10 islets per well) and INS-1 cells (2×10^5 cells per well) were cultured in the different treatment media (described previously) in 24-well plates. Cells were then washed and preincubated for 30 min in Krebs-Ringer bicarbonate buffer containing 3 mM glucose. After preincubation, the buffer was replaced with fresh Krebs-Ringer bicarbonate buffer containing 3 or 20 mM glucose. After 20-min incubation, the media were collected, and the insulin content within the media was measured using an insulin RIA kit (Beijing Atom HighTech Co. Ltd., Beijing, China). To measure the total intracellular protein content, cells were collected using radioimmunoprecipitation assay lysis buffer (Shenneng Bo Cai Co. Ltd., Shanghai, China) containing 1× PBS, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 0.5% sodium deoxycholate, and 1 mM sodium orthovanadate. Intracellular protein concentration was determined using a BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA). Insulin levels within the media (insulin secretion) were normalized based on the respective intracellular protein content. Each experiment was repeated four times.

For the total intracellular insulin content, the acid/ethanol method described by Hamid *et al.* (29) was used, and insulin was measured by RIA (as described previously). Total protein content was determined as described previously. The intracellular insulin content was normalized based on the respective cellular protein content. Each experiment was repeated four times.

RNA isolation and real-time PCR.

Total RNA was extracted from all cells (1×10^6 cells per well) and islets (100 islets) using the TRIzol (Invitrogen Corp., Carlsbad, CA) method. First-strand cDNA was generated using a commercial Takara RT kit (TaKaRa, Otsu, Shiga, Japan), and amplified by real-time PCR (30) using a QuantiTect SYBR Green kit (QIAGEN, Hilden, Germany) and the ABI 7700 Prism real-time PCR instrument and software (Applied Biosystems, Foster City, CA). Primer sequences used in the PCR are provided in Table 1. All quantifications were performed with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. The PCR was performed for 40 cycles at 95 C for 15 sec, 60 C for 30 sec, and 72 C for 30 sec. The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (31, 32), and the results were expressed as extent of change with respect to control values.

In addition, mRNA levels of PDX-1, PPARα, GLUT2, and insulin within the Pdx-1 no. 6 cells were determined by RT-PCR using a GeneAmp 9700 PCR instrument (Applied Biosystems). The PCR conditions were as follows: 1 cycle at 95 C for 5 min; 35 cycles at 94 C for

TABLE 1. Sequence information on the primers used for real-time RT-PCR

Genes	Sequences (5′–3′)	Product size (bp)	Annealing temperature (C)	GenBank accession no.
PPARα	TGTCACACAATGCAATCCGTTT TTCAGGTAGGCTTCGTGGATTC	150	60	NM_013196
PDX-1	AAACGCCACACACAAGGAGAA AGACCTGGCGGTTCACATG	150	60	NM_022852
GLUT2	CAGCTGTCTCTGTGCTGCTTGT GCCGTCATGCTCACATAACTCA	150	60	NM_012879
Insulin	TCTTCTACACACCCATGTCCC GGTGCAGCACTGATCCAC	149	60	NM_019130
GAPDH	TGGTGGACCTCATGGCCTAC CAGCAACTGAGGGCCTCTCT	105	60	XM_344448

30 sec, 60 C for 30 sec, and 72 C for 30 sec; and 1 cycle at 72 C for 5 min. PCR products were resolved by electrophoresis on 2% agarose gels.

Protein analysis by Western blotting

To measure the expression of the GLUT2 protein, INS-1 cells were first extracted using radioimmunoprecipitation assay lysis buffer supplemented with 1 mM phenylmethylsulfonylfluoride. Nuclear extracts of INS-1 cells for the detection of PDX-1 were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Protein concentration was determined by BCA assay. Protein extracts (60 μg total protein for GLUT2 and 20 μg nuclear protein for PDX-1) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Billerica, MA). Membranes were incubated overnight at 4 C with rabbit anti-PDX-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or goat anti-GLUT2 antibody (Santa Cruz Biotechnology). Proteins were visualized by chemiluminescence (Amersham, Buckinghamshire, UK). Membranes were then reblotted with mouse anti-β-actin monoclonal antibody (Abcam, Cambridge, UK).

Immunoprecipitation (IP)

Total INS-1 cell lysate was prepared, and protein concentration was determined via BCA assay, as described previously. To initiate IP, 2 μg (10 μl) goat anti-PPARα antibody (Santa Cruz Biotechnology) was added into each Eppendorf tube containing 500 μl whole cell lysate (total 500 μG protein). Tubes were incubated at 4 C overnight with constant agitation.

After incubating with 20 μl Protein G agarose beads (Upstate, Lake Placid, NY) at 4 C for 2 h, samples were washed with cold IP buffer three times, then resuspended, and boiled in 60 μl double-concentrated electrophoresis sample buffer [125 mM Tris (pH 6.8), 4% sodium dodecyl sulfate, 10% glycerol, 0.006% bromophenol blue, and 2% β-mercaptoethanol]. PPARα was detected by Western blotting analysis using mouse anti-PPARα antibody (Abcam).

Immunofluorescence

The location and expression of PDX-1 and insulin were examined by immunofluorescence, according to methods reported by Wang *et al*. (28). INS-1 cells were plated on polyornithine-coated glass coverslips and treated as described previously. Rabbit anti-PDX-1 (Santa Cruz Biotechnology) and mouse anti-insulin (Dako, Glostrup, Denmark) antibodies were used. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (blue) (Vector Laboratories, Burlingame, CA), and the resultant immunofluorescence was viewed under a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). All images were acquired using the same intensity and photodetector gain to allow quantitative comparisons of the relative levels of immunoreactivity between sections.

