PPAR-α increases gluconeogenesis via glucose 6-phosphatase gene expression in fasting liver

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Directed by Professor **Yong-Ho Ahn**

The master's Thesis Submitted to the Department of Medical Science, the Graduate School of Yonsei University in the partial fulfillment of the requirements for the degree of Master of Medical Science

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This certifies that the Master's Thesis of Sool-Ki Kwon is approved.

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Again once, I would like to express special thanks to my parents. This thesis is dedicated to my mother and father.

Sool-Ki Kwon

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Abstract

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Glucose-6-phosphatase (G6Pase) plays a key role in maintaining blood glucose level. The enzyme is responsible for production of glucose in liver during fasting or type 2 diabetes mellitus (T2DM). Thus, understanding the molecular mechanism of G6Pase gene expression is crucial for the development of therapeutic drugs for T2DM. During fasting or in T2DM, peroxisome proliferator activated receptor-α (PPAR-α) is activated, which

may contribute to increased hepatic glucose output. However, it is not well established that the expression of G6Pase gene was regulated by PPAR-α.

In this study, we demonstrate that PPAR-α/RXR-α upregulates glucose 6-phosphatase gene expression. We have localized and characterized PPAR responsive element (PPRE) in the promoter region of rat G6Pase gene. Treatment of PPAR-α ligand, Wy14,643, to HepG2 cell lines and primary cultured hepatocytes increased G6Pase mRNA level. Serial deletion, mutation studies in the putative PPRE and electrophoretic mobility-shift assay studies indicated that the putative PPRE is present in the region between bases -268 and -256 of G6Pase promoter. Treatment of Wy14,643 to lean or ZDF rats resulted in the rise of G6Pase mRNA level. These results indicate that PPAR-α can directly upregulates hepatic G6Pase gene expression.

Key Words : Glucose 6-phosphatase, PPAR-α, gluconeogenesis, type 2 diabetes mellitus, liver

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Ⅰ. Introduction

The incidence of type 2 diabetes (T2DM) is increasing throughout the world [1]. T2DM has complex features of biochemical and clinical manifestations. One of them is increased hepatic glucose production [2] which is caused by the increased gluconeogenesis [3]. Gluconeogenesis is one of the metabolic adaptation during energy deprivation in mammals. During fasting in normal state, glucose which is formed from noncarbohydrate precursors in liver is supplied to other tissues for energy source. On the other hand, in T2DM, activation of gluconeogenesis is in part responsible for chronically

increased blood glucose level [4, 5]. In this state, free fatty acids are elevated and have been implicated as a causative link of insulin resistance appeared in obesity or T2DM [6, 7]. In addition, free fatty acids were shown to increase hepatic glucose production [8, 9] by activating key enzymes of gluconeogenesis, i.e., phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6 bisphosphatase (F1,6BPase) and glucose- 6-phosphatase (G6Pase) [10].

G6Pase hydrolyzes glucose-6-phosphate to glucose at the final step of gluconeogenesis. The enzyme is expressed mainly in liver, kidney, and β-cells of pancreas [11, 12]. The enzyme which is a multienzyme complex consists of catalytic and transporter subunit. The transporter includes T1 (glucose-6-phosphate transporter), T2 (inorganic phosphate transporter), and T3 (glucose transporter). G6Pase gene is positively controlled at the transcriptional level by glucocorticoids, cAMP, glucose, and fatty acids [13-15] whereas the gene is inhibited by insulin, tumor necrosis factor-α, and interleukin-6 [16-18]. G6Pase mRNA level is increased in animal models of diabetes [19-22]. Furthermore, modest overexpression of G6Pase in rat liver results in glucose intolerance and hyperinsulinemia [23].

The peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors acting as transcription factors. The receptors share a high degree of structural homology with other nuclear hormone receptors in the DNA-binding, ligand-binding, and cofactor-binding domain [24]. The receptor acts as heterodimer with retinoid X receptor (RXR). When ligands bind to the receptor, the heterodimer activates gene expression by binding to a *cis*-

element, known as peroxisome proliferator response element (PPRE) in the target gene promoters. In the transcriptional control by PPARα/RXRα, interaction with coregulator complexes is required.

PPAR-α is a nuclear receptor which regulates liver glucose homeostasis as well as lipid metabolism [25]. PPAR-α is widely expressed in liver, muscle, kidney, and intestine [26]. PPAR-α. lowers circulating triglyceride levels by increasing fatty acid oxidation and reduces adiposity, which improves insulin sensitivity [27-29]. Furthermore, PPAR-α agonists have significant anti-inflammatory responses that may play a protective role in the cardiovascular system [30]. In the liver, PPAR-α activates fatty acid catabolism, stimulates gluconeogenesis, ketone body synthesis, and lipoprotein assembly [31-34]. Severe hypoglycemia observed in PPAR-α-deficient mice upon fasting, characterized by a 50% drop in blood glucose concentration after 24 hours of fasting, suggested a role for PPAR-α in glucose homeostasis [35]. However, molecular mechanism by which PPAR-ά upregulates G6Pase gene expression has not been explored.

In this study, we have identified functional PPRE in the promoter of G6Pase gene. We also demonstrated that PPAR-α directly mediates the upregulation of G6Pase gene in the liver of fasting and animal model of T2DM.

