Insulin-like growth factor (IGF)-binding protein-2 (IGFBP-2), one of the most abundant circulating IGFBPs, is known to attenuate the biological action of IGF-1. Although the effect of IGFBP-2 in preventing metabolic disorders is well known, its regulatory mechanism remains unclear. In the present study, we demonstrated the transcriptional regulation of the Igfbp-2 gene by peroxisome-proliferator-activated receptor (PPAR) α in the liver. During fasting, both Igfbp-2 and PPARα expression levels were increased. Wy14643, a selective PPARα agonist, significantly induced Igfbp-2 gene expression in primary cultured hepatocytes. However, Igfbp-2 gene expression in Ppara null mice was not affected by fasting or Wy14643. In addition, through transient transfection and chromatin immunoprecipitation assay in fasted livers, we determined that PPARα bound to the putative PPAR-responsive element between −511 bp and −499 bp on the Igfbp-2 gene promoter, indicating that the Igfbp-2 gene transcription is activated directly by PPARα. To explore the role of PPARα in IGF-1 signalling, we treated primary cultured hepatocytes with Wy14643 and observed a decrease in the number of IGF-1 receptors (IGF-1R) and in Akt phosphorylation. No inhibition was observed in the hepatocytes isolated from Ppara null mice. These results suggest that PPARα controls IGF-1 signalling through the up-regulation of hepatic Igfbp-2 transcription during fasting and Wy14643 treatment.

Key words: fasting, gene expression, insulin-like growth factor-1 (IGF-1), insulin-like growth factor-binding protein 2 (IGFBP-2), liver, peroxisome-proliferator-activated receptor α (PPARα).

INTRODUCTION

The insulin-like growth factor (IGF)-binding protein (IGFBP) is known as a carrier protein for IGF-1 [1]. There are currently seven characterized IGFBPs: IGFBP-1 to IGFBP-7. IGFBPs also function as modulators of IGF bioactivity and availability [2]. The binding affinity of IGFBPs for IGFs is higher than that of type I IGF receptors. The biological function of IGFBPs was identified when it was demonstrated that IGFBPs are capable of important biological roles that are independent of their ability to bind to IGFs [3]. IGFBP-2, an abundantly secreted protein [4], is expressed in the adult liver, adipocytes and central nervous system, and plays a key role in the regulation of metabolic homeostasis and insulin sensitivity [5,6].

IGF-1 not only plays a key role in regulating growth and development, but also has a variety of pathophysiological functions related to cardiovascular disease, cancer, diabetes, liver cirrhosis and Laron syndrome [7,8]. It is a single-chain polypeptide hormone with endocrine, paracrine and autocrine properties and is produced mainly in the liver. However, its synthesis has also been shown to occur in a variety of other tissues, such as the kidney, pancreas, skin and testes [7,9]. Because IGF-1 is known to be involved in the regulation of hepatic glucose and lipid metabolism [15], IGF-1R is expressed in various cell types, including the heart, kidney and intestines [15,16]. PPARα is stimulated by ligands such as Wy14643 in addition to fenofibrate and the fasting state, elevating fatty acid oxidation, ketogenesis, bile acid synthesis and gluconeogenesis, as well as improving inflammation and insulin sensitivity in response [16,17]. Previous reports have shown that PPARα promotes apoptosis and inhibits cellular functions by IGF-1R signalling and Akt phosphorylation in various cancer cells [18–20]. Although PPARα is associated with an IGF-1-dependent pathway in vitro, any potential link between PPARα and IGF-1 signalling has yet to be determined in an in vivo system.

Abbreviations: G6pc, glucose-6-phosphatase catalytic subunit; HEK, human embryonic kidney; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; IGFBP, insulin-like growth factor-binding protein; mTOR, mammalian target of rapamycin; Pck1, phosphoenolpyruvate carboxykinase 1; PPAR, peroxisome-proliferator-activated receptor; PPRE, peroxisome-proliferator-responsive element; qPCR, quantitative PCR; S6K, S6 kinase; WT, wild-type.