Nuclear extract preparation and EMSA

Nuclear proteins from INS-1 cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce), and the protein concentration was determined using the BCA protein assay kit. The EMSA was performed using a commercial Lightshift Chemiluminescent EMSA Kit (Pierce), in accordance with the manufacturer's instructions. Probes end labeled with biotin (Invitrogen) and containing a consensus TAAT sequence (*underlined*) in the A1 site of the insulin promoter were used to assess PDX-1 DNA binding activity (sense: GCCCTTAATGGGCC; antisense: CGGGAATTACCCGG) (33).

PPARα DNA binding activity was examined with Gel Shift Assay Core System (Promega, Madison, WI). A double-stranded 28-oligomer oligonucleotide containing the most common PPRE consensus sequence [(*underlined*) sense: TGGACCAGGACAAAGGTCACGTTCGGGA; antisense: ACCTGGTCCTGTTTCCAGTGCAAGCCCT] (34), or corresponding to the PPRE of PDX-1 (GenBank, U39640: sense, GCAGGGCCAGGCCAATGGTGGCCCCAGGCT; antisense, CGTCCCGGTCCGGTTACCACCGGGGTCCGA) was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The binding reactions were performed at 37 C for 45 min, and the products were separated by electrophoresis on a 4% nondenaturating polyacrylamide gel. The protein-DNA complexes were visualized by autoradiography.

The specificity of the DNA-protein binding was determined by competition reactions in which a 200-fold molar excess of unlabeled oligonucleotide (specific competitor) was added to the binding reaction before the addition of the labeled probe.

Statistical analysis

Each experiment was performed at least three times. All values were given as mean ± SD. Statistical analyses were performed using a one-way ANOVA, with a significance level set at 5%.

Results

Ligand activation of PPARα ameliorates GSIS impaired by palmitate in pancreatic islets

To investigate the effects of activated PPARα on β-cell insulin secretion under elevated or normal palmitate concentrations, we cultured isolated rat pancreatic islets in media with (0.2 or 0.4 mM) or without palmitate, and treated with and without 5 μM fenofibrate (a PPARα agonist) for 24 h. The cells were then stimulated with 3 and 20 mM glucose, with the amount of insulin subsequently secreted measured as basal insulin secretion (BIS) and GSIS, respectively (Fig. 1A). The insulin secretion index (ISI) was then derived as follows: the amount of insulin after 20 mM glucose

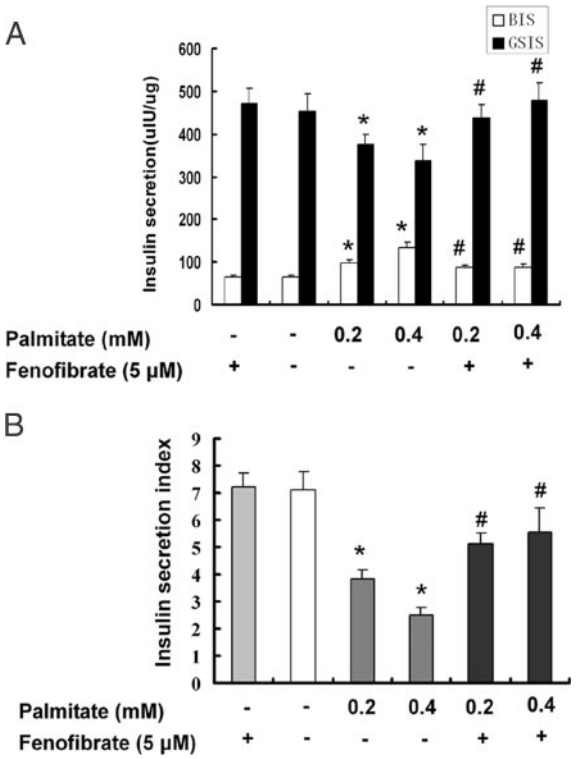

FIG. 1. Ligand activation of PPARα ameliorates GSIS impaired by palmitate in pancreatic β-cells. Isolated rat pancreatic islets were treated with the PPARα agonist, fenofibrate, in the absence or presence of either 0.2 or 0.4 μM palmitate for 24 h. Insulin secretion was measured by RIA. Insulin concentration was adjusted for the intracellular protein content. A, The *white column* represents BIS induced by 3 mM glucose. The *black column* represents GSIS induced by 20 mM glucose. B, The ISI was derived as follows: GSIS/ BIS. The data presented are based on the results of four separate experiments and expressed as mean ± SD. *, $P < 0.05$ *vs*. controls. #, $P < 0.05$ *vs*. cells treated with equimolar palmitate.

stimulation (GSIS) divided by the amount of insulin after 3 mM glucose stimulation (BIS) (Fig. 1B).

Compared with the control (no palmitate: GSIS, 453 ± 40.7 μIU/μg; and BIS, 63.84 ± 4.43 μIU/μg), increasing palmitate concentrations significantly reduced GSIS to 375 ± 24.9 μIU/μg (0.2 mM palmitate) and 336 ± 40.6 μIU/μg (0.4 mM palmitate). In addition, elevated palmitate enhanced BIS (0.2 mM, 98 ± 7.1 μIU/μg; and 0.4 mM, 134 ± 12.3 μIU/μg). Accordingly, palmitate decreased the ISI compared with the control in a dose-dependent manner (0.2 mM: ISI = 54%, $P < 0.05$; and 0.4 mM: ISI = 35%, $P < 0.05$).

When the palmitate-cultured cells were treated with fenofibrate, GSIS levels were restored to 437 ± 30.8 μIU/μg (0.2 mM palmitate) and 479 ± 41.2 μIU/μg (0.4 mM palmitate). BIS levels were also restored to normal in palmitate-cultured cells treated fenofibrate. The ISI value was 1.32-fold higher in fenofibrate-treated cells cultured in 0.2 mM palmitate compared with those without fenofibrate ($P < 0.05$), and 2.21-fold higher in 0.4 mM palmitate-cultured cells ($P < 0.05$). These results suggest that the activation and binding of PPARα restore the palmitate-induced suppression of GSIS in rat pancreatic islets.