Ⅱ. Material and Methods

1. Animals and materials

Male Sprague-Dawley rats (weighing approx. 200g each) were fed a fat-free, high carbohydrate diet for the indicated period. Lean and obese Zucker diabetic fatty (ZDF) rats were kindly donated by Dr. Seong JK, Seoul National University, Korea. Diabetes was confirmed by checking the plasma glucose levels through an oral glucose tolerance test (OGTT)[36]. All the materials for the diet were purchased from Harlem Teklad Co. (Madison, WI, USA). Wy14,643 in 20 mM in 19% BSA and 5% dimethyl sulfoxide and 9-*cis* retinoic acid (9-*CR*) in 2 mM in 50% ethanol and 50% dimethyl sulfoxide were diluted to the final concentration of 20 μM and 1 μM respectively. Wy14,643 and 9-*CR* were purchased from Sigma-Aldrich (St. Louis, MO). PPAR-α antibody and RXR-α antibody were purchased from Chemicon and Santa Cruz, respectively. [γ-^{32}P]ATP, [α-^{32}P]dCTP were purchased from Perkinelmer Life Science. Rediprime labeling kits, and a rapid hybridization solution were purchased from Amersham Biosciences.

2. Cell culture

HepG2 human hepatoma cell line (American Type Culture Collection number HB-8065) was maintained in Dulbecco's Modified Eagle's medium (DMEM, Hyclone) with 10% (v/v) fetal bovine serum (FBS), 100 unit/㎖

penicillin and 100 ㎍/㎖ streptomycin at 37 ℃ and 5% CO_2.

3. Isolation of total RNA, northern blot analysis, and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the liver of Sprague-Dawley rat using Trizol reagent (Invitrogen) according to the manufacture's instructions. For northern blotting, DNA probes were radio-labeled by filling-in with [α-^{32}P]dCTP (Perkinelmer Life Science, Boston, MA, USA) using the Klenow fragment of DNA polymerase 1 (Roche Diagnostics GmbH, Germany). Labeling was performed using the Rediprime labelling Kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Twenty μg of each sample was denatured and subjected to electrophoresis in a 0.9% denaturing formaldehyde-agarose gel and transferred to a nylon membrane. The membranes were hybridized with the probe for 4 h at 65 °C with Rapid-Hybrid buffer (Amersham Biosciences). After hybridization, the membrane was washed twice with a high salt washing buffer (0.1% SDS, 2x SSC) at room temperature for 30 min, followed by a low salt washing buffer (0.1% SDS, 0.2x SSC) at 65 °C for 15 min. The membrane was exposed to Kodak BioMax film using an intensifying screen at –70 °C. For RT-PCR, the first strand of cDNA was synthesized from 1 ㎍ of total RNA using using ImProm II™ reverse transcriptase (Promega). One ㎕ of the reverse transcription reaction mixture was amplified with primers specific for G6Pase, PPAR-α and β-actin in a total volume of 50 ㎕. Linearity of the PCR was tested with amplification cycles between 25 and 30.

According to the test amplification profile, samples were amplified using the following parameters: 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec. β-actin was used as an internal control for quality and quantity of RNA. The PCR primers used were as follows: rat G6Pase, forward 5'-GTG GGT CCT GGA CAC TGA CT-3' and reverse 5'-CAA TGC CTG ACA AGA CTC CA-3'; rat PPAR-α, forward 5'-GGT CCG ATT CTT CCA CTG C-3' and reverse 5'-TCC CCT CCT GCA ACT TCT C-3'; rat β-actin forward 5'-TT G TAA CCA ACT GGG ACG ATA TGG-3' and reverse 5'-CGA CCA GAG GCA TAC AGG GAC A AC-3'.

4. Construction of reporter plasmids

Rat G6Pase promoter (-1194/+88 bp) and human PEPCK (-599/-61 bp) were constructed by PCR-based amplification from rat genomic DNA and inserted into the pGL3 basic vector (pGL3b). Serial deletion constructs from G6Pase promoter construct as above, pG6P-483, pG6P-231, pG6P-159 were prepared by amplifying the regions of -483/+88, -231/+88 and -159/+88 respectively, and were subcloned into the pGL3b. The PPRE point mutation was introduced into the putative PPAR-α binding site, designated PPRE, by QucikChange site-directed mutagenesis kit (Stratagene) to generate pG6P-mut. Following primers were used: sense: 5'-AGG AAG GCA TAC ACC CCT TAG CAC TGT CAA-3', antisense 5'-GGG GTG TAT GCC TTC CTG GTC CTC GAA GCC-3').

5. Transient transfection assay

Transient transfections were performed using TransIT-LT1 transfection reagent (Mirusbio, Japan) according to the manufacturer's protocol. Briefly, cells were plated in 6-well tissue culture dishes at a density of $1x10^6$ cells/well in 2 ㎖ medium. Six ㎕ of TransIT-LT1 transfection reagent were gently mixed in 200 ㎕ of OPTI-MEM (Gibco-BRL, Grand Island, NY, USA) and incubated for 15 min at room temperature. And then, 0.5 ㎍ of pGL3basic, G6Pase, and PEPCK-promoter-luciferase constructs, 0.1 ㎍ of pCMV-β-galactosidase expression plasmid, the indicated amounts of pCMX, PPAR-α, and RXR-α expression plasmid, and 206 ㎕ of transfection reagent mixtures were mixed and incubated for 15 min. The transfection mixtures were added to the cells and incubated at 37 ^oC in a humidified incubator. After 24 h, the medium was replaced with DMEM containing appropriate ligands or their solvent (the final concentration of 20 μM for Wy14,643 and 1 μM for 9-*CR* respectively). After 24 h of ligands treatment, cells were harvested for luciferase and β-galactosidase assay (Promega). The luciferase activity was normalized by β-galactosidase activity.