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In the present study, we identified PPARα as a key regulator of Igfbp-2 gene transcription in the fasting state and revealed up-regulation of IGFBP-2 by Wy14643 exposure. IGF-1 sensitivity was prevented via down-regulation of the IGF-1-dependent pathway under fasting in vivo and in vitro conditions. These results suggest that regulation of the IGFBP-2–IGF-1 network by a PPARα agonist may provide a novel molecular mechanism for improving physiological changes via controlling IGF-1 bioactivity.

EXPERIMENTAL

Materials

Wy14643 (Sigma–Aldrich) and recombinant human IGF-1 (Life Technologies) were dissolved in the recommended solvents. Antibodies against p-IGF-1R, p-Akt, Akt, p-mTOR and p-S6 kinase (S6K) were purchased from Cell Signaling Technology and anti-PPARα, IGFBP-2, IGF-1R and β-actin were from Santa Cruz Biotechnology.

Experimental animals

Male C57BL6 mice (Jung-Ang Experimental Animals, Seoul, Republic of Korea) and Ppara null mice at 8-weeks-old were used in the experiments, as described previously [21]. For fasting and feeding experiments, mice were fed or fasted for 24 h.

All animal experiments were performed in accordance with the rules and regulations of the Institutional Animal Use and Care Committee (IAUCC), Keimyung University School of Medicine.

Isolation and culture of primary mouse hepatocytes

Mouse primary hepatocytes were isolated from wild-type (WT) and Ppara null mice. The hepatocytes were used for quantitative PCR (qPCR) and immunoblot analyses. The hepatocyte isolation method was described previously [22].

qPCR analysis

Total RNA was isolated from mouse primary hepatocytes and livers using the TRIzol method (Invitrogen). cDNA was synthesized using a SuperScript III First-Strand cDNA Synthesis kit (Invitrogen) and used for qPCR with LightCycler Real-Time PCR systems (Roche Applied Science). All data were normalized to ribosomal L32 expression. The following primer sets were used: Ppara: forward, 5′-AGAGCCCATCTGTCTCTCTC-3′; reverse, 5′-ACTGGTAG-TCTGCAAACACAAA-3′; Igfbp-1: forward, 5′-ATCGCC-CATCCCTGTGGGAC-3′; reverse, 5′-TGCAGCTAATCTCCTAGCACC-3′; Pck1 (phosphoenolpyruvate carboxykinase 1): forward, 5′-CCACAGCTGCTGCAAAACA-3′; reverse, 5′-GAAGGGTCG-GATGGGACAAA-3′; L32: forward, 5′-ACATTTGCGCTGACTGT-3′; reverse, 5′-ATCCCTCTGGATGATCTT-3′; human IGF-2: forward, 5′-GCAATGGCGATGACACCTCA-3′; reverse, 5′-CAGCTCTCTTCAACCAGCAT-3′; human ACTB (β-actin): forward, 5′-GGCATCTCCACCTGGAAGTA-3′.

Immunoblotting

Mouse primary hepatocytes were isolated and processed according to a method described previously [21]. The membranes were probed with the indicated antibodies and then developed using an enhanced chemiluminescent Western blot detection kit (GE Healthcare). The intensities of the bands were calculated using ImageJ software (NIH), verifying non-saturation and subtracting the background. Values are expressed as the integrals (target area density) of each band (normalized to total indicated protein band).

ChIP assay

The ChIP assay was performed as described previously [21]. Briefly, mice were fasted for 24 h and refed for 12 h. Livers were then collected from the mice and fixed with paraformaldehyde for 5 min prior to performing the ChIP assay using anti-PPARα. Liver tissue (25 mg) was used for each ChIP/antibody sample. The final DNA extractions were quantified by PCR with primers for the putative peroxisome-proliferator-responsive element (PPRE; −500/−300) region of the Igfbp-2 promoter. PPRE on the glucose-6-phosphatase catalytic subunit (G6pc) promoter was used as a positive control for the ChIP experiment with an anti-PPARα antibody. Raw C_t values obtained from the ChIP samples were divided by C_t values obtained from the appropriate input samples to analyse the percentage input values. The specific primers used for PCR were as follows: mouse Igfbp-2c, forward 5′-TCATTACCTGACGCGGTGACG-3′ and reverse 5′-CCCCGGGAAACACAAAACGAGCA-3′; mouse G6pc-Ppre, forward 5′-GCTGTTTTTGTGCTGCTGT-3′ and reverse 5′-TGCTATAGTGTGCTGCTGT-3′; mouse Gapdh, forward 5′-CCTGGAGAAACCTGCAAGTA-3′ and reverse 5′-TGGAAGAGTGAGGTTGCTGT-3′.