Ligand activation of PPARα increases the PDX-1 expression in pancreatic islets

To gain insight into the functional changes in β-cells during ligand activation of PPARα in palmitate-treated pancreatic islets, we performed real-time PCR for PDX-1 mRNA, and Western blotting analyses for PDX-1 nuclear expression. The levels of PDX-1 mRNA in palmitate-treated cells were significantly lower ($P < 0.05$) than those in the cells that were not cultured in palmitate-supplemented media and/or treated with 5 μM fenofibrate (Fig. 2A). This result was confirmed by Western blotting (Fig. 2B).

Ligand activation of PPARα by fenofibrate blunted the suppressant effects of palmitate on GSIS, and was also associated with overexpression of PDX-1 mRNA and protein (Fig. 2, A and B). Fenofibrate-treated cells not exposed to palmitate showed a slight, but not marked, enhancement in PDX-1 mRNA levels ($P > 0.05$).

Due to the involvement of PDX-1 in the transcriptional regulation of the insulin and GLUT2 genes within β-cells, we also investigated the effects of PPARα activation on the levels of insulin and GLUT2 mRNA. The mRNA levels of insulin and GLUT2 were lower in the palmitate-cultured cells (0.2 or 0.4 mM) (insulin: 43.6–58.9% of control, $P < 0.05$; GLUT2: 50.5–71.4% of control, $P < 0.05$) (Fig. 2, C and D). The expression levels of insulin mRNA and GLUT2 mRNA were restored to normal with 5 μM fenofibrate treatment. Fenofibrate treatment also induced a small increase in the expression of insulin and GLUT2 ($P > 0.05$) in cells not exposed to palmitate.

Effects of PPARα ligand activation in INS-1 cells

Our findings in isolated rat pancreatic islets prompted us to investigate further the regulatory role of PPARα using INS-1 β-cells. The INS-1 cells were cultured in media with (0.2 or 0.4 mM) or without palmitate, and were treated with and without 5 μM fenofibrate (a PPARα agonist) for 24 h.

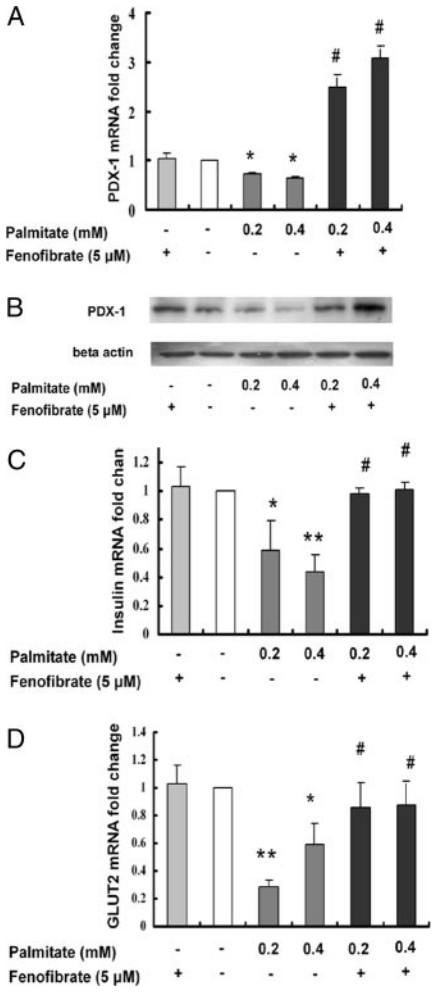

FIG. 2. Activation of PPARα up-regulates PDX-1 expression in pancreatic β-cells cultured in palmitate-enriched media. Isolated rat pancreatic islets (100 islets) were treated with the PPARα agonist, fenofibrate in the absence or presence of either 0.2 or 0.4 μM palmitate for 24 h. The mRNA levels of PDX-1 (A), insulin (C), and GLUT2 (D) were determined by real-time PCR. B, The protein expression of PDX-1 was detected by Western blotting. All values were normalized to 100% for the value of control and expressed as the percentage of control. Data are expressed as mean ± SD. The data presented are based on the results of four separate experiments. *, $P < 0.05$, **, $P < 0.01$ *vs.* controls. #, $P < 0.01$ *vs.* cells treated with palmitate.

Changes in insulin secretion in the INS-1 cells were consistent with those observed in the rat islets (Fig. 3, A1 and A2), *i.e.* GSIS and ISI decreased, whereas BIS increased in palmitate-treated cells. In addition, both GSIS and BIS levels were restored by fenofibrate treatment ($P < 0.05$). These results allowed us to use INS-1 cells as a model for further characterization and better understanding of PPARα regulation.

We first assessed PPARα protein expression using IP (Fig. 3B). The weakest band represented the cells that had been supplemented with 0.2 mM palmitate alone. By contrast, the addition of fenofibrate strengthened the band intensity regardless of palmitate presence or concentration.

As shown in Fig. 3C, the PDX-1 mRNA levels were significantly lower ($P < 0.05$) in palmitate-cultured cells compared with cells not cultured with palmitate. Fenofibrate treatment of palmitate-cultured cells restored PDX-1 mRNA