6. *In vitro* translation and western blot analysis

pcDNA3.1, pcDNA3.1-rPPAR-α and pcDNA3.1-rRXR-α were expressed in TNT-coupled reticulocyte lysate system (Promega) according to the manufacture's protocol. pcDNA3.1-rPPAR-α and pcDNA3.1-rRXR-α were

tagged with V5 and flag respectively. *In vitro* translations were confirmed by western blot analysis using V5 (Chemicon) and flag (Sigma) antibodies. Briefly, total recombinant proteins by *in vitro* translation were fractionated on 8% SDS-PAGE and transferred to nitrocellulose membranes. Then, the blot was incubated with an indicated antibody followed by a secondary antibody conjugated to horseradich peroxidase. Reactivity was then detected with the Enhanced Chemiluminiscence (ECL) kit (Amersham Biosciences).

7. Electrophoretic mobility shift assay (EMSA)

The single-stranded G6Pase-PPRE probes for the gel shift assays were labelled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase and annealed with five fold molar excess of cold complementary oligonucleotides, which was heated at 90 ℃ for 5 min and slowly cooled down to room temperature. The labeled double-stranded oligonucleotides were purified by using spin column (BioRad) with Sephadex G25 (Amersham Biosciences). pCMX, PPAR-α and RXR-α proteins were prepared by *in vitro* translation as described above. One hundred thousand cpm of labeled probe and 8 ㎕ of *in vitro* translated proteins were incubated for 40 min on ice with 15 ㎕ binding buffer containing 50 mM 〔2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid〕(HEPES), pH 7.9, 300 mM KCl, 1mM dithiothreitol (DTT), and 2 ㎍ poly(dI-dC). The protein-DNA complexes were resolved form the free probe by electrophoresis at 4 ^{o}C on a 4% polyacrylamide (PAGE) gel in 0.25X TBE buffer (Tris-borate EDTA, 1 X

TBE contained 9 mM Tris, 90 mM boric acid, 20 mM EDTA, pH 8.0). The dried gels were exposed to X-ray film at -70 ^{o}C with intensifying screens. The oligonucleotides used for EMSA were shown in Table 1.

8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously with minor modifications [36]. Rats were anesthetized and perfused with 100 ㎖ of ice-cold phosphate buffered saline (PBS) and liver was fixed by perfusing the portal vein with 200 ㎖ of 3% formaldehyde in ice-cold PBS. Liver was harvested, homogenized, and sonicated. The sonicated supernatant was divided into aliquots (100 ㎕) and diluted with 10 fold volumes of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1) and 167 mM NaCl) and precleared with 50% slurry of protein A-agarose/herring sperm DNA for 1 h. For immunoprecipitation, antibody was added and agitated overnight at 4 ^{o}C. The complexes were collected with protein A-agarose/herring sperm DNA and eluted from the beads. The eluted DNA was amplified by PCR using the primers which were appropriated for each set. These primers were as follows: for G6Pase-Chip; sense 5'-TCT CCA AAC AAA TAC AAT TG-3' and antisense 5'-TCA TCA GTA GGT TGA TGC AA -3', for G6Pase-control; sense 5'-AAA AGT GAC TGG TCA GGC TG-3' and antisense 5'-GGA ATG GAC TCC ATG AGT CA-3'. For PEPCK-Chip Sense 5'-GTA ACA CAC CCC AGC CAA CT -3' and antisense 5'- CTC TTG CCT TAA TTG TCA GG-3'

9. Statistical analysis

All transfection studies were performed in 3-5 separate experiments, where triplicate dishes were transfected. The data were represented as mean ± S.D. Statistical analysis was carried out using Microsoft Excel (Microsoft).

Table 1. The sequences of the oligonucleotides used in EMSA

Name	5’ -> 3’ sequence	
G6Pase-PPRE	Sense	GGC TTC GAG GAC CAG GAA GGA GGT CAC CCC TTA GC
	antisense	GCT AAG GGG TGA CCT CCT TCC TGG TCC TCG AAG CC
G6Pase-mut	Sense	GGC TTC GAG GAC CAG GAA GGC ATA CAC CCC TTA GC
	antisense	GCT AAG GGG TGT ATG CCT TCC TGG TCC TCG AAG CC
PEPCK-PPRE	Sense	CTT TGA CTT GAC CTT TGA CTA TGG GGT GAC
	antisense	GTC ACC CCA TAG TCA AAG GTC ACA GTC AAA G

Ⅲ. Results

1. Effect of fasting on PPAR-α and G6Pase mRNA levels in rat liver.

RT-PCR (Fig. 1A) and northern blot analysis (Fig. 1B) of G6Pase and PPAR-α revealed that both G6Pase and PPAR-α mRNA level in liver were increased during fasting when compared to ad *libitum* or refed rats. These results suggest that there is a positive correlation between the expression of PPAR-α and G6Pase gene. Treatment of Wy14,643 and 9-*CR* known as lignads for PPAR-α and RXR-α respectively to primary cultured hepatocytes resulted in the increase in G6Pase mRNA level which was measured by RT-PCR (Fig. 2A) or northern blot (Fig. 2B). As shown, G6Pase mRNA was increased by Wy14,643, but not by 9-*CR*. Combined treatment of Wy14,643 and 9-*CR* also increased G6Pase mRNA level. These data suggested that PPAR-α is a major determinant in the upregulation of G6Pase gene transcription.