ELISA for IGFBP-2

Serum was obtained from mice and primary hepatocytes. Secreted IGFBP-2 was quantified using mouse serum and the Quantikine mouse IGFBP-2 kit (Raybiotech). Serum (50 μl) was diluted with 50 μl of dilute buffer. Then, 80 μl of 12.5 % 1 M formic acid and 87.5 % ethanol mixture were added and incubated for 30 min at room temperature to free IGFBP-2. The prepared samples were used in each well of the Quantikine kit, as instructed in the manual. Conditioned medium was assayed for secreted IGFBP-2 using the IGFBP-2 ELISA kit from RayBiotech, according to the manufacturer’s protocol.

Statistical analysis

Results are expressed as means ± S.D. Differences between groups were detected by one-way analysis of variance or a paired Student’s t test. Differences were considered statistically significant at P < 0.05.

RESULTS

Gene expression profiling in fasting compared with refeding conditions

To uncover the physiological relevance in the livers of fasted and refed WT mice, we first evaluated general gene expression profiles under different nutritional conditions. Previously, high-throughput screening analysis in fasting and refed mouse livers [23] had revealed that Igfbp-2 gene expression was higher in the liver of fasting mice than refed mice liver (Figure 1A). Ppara, Pck1 and Igfbp-2 mRNA levels were up-regulated in the fasting mice, whereas Fasn (fatty acid synthase), Gck (Glucokinase) and Srebf-1 (sterol regulatory element binding transcription factor-1) mRNA were increased after refeding.
Figure 1  Microarray analysis to identify *Igfbp-2* gene expression level and qPCR analysis in fasting condition

(A) Hierarchical gene clustering was generated with the TMA Microarray Software Suite (MeV) from fasted and refed livers. A heat map showing significant changes in a group of selected genes from the livers of mice that were fasted for 24 h and refed for 12 h. The ratios of gene profiles are presented as a heat map (left panel) and gene expression pattern (right panel). Fold change and P-values of the genes in the clusters are given on the right. (B-E) mRNA levels of *Igfbp-1, Igfbp-2, Ppara* and *Pck1* in the liver of WT mice were measured by qPCR under the indicated conditions. **P < 0.01 and ***P < 0.001 compared with fed mice.
To further evaluate the microarray data, we assessed qPCR analysis in the livers of fed and fasted WT mice. As expected, mRNA levels of Igfbp-2, Ppara, and Pck1 were increased significantly by the fasting state, relative to the fed state (Figures 1B–1E). In contrast, Igfbp-1 gene expression was not observed in fasted WT mice. Expression of other PPAR isoforms, such as Pparα1, PPARγ2 and PPARδ, did not change during fasting (Figures 1F–1H). Overall, these findings demonstrated that Igfbp-2 was up-regulated by the fasting condition.

Induction of IGFBP-2 by fasting and Wy14643 is dependent on PPARα

Next, we examined whether PPARα plays a role in regulating Igfbp-2 gene expression in primary cultured hepatocytes. Wy14643 treatment in hepatocytes resulted in a significant increase in the mRNA levels of Igfbp-2 and Ppara in a dose-dependent manner, but did not affect Igfbp-1 (Figure 2A). In addition, Wy14643 treatment increased Ppara mRNA levels in the hepatocytes of WT mice but not in Ppara null mice (Figure 2B). Moreover, Igfbp-2 mRNA expression increased in fasting WT mice compared with fed or refed mice, in a fashion similar to that shown in Figure 1. However, this phenomenon was not observed in Ppara null mice (Figures 2C and 2D). Next, we measured the level of secreted IGFBP-2 in the serum of WT and PPARα null mice subjected to feeding and fasting. The level of IGFBP-2 in the serum was increased in the fasted WT mice (Figure 2E) but not in the Ppara null mice. This indicates that IGFBP-2 secretion as well as mRNA expression of Igfbp-2 is also increased by PPARα. In accordance with mRNA level, the protein level of IGFBP-2 increased prominently in Wy14643-treated WT mice. Again, this increase in protein level was not observed in Wy14643-treated Ppara null mice (Figure 2F). Other PPAR isoforms were not affected by Wy14643 treatment in the primary hepatocytes (Figures 2G–2I). Collectively, these results indicate that PPARα is a key mediator for up-regulating IGFBP-2 expression in primary cultured hepatocytes.