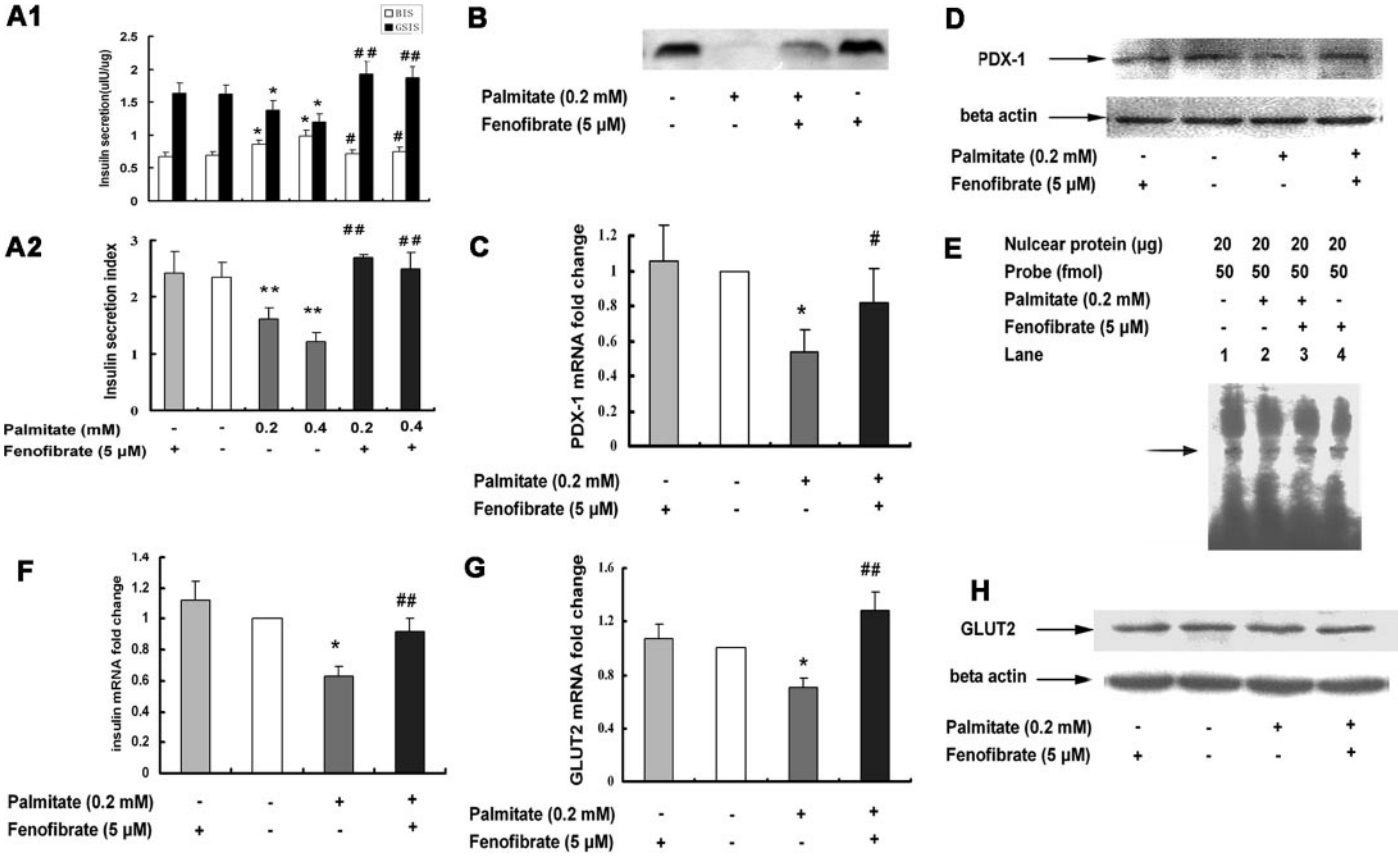

FIG. 3. The effects of activation of PPARα in INS-1 cells. INS-1 cells were treated with the PPARα agonist, fenofibrate in the absence or presence of either 0.2 or 0.4 μM palmitate for 24 h. A, The insulin concentrations within the different media were assayed by RIA, and were adjusted by intracellular protein content. A1, The *white column* represents BIS induced by 3 mM glucose. The *black column* represents GSIS induced by 20 mM glucose. A2, The ISI was derived as follows: GSIS/ BIS. Results are displayed as mean ± SD (n = 4). *, $P < 0.05$, **, $P < 0.01$ *vs.* control. #, $P < 0.05$, ##, $P < 0.01$ *vs.* cells treated with palmitate. B, The protein expression of PPARα was detected by IP. Results are based on those from three separate experiments. C, F, and G, The mRNA expression levels of PDX-1, insulin, and GLUT2 were measured by real-time PCR. The mRNA level was expressed as the percentage of control (100%). Results are displayed as mean ± SD (n = 4). *, $P < 0.05$, *vs.* control. #, $P < 0.05$, ##, $P < 0.01$ *vs.* cells treated with palmitate. D and H, The total protein expression of GLUT2 and the nucleic protein expression of PDX-1 were measured by Western blotting. Results are based on those from three separate experiments. E, The DNA binding activity of PDX-1 with insulin I promoter was measured by EMSA. The specific binding band is marked with an *arrow*. Results are based on those obtained from four separate experiments.

levels. Western blot analysis of nuclear PDX-1 expression confirmed this result (Fig. 3D); a stronger band was present for the cells treated with a combination of fenofibrate and palmitate, compared with the cells cultured with palmitate only.

Immunofluorescence with double staining for PDX-1 and insulin showed that the change in PDX-1 protein occurred principally within the cell nucleus (sections were costained with PDX-1 and insulin, with the nucleus stained in blue with DAPI). As shown in Fig. 4, intense staining for PDX-1 was observed in the nucleus, whereas staining for insulin was observed in the cytoplasm of cells treated with fenofibrate with or without palmitate. These observations were in sharp contrast to the weaker staining observed in the cells treated with palmitate alone. These results indicate that activation of PPARα restored the expression of PDX-1.

To confirm the functional changes of downstream of PDX-1 in accordance with the PPARα activation observed in our study, we tested the binding activity of PDX-1 with its direct downstream targets, insulin and GLUT2. First, the DNA binding activity of PDX-1 nuclear protein to the insulin I promoter was tested by EMSA, using the probes described in *Materials and Methods*. The results (Fig. 3E) showed a stronger band for the cells exposed to palmitate and fenofibrate, compared with those exposed to palmitate alone. This demonstrated that PPARα activation increased the DNA binding activity of PDX-1 protein with the insulin promoter.

Second, gene transcription levels of insulin and GLUT2 were assessed using real-time PCR. The differing levels of insulin mRNA expression were consistent with the different levels of PDX-1-insulin binding activity observed (Fig. 3F).