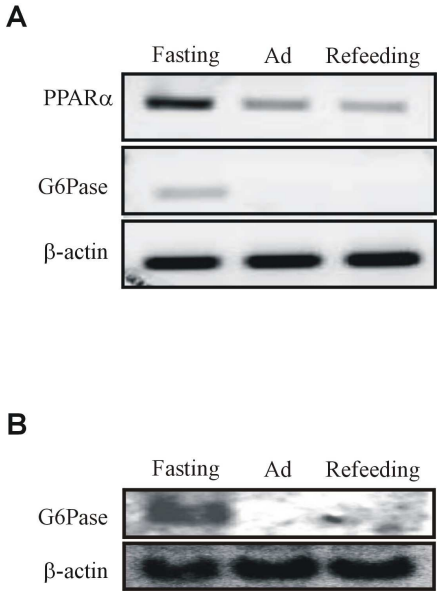


Fig. 1. The expression of PPAR-α and G6Pase mRNA in the fasting and refeeding liver. *A*: PPAR-α and G6Pase mRNA levels measured by RT-PCR. *B*, Northern blot of G6Pase mRNA levels. Rats were fasted for 24 h and ad *libitum*. For refeeding group, rats fasted for 24 h were refed with a fat-free, high-carbohydrate diet for 6 h. For northern blotting, total RNA (20 ㎍) isolated from liver tissue was subjected to 0.9% formaldehyde-agarose gel electrophoresis. The RNA in the gel was transferred to a nylon membrane and hybridized to ^{32}P-labeled cDNAs for G6Pase or β-actin. The blots are representatives of two different experiments. For RT-PCR, total RNA (3 ㎍) isolated from liver was used. Ad: ad *libitum*.

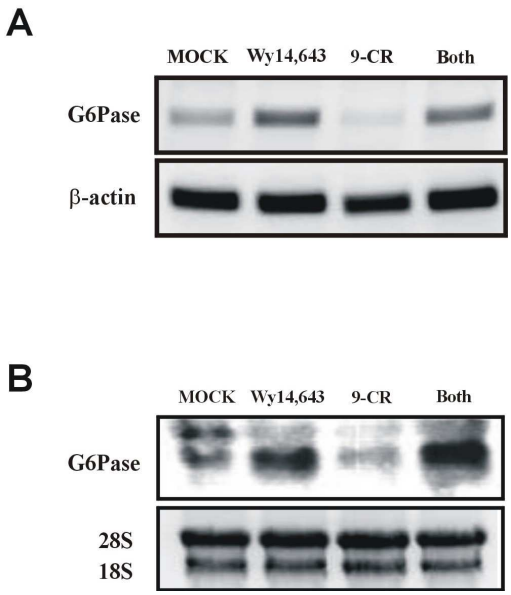


Fig. 2. The effect of PPAR-α and RXR-α agonists on the G6Pase mRNA levels in primary cultured hepatocytes. *A*, G6Pase mRNA levels measured by RT-PCR. *B*, Northern blot of G6Pase. Hepatocytes isolated from rat were plated for 5 h and cultured further for 16 h in the presence of 2% FBS, 10 μM dexamethasone, 2mM glutamate and 1mM sodium pyruvate and then incubated for 12 h in the presence or in the absence of insulin (100 nM) and glucose (25 mM). Wy14,643 (20 μM) and 9-*CR* (1 μM) were treated to primary cultured hepatocytes. Total RNA of 3 μg or 20 μg was used for RT-PCR or northern blotting, respectively. All the mRNA levels were normalized to α-actin for PCR products and 18s, 28s rRNA for northern blot analysis.

2. Effect of PPAR-α/RXR-α on the promoter activities of gluconeogenic genes in the HepG2 cell lines.

As shown in Fig. 3, the promoter activities of G6Pase and PEPCK gene were activated by ectopic expression of PPAR-α/RXR-α in HepG2 cell lines in the presence of their respective ligands. The reporter activities of G6Pase and PEPCK promoter were increased by 25 and 7 folds, respectively, suggesting that the promoters are activated by PPAR-α/RXR-α. However, the stimulatory effect of PPAR-α/RXR-α on the PEPCK promoter construct is weaker than that of G6Pase.

3. Localization of PPRE in rat G6Pase promoter.

A computer search for PPRE homology sequence in the G6Pase promoter suggested that -268/-256 bp regions of G6Pase promoter may play as a functional PPRE (Fig. 4A). To confirm the identity of the PPRE, serial deletion constructs of the G6Pase promoter (from -1194, -483, -231, and -159 to +88, Fig. 4B) were prepared. As shown in Fig. 4B, PPAR-α responsiveness is decreased when the promoter region is deleted down to -231 bp. These results suggest that PPRE may be located between -483 and -231 bp. Introduction of mutation at the putative PPRE region resulted in 75% decrease in promoter activity when compared to promoter construct (pG6P-1194), suggesting that -268 bp/-256 bp region may be a functional PPRE.

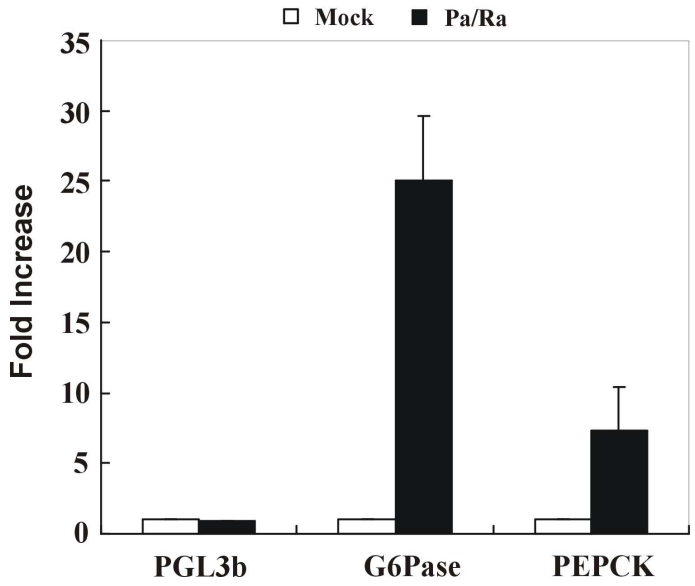


Fig. 3. Effect of PPAR-α ligand on the promoter activities of G6Pase and PEPCK gene. Luciferase reporter constructs of G6Pase (-1194/+88 bp) or PEPCK (-599/-61 bp) were cotransfected with PPAR-α/RXR-α in HepG2 cells. After 24h, 20μM of Wy14,643 and 1μM 9-CR was treated to the cells and it was maintained for 24h. Normalized luciferase activities were shown as means ± standard deviation of three independent experiments in triplicates and are expressed as the fold increase relative to the basal activity, 9-*CR*, 9-*cis* retinoic acid.