PPARα regulates the transcriptional activity of IGFBP-2

To identify the fundamental molecular mechanism through which PPARα regulates Igfbp-2 gene transcription, the mouse Igfbp-2 (mIgfbp-2) gene promoter was transiently transfected into human embryonic kidney (HEK)-293T-cells. As shown in Figure 3(A), computer analysis with consensus PPRE sequence showed a highly conserved putative PPRE on the Igfbp-2 promoter. A proposed PPARα-binding element is shaded in the Igfbp-2 promoter between −511 bp and −499 bp. The transcriptional activity of mIgfbp-2 in response to Wy14643 treatment was highest with a full-length gene promoter and was diminished markedly with deletion up to −444 bp (Figure 3B). Moreover, internal deletion of the putative PPRE between −511 bp and −499 bp from the full-length mIgfbp-2 resulted in decreased promoter activity (Figure 3C). These data collectively suggest that the putative PPRE is located between the −511 and −499 bp regions of the mIgfbp-2 gene promoter. We next performed ChIP assays in mouse livers to verify PPARα binding to the mIgfbp-2 gene promoter at the chromatin level. PPARα occupancy was greater on the mIgfbp-2 promoter (Figure 3D). A known PPRE region on the G6pc gene promoter was used as a positive control in the ChIP assay (Figure 3E). These results demonstrate that Igfbp-2 gene expression is regulated through the direct binding of PPARα to the Igfbp-2 promoter.

IGFBP-2 expression is not affected by PPARγ agonist

Finally, we asked whether Igfbp-2 expression was up-regulated by PPARγ agonists. Rosiglitazone, a PPARγ agonist, was added to primary hepatocytes for 6h and Igfbp-2 mRNA levels were measured. As shown in Figure 5(A), Igfbp-2 expression was not up-regulated by rosiglitazone in primary hepatocytes of WT or Ppara null mice. Treatment with rosiglitazone in the HepG2 cell line also showed minor effects (Figure 5B). These findings indicate that PPARγ is not involved in Igfbp-2 expression.

DISCUSSION

In the present study, we have shown that the fasting state and Wy14643 exposure elevated Igfbp-2 gene transcription and secretion by promoting PPARα in primary hepatocytes, inhibiting IGF-1 signalling and attenuating an IGF-1-dependent pathway. However, the inhibitory action of Wy14643 on the IGF-1 signalling pathway was disrupted in Ppara null mice. Our study showed that Wy14643 exposure increased Igfbp-2 gene transcription through PPARα recruitment. Based on these findings, we propose that up-regulation of Ppara by physiological changes and ligand exposure may prevent hepatic IGF-1 signalling by activating Igfbp-2 expression.

Changes in IGFBP-2 levels are associated with various physiological and pathological states, such as exercise, pregnancy, aging, hormones, diabetes, obesity, insulin resistance and tumours [24–26]. Previous studies have shown that expression of the Igfbp-2 gene is promoted significantly in the liver during fasting [27,28] and that insulin markedly attenuates Igfbp-2 gene expression in myoblast cells and in the heart [29,30]. These findings suggest that IGFBP-2 is altered by physiological states and insulin. However, the biological link between IGFBP-2 and other transcription factors in response to fasting states has not been fully addressed. Our results demonstrate that fasting states elevate IGFBP-2 and PPARα significantly compared with feeding states (Figures 1C and 1D). Remarkably, fasting states and Wy14643 treatment effectively promoted Igfbp-2 gene expression in WT mice, but not in Ppara null mice (Figure 2C). These findings suggest that IGFBP-2 may be involved in fasting conditions and that Wy14643 plays an important role in controlling Igfbp-2 gene expression in primary hepatocytes.