PPARα activation also increased the expression of GLUT2 mRNA (Fig. 3G), but not the expression of GLUT2 protein (Fig. 3H) when the duration of treatment was 24 h. We postulated that this result may have been influenced by an insufficient treatment duration. Therefore, the treatment duration was lengthened to 48 h.

All results in the 48-h treatment experiment showed that the INS-1 cells cultured in the palmitate-supplemented media reduced the ISI value, and reduced the expression of

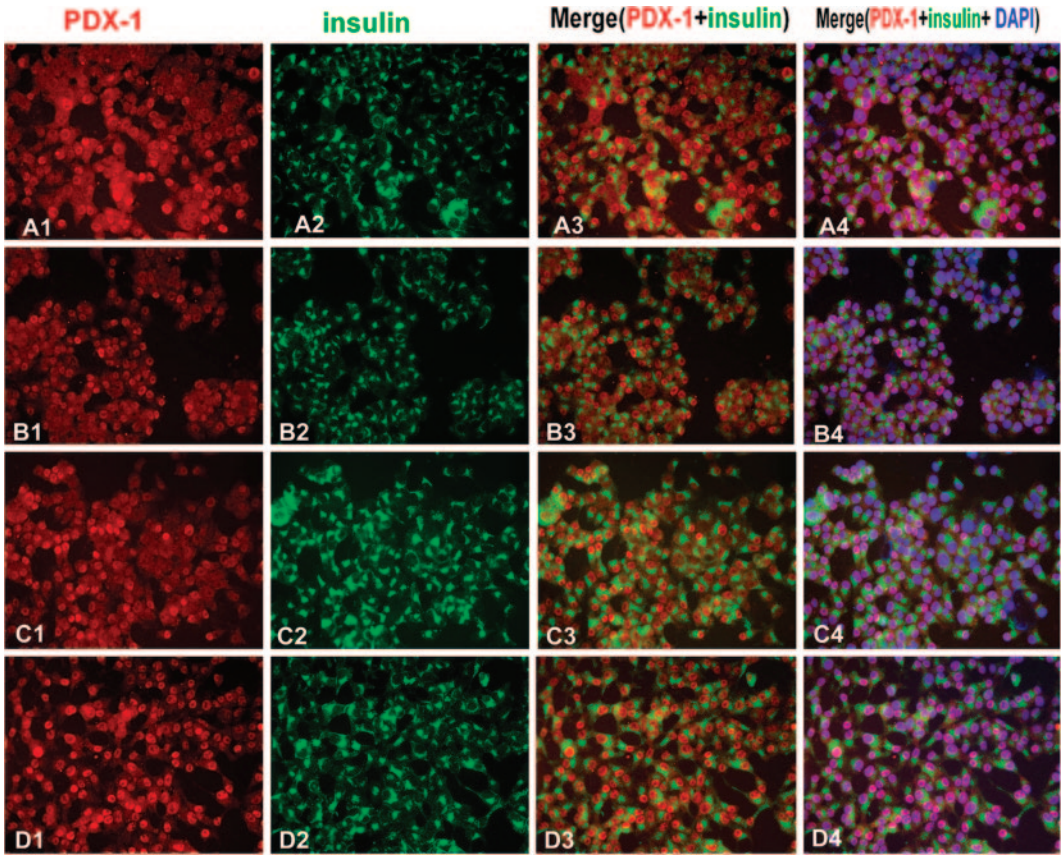

FIG. 4. The immunofluorescence images of PDX-1 and insulin in INS-1 cells treated with fenofibrate in the presence or absence of palmitate. INS-1 cells were treated with the PPARα agonist, fenofibrate, or palmitate for 48 h. The staining of PDX-1 and insulin was detected in INS-1 cells by double immunofluorescence with anti-PDX-1 and anti-insulin antibody. The *red color* depicts PDX-1 protein (tetraethyl rhodamine isothiocyanate-conjugated), which is located in the nucleus and cytoplasm, although mostly in the nucleus. The *green color* depicts insulin protein (fluorescein isothiocyanate-conjugated), which is expressed in the cytoplasm. The *blue color* in the images depicts the nucleus, stained by DAPI. The merged images by PDX-1 and insulin were obtained after superposition of the *green* and *red* channels. The merged images by PDX-1, insulin, and DAPI were obtained after superposition of the *green, red,* and *blue* channels. A1–4, Control INS-1 cells. B1–4, Palmitate-treated INS-1 cells, C1–4, Palmitate and fenofibrate-treated cells together. D1–4, Fenofibrate-treated cells. Representative images of four experiments performed on different experimental days are shown. Magnification, ×400.

PPARα, insulin, and GLUT2 ($P < 0.05$). By contrast, treatment of the palmitate-cultured cells with fenofibrate restored these values to normal (Fig. 5).

The effects of a PPARα antagonist in INS-1 cells

To examine further the regulatory role of PPARα on PDX-1, we incubated INS-1 cells for 48 h with the PPARα antagonist, MK886 (35), in the presence or absence of 0.2 mM palmitate.

BIS levels increased, and GSIS decreased in INS-1 cells treated with palmitate alone, with MK886 alone, and with the combination of both treatments ($P < 0.05$). The ISI values (Fig. 5A) were reduced by treatment with MK886 alone (reduced by 31% of control; $P < 0.05$) and also when palmitate was added (reduced by 42% of control; $P < 0.05$). The PPARα agonist increased ISI more effectively in INS-1 cells than those cultured in palmitate-rich media ($P < 0.05$ *vs.* control in both cases). These results demonstrate again that PPARα plays a key role in insulin secretion.

While testing the effect of the PPARα antagonist on the expression of PPARα, real-time PCR revealed a reduction of 63.5% in PPARα mRNA levels in cells treated with MK886 alone ($P < 0.05$), and a 60% reduction in cells treated with a combination of MK886 and palmitate (Fig. 5B; $P < 0.05$) compared with the control. As expected, treatment with the PPARα agonist restored PPARα mRNA expression to normal.