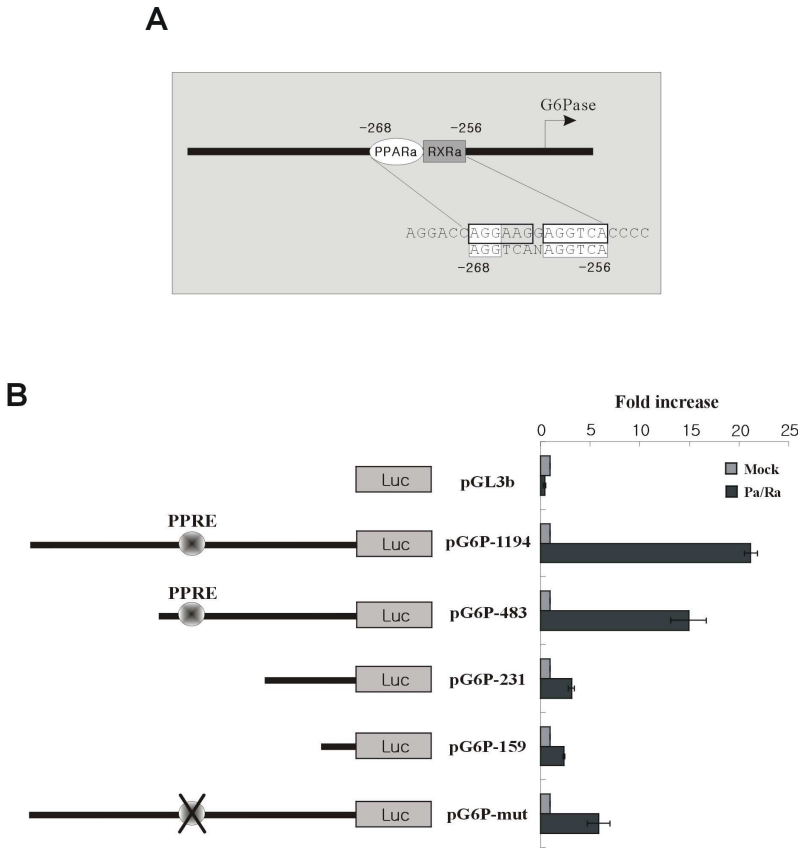


Fig. 4. Localization of PPRE in the rat G6Pase promoter. *A*, A schematic showing putative PPRE in the G6Pase promoter. *B*, Effect of deletion on the PPAR-α dependent promoter activities of G6Pase. Scheme of deletion constructs were shown in the left panel and PPAR-α dependent activities were shown in the right. The indicated numbers represent the number of

nucleotides from mRNA start codon. Promoter activities were measured by cotransfecting PPAR-α/RXR-α with deletion constructs into HepG2 cell lines which were plated at a density of 2×10^5 cells/35-mm dish. The results were shown as relative luciferase activities. Normalized luciferase activities are shown as mean ±S.D. of three independent experiments in a triplicate.

4. Identification of PPRE and binding of PPAR-α/RXR-α to the G6Pase promoter.

Fig. 5*A* shows a putative PPRE in the G6Pase promoter. To confirm the binding of PPAR-α/RXR-α heterodimer, electrophoretic mobility shift assay (EMSA) was performed (Fig. 5*B*) using recombinant PPAR-α and RXR-α which were prepared by *in vitro* translation. The oligonucleotide probe (-281/-247) formed DNA-protein complex (*lane 5*). Addition of 100 fold excess amount of unlabeled competitor from PEPCK-PPRE (38) diminished the formation of protein-DNA complex (*lane 7*). Mutant probe (*lane 8*) failed to compete the binding of PPAR-α/RXR-α heterodimer to the probe. Addition of V5 antibody decreased the formation of protein-DNA complex (*lane 6*), suggesting that PPAR-α/RXR-α heterodimer binds to the probe. Introduction of mutation at the core site of putative PPRE (AGGT → CATA in Fig. 5*A*) abolished the binding of PPAR-α/RXR-α (Fig. 5*B, lane 11*). The quality of recombinant PPAR-α and RXR-α prepared by in vitro translation system was explored by western blot analysis as shown in Fig. 5*C*.