As mentioned previously, PPARα acts as a key regulator of diverse metabolic processes and also of metabolic homeostasis [15,16,31]. It has been shown that PPARα reduced cellular functions by attenuating IGF-1R and Akt activity in diverse cancer cells [18–20]. However, there is no evidence of a correlation between PPARα and the IGF-1 signalling system in primary hepatocytes. Our findings reveal a novel action of PPARα involving IGF-1 sensitivity via the IGF-1R–Akt signalling pathway. Our results demonstrate that the stimulation of IGF-1R, Akt, mTOR and S6K activity by IGF-1 exposure is markedly decreased by Wy14643 treatment. The inhibitory effects of
Figure 2  PPARα is involved in the induction of IGFBP-2 following the fasting state and Wy14643 treatment

(A) Mouse primary hepatocytes were treated with Wy14643 (Wy) for 6 h at the indicated concentrations. Total RNA was isolated and analysed using qPCR with the observed primers. (B) Mouse primary hepatocytes from WT and Pparα null mice were treated with or without Wy14643 for 6 h. The level of Pparα mRNA was measured by qPCR analysis. (C) The mRNA level of Igfbp-2 in liver from WT and Pparα null mice fed and fasted for 24 h was analysed by qPCR. (D) Protein level of IGFBP-2 in livers from WT and Pparα null mice fed and fasted for 24 h was analysed by Western blot analysis. (E) Secretion level of IGFBP-2 in vivo and in vitro. Mice were fed or fasted for 24 h and serum was collected from WT and Pparα null mice. Secretion levels of IGFBP-2 in feeding or fasting conditions were measured by ELISA. (F) WT and Pparα null mice were treated with or without Wy14643 for 6 h. Whole cell extracts were isolated from primary hepatocytes of the observed conditions and assessed by Western blot analysis with the indicated antibody. (G–I) Primary hepatocytes from WT and Pparα null mice were treated with or without Wy14643 for 6 h. The level of Pparγ1, Pparγ2 and Pparδ mRNA was measured by qPCR analysis. *P < 0.05 and **P < 0.01 compared with untreated control or fed WT mice.
Wy14643 were disrupted in PPARα null mice. Thus, PPARα may protect against IGF-1 sensitivity by attenuating the IGF-1-dependent pathway in primary hepatocytes.

It is well known that PPARα regulates the transcription of various target genes [16,21,32,33]. Although our results identify a novel link between PPARα and IGFBP-2 in primary hepatocytes, we do not rule out the possibility that PPARα may rely on other as-yet-unknown mechanisms that control the involvement of transcription co-activators or the completion of co-repressors, protein degradation and modification. Thus, further study is required to determine the detailed molecular network of PPARα and IGFBP-2 in primary hepatocytes.

Ppar isoforms activate target gene expression by binding to the PPRE on the promoter region(s) of target genes [34]. Among the various Ppar isoforms, Ppara expression is increased in the liver during fasting, resulting in the stimulation of Igfbp-2...
expression and the secretion of IGFBP-2. In the present study, we demonstrated that Ppara was the only PPAR isoform induced in fasted liver (Figures 1 and 5). Additionally, because PPARγ expression is very low in the liver during fasting, PPARγ may be not involved in the stimulation of Igfbp-2 gene transcription. These results indicate that IGFBP-2 is primarily regulated in the liver by PPARα during fasting.

In conclusion, we have shown that the fasting state promotes Igfbp-2 gene expression by up-regulating PPARα and that the PPARα-IGFBP-2 cascade attenuates IGF-1 sensitivity by controlling the IGF-1 signalling network. Our findings provide evidence of a novel pathway involved in the regulation of the hepatic IGFBP-2–IGF-1 network and/or PPARα in the fasting state as well as a potential therapeutic approach for the treatment of hepatic metabolic disorders.

**AUTHOR CONTRIBUTION**

Hye Kang and Mi-Young Kim contributed to the conception, design and performance of experiments, analysis and interpretation of data and writing the draft manuscript. Seung-Jae Kim, Yong-Deuk Kim and Young-Kyo Seo contributed to the performance of experiments, analysis and interpretation of data. Jae-Hoon Bae, Goo-Taeg Oh, Dae-Kyu Song and Yong-Ho Ahn contributed to the analysis and interpretation of data and a critical review of the manuscript before submission. Seung-Soon Im contributed to the conception and design of the experiments, the article and critically reviewed the manuscript before submission.

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