Importantly, a similar restoration effect of PPARα activation on PDX-1 expression was observed in the cells treated with the PPARα antagonist in the presence or absence of palmitate. In addition, the PDX-1 mRNA levels (Fig. 5C) were reduced to 46.5% compared with the control in the cells treated with MK886 alone ($P < 0.05$), and reached a maximum reduction (27% over control) in the cells treated with a combination of MK886 and palmitate ($P < 0.05$). Consistent with the reduction in PDX-1 mRNA expression, PDX-1 protein expression was also reduced (Fig. 5D). Weak bands were present for the cells exposed to palmitate alone and MK886 alone. The weakest band appeared in the cells treated with a combination of palmitate and MK886. Accordingly, the mRNA levels of insulin (Fig. 5E) and GLUT2 (Fig. 5G) decreased in the cells treated with MK886 alone (insulin: 34%

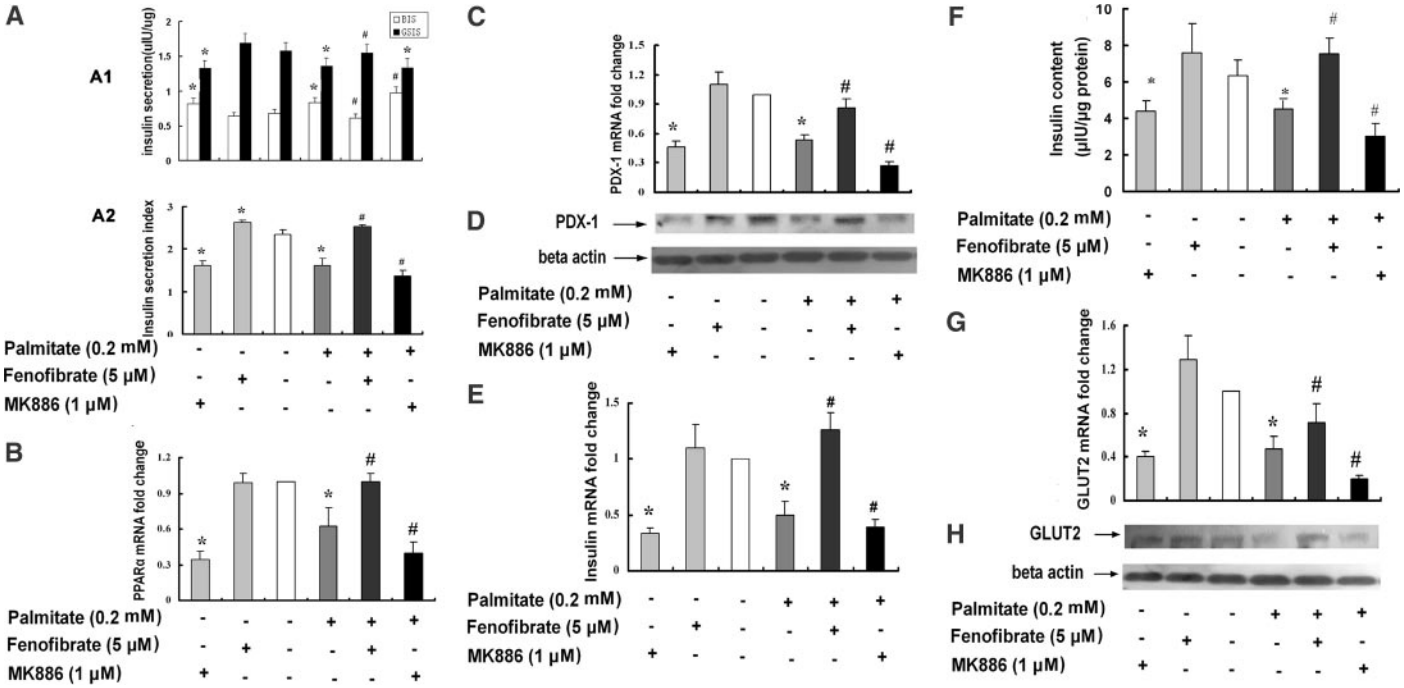

FIG. 5. The effects of a PPARα agonist and antagonist in INS-1 cells. INS-1 cells were treated with the PPARα agonist, fenofibrate, or the PPARα antagonist, MK886, in the absence or presence of palmitate for 48 h. A, The insulin concentration of the media were measured by RIA. A1, The *white column* represents BIS induced by 3 mM glucose. The *black column* represents GSIS induced by 20 mM glucose. A2, The ISI was derived as follows: GSIS/ BIS. Results are displayed as mean ± SD (n = 4). B, C, E, and G, The mRNA levels of PPARα, PDX-1, insulin, and GLUT2 were measured by real-time PCR. Duplicate samples of RNA were extracted. The mRNA level was expressed as the percentage of control (100%). Results are displayed as means ± SD (n = 4). F, Intracellular insulin content was determined by RIA and adjusted by protein concentration (n = 4). D and H, Nuclear protein for PDX-1 and total protein for GLUT2 were determined by Western blotting. The results were obtained from four independent experiments. *, $P < 0.05$ *vs.* controls. #$P < 0.05$ *vs.* cells treated with palmitate.

of control; GLUT2: 40% of control; $P < 0.05$), and in cells treated with a combination of MK886 and palmitate (insulin: 40% of control; GLUT2: 19% of control; $P < 0.05$). The same phenomenon occurred with respect to the intracellular insulin content (Fig. 5F) (reduced to 52% of control by MK886; reduced to 31% by MK886 and palmitate; $P < 0.05$) and GLUT2 protein expression (Fig. 5H). All effects mediated by the PPARα antagonist indicated that the PPARα regulation of PDX-1 occurred in INS-1 cells whether the concentrations of palmitate were normal or elevated.