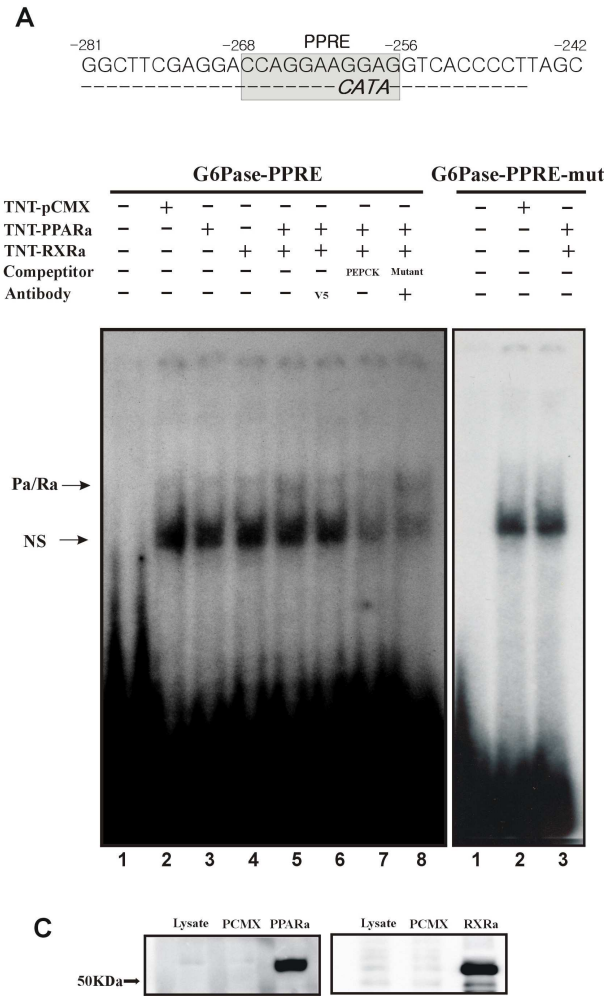


Fig. 5. PPAR-α/RXR-α binding to PPRE of G6Pase promoter *in vitro*. *A*, Nucleotide sequence of promoter region containing putative PPRE. *B*,

Identification of PPAR-α/RXR-α binding to the putative PPRE or mutant probe in the G6Pase promoter. *C*, Western blot of *in vitro* translated PPAR-α and RXR-α. The proteins were detected by anti-V5 antibody. EMSA was performed with in vitro translated-PPAR-α and RXR-α in 4% (w/v) non-denaturing polyacrylamide gel. Then, 100,000 cpm (0.1 pmole) of ^{32}P-labeled G6Pase promoter fragment (-281/-242) containing putative PPRE was incubated with 4 $\mu\ell$ of PPAR-α and RXR-α protein respectively. The DNA-protein complexes are indicated by an arrow. Results are the mean ± S.D. of three independent experiments performed in triplicate. Pα/Rα, PPAR-α/RXR-α.

5. PPAR-α/RXR-α heterodimer binding to putative G6Pase-PPRE in fasting state in vivo

To confirm whether PPAR-α/RXR-α complex binds directly to the rat G6Pase gene promoter region *in vivo*, we observed interaction between PPAR-α/RXR-α and the putative PPRE in the promoter region of G6Pase in liver using Chip assay. Chromatin was extracted from *ad libitum* or fasted rat liver, and the PPAR-α or RXR-α antibody was used to immunoprecipitate the fragmented chromatin followed by PCR amplifications with respective primers (Fig 6). In fasting state, the binding of PPAR-α/RXR-α to the rat G6Pase promoter was increased by fasting (Fig.6B), and it suggests that endogeneously increased PPAR-α forms a heterodimer with RXR-α and this PPAR-α/RXR-α binds to the putative PPRE of G6Pase gene promoter. Binding of PPAR-α/RXR-α to PEPCK promoter was slightly increased (Fig. 6C)

6. Binding of PPAR-α/RXR-α to putative G6Pase-PPRE in ZDF rats.

To confirm that increased PPAR-α cause the transcriptional up-regulation of G6Pase gene expression in the diabetic animal model, we experienced the ChIP assay using ZDF rats which showed elevated glucose tolerance(GTT) curve (Fig. 7C). As shown in Fig. 7B, binding of PPAR-α/RXR-α to PPRE in the G6Pase is increased, suggesting that PPAR-α binding is increased in the PPRE of G6Pase gene promoter in the ZDF rats. As a negative control, we used upstream region of G6Pase promoter which does not contain PPRE. As shown, there is no binding to the G6Pase control region (Fig. 7A).

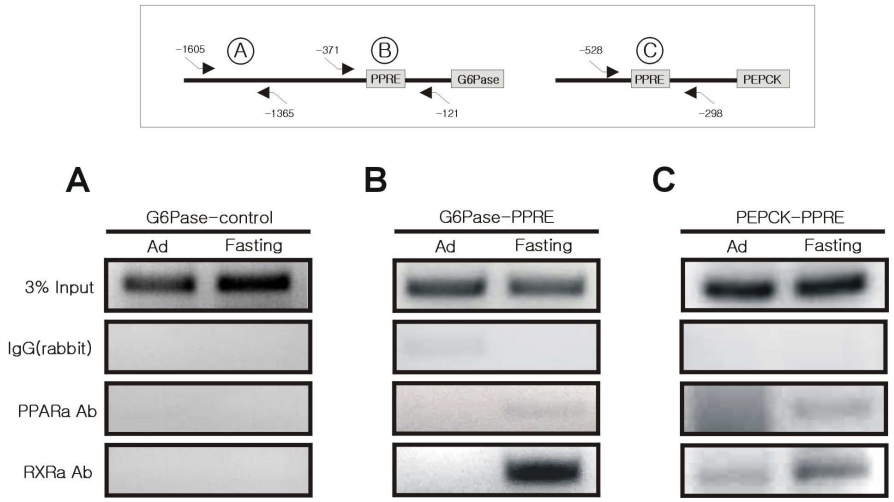
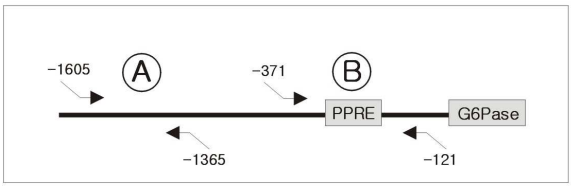
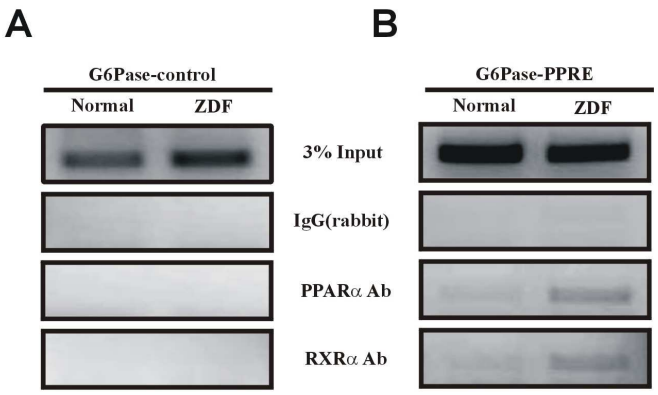


Fig. 6. Effect of fasting on the PPAR-α/RXR-α binding to PPRE of G6Pase promoter *in vivo*. Upper panel: Schematics of the promoter region of G6Pase and PEPCK gene in the ChIP assay. The regions between - 371 and -121 for G6Pase gene and between -528 and -298 for PEPCK gene were amplified. For the Chip assay, chromatin was extracted from livers of rats *ad libitum* or fasted for 48 h. and precipitated by PPAR-α antibody and putative PPRE was amplified by PCR. The quantity of DNA in the precipitation was normalized by input chromatin ($3/100^{th}$ of chromosomal DNA used for precipitation). The regions (A as a negative control, B of G6Pase, C of PEPCK) were amplified. Lower panel: (*A*) Negative control, (*B*) and (*C*) PPRE of G6Pase and PEPCK, respectively.


−1605
A
−371
B
PPRE
G6Pase
−1365
−121


A
B
G6Pase-control
Normal
ZDF
G6Pase-PPRE
Normal
ZDF
3% Input
IgG(rabbit)
PPARα Ab
RXRα Ab

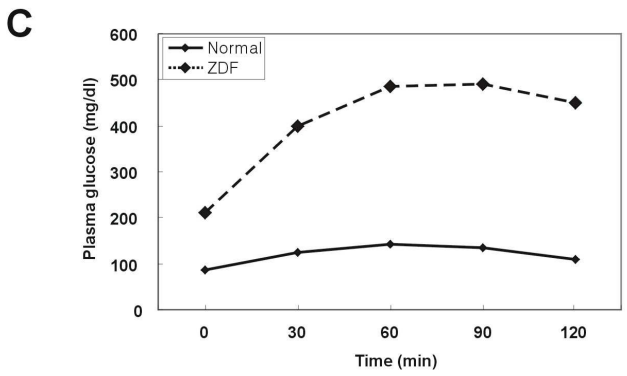

C
Normal
ZDF
Plasma glucose (mg/dl)
600
500
400
300
200
100
0
0
30
60
90
120
Time (min)

Fig. 7. Binding of PPAR-α/RXR−α to the putative PPRE in the G6Pase gene promoter *in vivo*. Upper panel: Scheme of G6Pase promoter amplified in the ChIP assay. The region between -1605/-1365 (A) and -371/-121 (B) were amplified. For ChIP assay, chromatin extracted from the livers of lean or ZDF rats were precipitated either by PPAR-α or RXR-α antibodies and G6Pase-PPRE. The DNA fragments were amplified by PCR. Detailed procedures are described in the EXPERIMENTAL PROCEDURES. A, Negative control. *B*, Chip assay of lean and ZDF rats. *C*, Glucose tolerance curve of lean and ZDF rats.

Ⅳ. Discussion

Gluconeogenesis occurs in the livers of animals when they are subjected to long term fasting [37] or diabetic states [38]. The key gluconeogenic enzymes, G6Pase and PEPCK are regulated either at transcriptional or translational levels. The promoters of these genes were cloned and numerous transcriptional factors regulating their gene expression in relation to the pathogenesis of T2DM have been characterized [39].

PPAR-α is a nuclear receptor which is mainly expressed in liver and brown adipose tissue [40]. It plays as an important lipid sensor and regulator of cellular energy-harvesting metabolism [41]. This conclusion is derived from PPAR-α knock-out (KO) mice which showed decreased levels of enzymes involved in fatty acid catabolic enzymes and showed an unresponsiveness to peroxisome proliferating agents [42]. In addition, the PPAR-α agonist, Wy14,643, decreased glycogen content in liver [43]. These results indicate that PAPR-α may be involved in hepatic glucose production directly or indirectly. Direct role of PAPR-α on the transcriptional activation of gluconeogenic genes is not well established, although there is a functional PPRE in the PEPCK gene, one of the key gluconeogenic enzyme. The *cis*-element PPRE on the PEPCK promoter region is mainly responsible for the regulation in the adipocytes [44].

In this study, we showed that there is a direct regulatory link between PPAR-α and G6Pase, another key gluconeogenic enzyme. In the liver of

fasted rats, both PPAR-α and G6Pase mRNA level were increased (Fig. 1). Treatment of the PPAR-α ligand resulted in the increase in G6Pase mRNA levels. However, 9-*CR* alone did not increased G6Pase mRNA levels, suggesting that PPAR-α plays a major role in the upregulation of the G6Pase gene. Combined treatment of Wy14,643/9-*CR* further increased G6Pase mRNA levels, indicating that these two ligands act in a synergistic manner (Fig. 2*B*).