Overexpression of PDX-1 protein has no effects on PPARα

All the aforementioned data indicated that PPARα regulates the PDX-1/insulin-GLUT2 signaling pathway. To confirm this possibility and exclude the possibility of PDX-1 affecting PPARα, we conducted another experiment using an INS-1 stable cell line, Pdx-1 no. 6. The expression of PDX-1 is relatively weak in this cell line, but it can be induced by the presence of doxycycline.

Our results revealed a doxycycline- dependent, dose-related expression of PDX-1 mRNA and protein in Pdx-1 no. 6 cells (Fig. 6). These results also showed parallel increases in the mRNA expression of insulin and GLUT2, suggesting that PDX-1 expression was induced and performed its function well in the current experiment. More notably, no change in the PPARα mRNA level was observed regardless of the level of PDX-1 expression. When PDX-1 expression was lacking, PPARα could not regulate insulin and GLUT2 expression, suggesting that PPARα was not influenced by PDX-1, and that the regulation of insulin and GLUT2 by PPARα was, at least partly, via PDX-1.

PPARα activation increases its binding activity with PDX-1 promoter

To investigate the effect of PPARα activation on its DNA binding activity, we performed EMSA experiments using

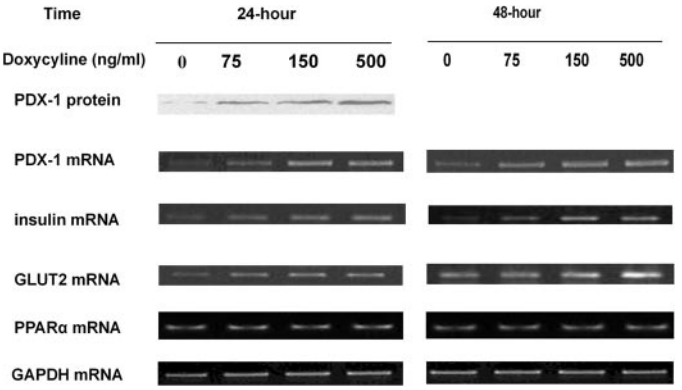

FIG. 6. Overexpression of PDX-1 protein has no effect on PPARα. Pdx-1 no. 6 cells were cultured in media with or without increasing concentrations of doxycycline for 24 and 48 h. The PDX-1 protein expression was determined by Western blotting. The mRNA expression levels of PPARα, PDX-1, insulin, GLUT2, and GAPDH mRNA were semiquantitatively evaluated by RT-PCR.

two pairs of probes. The first probe pair was previously verified by Roduit *et al.* (34). The design of the second probe was based on the PDX-1 promoter. As shown in Fig. 7, the two EMSA experiments showed similar results. Weak bands were present for the cells treated with palmitate alone (Fig. 7, A, lanes 3 and 4, and B, lanes 2 and 3), whereas the stronger bands were observed for the cells treated with a combination of PPARα activator (fenofibrate) and palmitate (Fig. 7, A, lane 1, and B, lane 4).

The binding specificity of PPARα-DNA was verified by competition experiments, whereby the bands disappeared with a 200-fold excess of unlabeled PPRE oligonucleotides (Fig. 7, A, lane 5, and B, lane 5), yet were still detected in a 200-fold excess of unlabeled nuclear factor-κB oligonucleotides (data not shown). In addition, the band intensity was dependent on the amount of PPARα protein present (Fig. 7, A, lanes 3 and 4, and B, lanes 2 and 3), presenting in a dose-dependent manner. These data have revealed that PPARα activation may increase its DNA binding activity with the PDX-1 promoter in cells treated with palmitate. In turn, this provides direct evidence for the regulation of PDX-1 by PPARα in INS-1 cells.

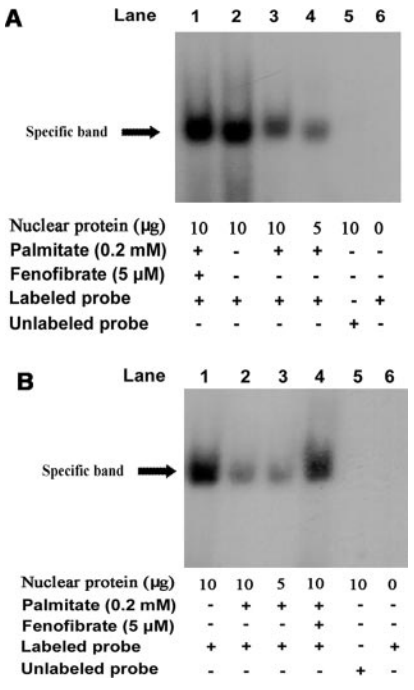

FIG. 7. PPARα activation enhances its DNA binding activity in palmitate-treated INS-1 cells. PPARα-DNA binding activity was assessed by EMSA with labeled oligonucleotide probe containing PPRE sequence. Two pairs of labeled probes were used. One is commonly accepted as assessing the DNA binding activity of PPARα (A). The other is designed in the promoter of PDX-1 containing a putative PPRE sequence (B). Nuclear extracts were prepared from INS-1 cells treated with and without palmitate and fenofibrate (lanes 1–4) for 48 h. Competition experiments with a 200-fold of unlabeled PPRE oligonucleotide (A, lane 5, and B, lane 5) were done with the same extracts that were used in A, lane 2, and B, lane 1, respectively. As negative controls, no nuclear extracts were added in A, lane 6, and B, lane 6, with each probe. DNA-protein complexes were visualized by autoradiography. The specific band is marked with an *arrow*. The results shown were one of three representative experiments.

Discussion

In the present study, we found that PDX-1, which would normally activate insulin and GLUT-2 expression, is a possible target for PPARα action in INS-1 cells. The down-regulation of PDX-1 by preventing PPARα may reduce intracellular insulin content in β-cells. As a result, insulin secretion is decreased. Fenofibrate, a PPARα agonist, may promote insulin secretion impaired by palmitate by causing up-regulation of PDX-1. This finding provides new evidence that fenofibrate may be beneficial for treating β-cell dysfunction.