In the promoter reporter assays (Fig. 3), the promoter of G6Pase gene is activated 100 folds whereas that of PEPCK is only activated 7 fold by their ligands. This result parallels with that observed in the ChIP assay. It showed a considerably weaker binding of PAPR-α/RXR-α to the PEPCK gene promoter compared to the G6Pase gene promoter (Fig. 6). Although the PEPCK gene promoter has an intermediate-affinity PPRE [45] and this region is responsible for binding and upregulation of the gene by PPAR-γ in adipose tissue [44], PEPCK gene expression was not altered in wild type and PPAR-α KO mice in livers of both the fed and fasted mice [35]. The lack of PPAR-α responsiveness of PEPCK might be due to the competition between other transcription factors such as HNF4 and RXR/RAR [46]. These results suggest that PPRE plays relatively minor role in PEPCK gene expression. Contrary to PEPCK-PPRE, the G6Pase gene promoter is well activated by PPAR-α/RXR-α *in vitro* (Fig. 3). The binding of PPAR-α/RXR-α to the G6Pase promoter is increased in fasting or in the ZDF rats (type 2 diabetic rat model) (Fig. 6 and 7). These results suggest that, contrary to PEPCK, G6Pase may be directly

activated by PPAR-α.

During fasting or in T2DM, free fatty acids (FFAs) which are increased in β-oxidation, have been implicated in hepatic gluconeogenesis [47-49]. Several putative mechanisms are proposed on the link between FFA and hepatic glucose production. Among these, FFA upregulates the genes expression involved in the gluconeogenesis by activating transcriptional factors like SREBP-1, PPARα, or HNF-4 [48-51]. Indeed, there are seven potential HNF-4 binding sites in the promoter region of G6Pase [52]. These sites are responsible for FFA-mediated activation of G6Pase. In addition, upregulation of gluconeogenic genes, i.e., PEPCK [48] and G6Pase [50] by FFAs is has been shown to be PAPR-α dependent [51]. To explore the relationship between HNF-4 and PPAR-α in the activation of G6Pase gene expression, we have overexpressed HNF-4α and PPAR-α in the HepG2 cell lines. As shown, the addition of HNF-4α to PPAR-α group did not affect the promoter activities of G6Pase gene (Suppl.1). Short chain FA upregulates the G6Pase gene expression through HNF-4α binding to the region of -668/-647 in the promoter [53], whereas the PPRE region overlaps with HNF-4 binding region -433/-457 in the PEPCK promoter region [54, 55]. HNF-4α may compete with the putative PPRE which was observed in the PEPCK gene promoter. Theses results indicate that the activation of G6Pase gene expression by HNF-4α may be different from that of PEPCK. These assumption are further supported by the results shown on in Fig. 3, 6, and 7.

Current study provides evidences that up-regulation of the G6Pase gene can

be mediated by the direct action of PPAR-α/RXR-α and adds to the current understanding of the transcriptional control of G6Pase gene expression. By studying the diverse effects of PPAR-α, it will be possible to develop selective modulators of PAPR-α, which is more potent and less toxic to patients with type 2 diabetes and dyslipidemia.

Ⅴ. Conclusion

We have localized the PPRE(-268/-256) in the rat G6Pase promoter. The region was regulated by PPARa, which may play an important role in the regulation of blood glucose level. Wy14,643 treatment regulted in the increase in G6Pase mRNA in the isolation hepatocyte system. From these results, Wy14,643 may play an pmportant role in regulation blood glucose level by activating hepatic G6Pase level.

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Abstract (in Korean)

금식시 간장에서 Glucose 6-phosphatase 유전자 발현을 통한 PPAR-α의 당신생과정 증가

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권 슬 기

Glucose 6-phosphatase(G6Pase)는 혈당을 조절하는데 중요한 역할을 하는 효소로서, 금식시 또는 제2형당뇨 상태에서 포도당을 생성 하도록 반응한다. 따라서, G6Pase의 발현 조절에 대해 이해 하는 것은 제2형당뇨 치료약을 개발 하는 데에도 매우 중요하다.

금식시 또는 제2형당뇨 상태에서 peroxisome proliferator activated receptor-α(PPAR-α)는 활성화 되고, 이것은 간장에서 포도당을 방출 하는데 기여한다. 그러나, PPAR-α에 의한 G6Pase의 조절은 잘 알려져 있지 않다.

본 연구에서는 PPAR-α/RXR-α에 의해 G6Pase의 발현이 증가 되는 것을 규명 하였고, 백서의 G6Pase promoter 부위에서 PPAR-α/RXR-α에 반응하는 부위를 찾아 내었다. PPAR-α lignand

인 Wy14,643을 primary hepatocyte에 처리 하였을 때 G6Pase의 mRNA양이 증가 하는 것을 관찰 하였다. Promoter의 염기 서열을 순차 적으로 제거 하거나 PPAR-α 반응부위라고 예상된 지점을 돌연변이 시켜 활성을 측정해 보고, EMSA를 수행한 결과 백서의 G6Pase promoter의 -268/-256 부위가 PPAR-α 반응 부위라는 것을 확인 하였다. 또한 제2형 당뇨 백서인 ZDF 백서의 간장 조직을 이용하여 Chip assay를 수행한 결과 G6Pase promoter 부위에 대한 PPAR-α/RXR-α의 결합력이 lean 백서에 비해 더 강하다는 것을 확인 하였다. 이상의 결과로 간장에서 G6Pase의 발현을 PPAR-α가 직접적으로 상승 조절 시킨다는 것을 알 수 있었다.

핵심 되는 말 : Glucose 6-phosphatase, PPAR-α, 당신생과정, 제2형당뇨, 간장