The experiments reported here used pancreatic islets isolated from rats. Previous studies using this method have reported difficulties, including the preparation of large numbers of viable islets, cellular heterogeneity, and the availability of the other pancreatic endocrine tissues (36). In contrast, INS-1 cells represent a stable and highly differentiated rat insulinoma cell line that maintains β-cell characteristics, such as glucose responsiveness (37), and has been used as a model for β-cell function (38, 39). In our study we confirmed that comparable results were obtained, with respect to insulin secretion and gene expression, with the INS-1 cells and isolated rat islets.

We investigated the role of PPARα in β-cells exposed to a FFA, in this case using palmitate as a FFA (3). Our results were consistent with those from other studies, whereby reduced PPARα expression in palmitate-exposed cells was often accompanied by impaired insulin secretion. In β-cells cultured under identical conditions, PPARα activation by fenofibrate increased the expression of PPARα mRNA and protein, leading to an increase in insulin secretion. These findings are consistent with results obtained using insulin-resistant rodents or primary cultures of human pancreatic islets. In those systems, PPARα agonists repaired fatty acid-induced impairment of GSIS (17, 18). However, in contrast, Yoshikawa *et al.* (40) reported that the exposure of rat pancreatic islets to bezafibrate, another PPARα agonist, lowered GSIS. A possible explanation for this difference may be the concentration and category of the PPARα agonist used. Yoshikawa *et al.* (40) used 300 μM bezafibrate, whereas this study used 5 μM fenofibrate.

The results of this study have led us to propose that PPARα may regulate the expression of PDX-1 in INS-1 cells. To date, such a regulatory relationship has not been confirmed, although a few reports have suggested its existence. Yoshikawa *et al.* (3) reported that FFA suppression of β-cell insulin secretion was probably due to the inhibition of PPARα or PDX-1. Accordingly, β-cell function was improved in rats treated with a PPARα agonist (17), and in mice with PDX-1 overexpression (41). Our results indicated that PPARα may regulate the expression of PDX-1 in INS-1 cells, where PPARα activation causes up-regulation of PDX-1. This conclusion is based on observations that PPARα activation enhanced the PDX-1 mRNA and protein expression, increased the levels of PDX-1 binding activity with the insulin promoter, and the overexpression of downstream targets of PDX-1, such as insulin and GLUT2. Further support for this relationship is gained from the results of experiments using the PPARα antagonist, MK886, in which we detected an

inhibition of these cellular events. A similar β-cell dysfunction has been reported in mice with a PPARα (4) or PDX-1 (19) gene knockout, and in INS-1 cells unable to express PDX-1 (42).

Another major finding in our study is that a regulatory relationship between PPARα and PDX-1 also exists in β-cells cultured in media that were not enriched with palmitate or in a normal physiological environment. Apart from the observation that treatment of islets or INS-1 cells with fenofibrate alone yielded some evidence of this regulatory relationship, we observed a marked inhibitory effect on PDX-1 in the cells treated only with a PPARα antagonist. Further support for this contention comes from our studies conducted in Pdx-1 no. 6 INS-1 cells. In these cells, PPARα under PDX-1 slight expression had no marked effects on PDX-1 downstream targets, insulin and GLUT2. This observation is especially meaningful because it confirms that the regulation of insulin and GLUT2 by PPARα is, at least partly, via PDX-1. PDX-1 did not mediate PPARα but positively regulated the expression of the insulin and GLUT2 genes. Therefore, we concluded that PPARα may regulate the expression of PDX-1.

Because a functional PPRE sequence has been identified in the rat GLUT2 promoter (43), the issue occurs as to whether GLUT2 is regulated directly by PPARα or PDX-1 in INS-1 cells. Although we did not investigate this issue in our study, a study by Kim *et al*. (43) showed that the functional PPRE sequence in the rat GLUT2 promoter was not activated by coexpression of PPARα and retinoid X receptor-α but was activated by coexpression of PPARγ and retinoid X receptor-α, as well as PPARγ ligands. These results suggest that the PPRE of the GLUT2 promoter is not specific to PPARα but is specific to PPARγ. Therefore, we propose that the changes of GLUT2 expression that were observed in our experiment were not a result of direct regulation by PPARα but were a result of the transcriptional activation of PDX-1.

On the basis of all our data, we suggest that palmitate may reduce the expression of PDX-1, which is regulated by PPARα. This, in turn, decreases the transcriptional activation of insulin and GLUT2, and reduces intracellular insulin content in β-cells. Accordingly, we propose that this may be one of the mechanisms by which palmitate impairs GSIS. Activation of PPARα by fenofibrate increased the expression of PPARα and its regulation of PDX-1, as well as PDX-1 downstream targets. The effect of this was the amelioration of the impaired insulin secretion caused by palmitate exposure. The question then remained whether the regulation of PPARα on PDX-1 was direct or indirect.

To explore this question, we used an EMSA experiment using two pairs of probes. Using the first pair of probes (a functional PPRE sequence), a consistent change similar to PPARα mRNA and protein expression was observed, suggesting that the binding activity of PPARα was also involved in insulin secretion. To investigate whether direct binding occurs between the PPARα protein and PDX-1 promoter, we designed a second probe that contained a putative PPRE similar to the consensus sequence (44). Specific bands were detected by EMSA in every group, and the change was similar to that observed using the first pair of probes. Although the function of PPRE in the second pair of probes was not identified in our present experiment, it provided the possibility that PPARα might regulate PDX-1 by direct transcriptional binding with the PDX-1 promoter. More research should be done to elucidate the exact relationship between PPARα and PDX-1.

In conclusion, we have demonstrated that PPARα most likely regulates the expression of PDX-1 in INS-1 cells, and that this may be a new explanation for the mechanism of β-cell insulin secretion impairments by palmitate. Activation of PPARα may promote insulin secretion in β-cells under elevated palmitate conditions by causing up-regulation of PDX-1. These findings may have potential clinical implications for the use of PPARα agonists in the treatment of type 2 diabetes.

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