Regular Article

Insulin Represses Fasting-Induced Expression of Hepatic Fat-Specific Protein 27

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The fat-specific protein 27 (Fsp27) gene belongs to the cell death-inducing DNA fragmentation factor 45-like effector family. Fsp27 is highly expressed in adipose tissue as well as the fatty liver of ob/ob mice. Fsp27 is directly regulated by the peroxisome proliferator-activated receptor γ (PPARγ) in livers of genetically obese leptin deficient ob/ob mice. In the present study, Fsp27 was markedly induced by 24 h fasting in genetically normal mouse livers and repressed by refeeding a high sucrose diet. In contrast with the liver, Fsp27 expression was decreased in adipose tissue by fasting and increased by refeeding. Interestingly, fasting-induced Fsp27 liver expression was independent of PPARγ. Moreover, Fsp27 expression was induced in the insulin-depleted livers of streptozotocin-treated mice. Finally, Fsp27 expression was repressed by direct injection of glucose or insulin in fasting mice. These results suggest that insulin represses Fsp27 expression in the fasting liver.

Key words insulin; peroxisome proliferator-activated receptor γ (PPARγ); fatty liver; fat-specific protein 27 (Fsp27)

Fat-specific protein 27 (Fsp27) was initially identified from mouse adipocyte TA cell lines as a mature adipocyte-specific gene and belongs to the cell death-inducing DNA fragmentation factor 45-like effector (CIDE) family based on protein sequence homology. The CIDE family consists of three proteins, CIDEA, CIDEB and Fsp27/CIDEC. The human homolog of mouse Fsp27 was reported as CIDEC. Fsp27 was found to be highly expressed in white and brown adipose tissue and localized to lipid droplets (LDs) in adipocytes through amino acids 173–220 of the Fsp27 protein. In earlier studies, Fsp27 promoted the formation of LD–LD fusion and enlarged unilocular LDs in cooperation with perilipin 1, another LD-associated protein. Fsp27-null mice displayed protection from diet-induced obesity and insulin resistance, a small mass of white adipose tissue, and the presence of multilocular LDs. Adipocyte-specific Fsp27-null mice also had a small white adipose tissue mass and hepatosteatosis.

In an earlier study, hepatic peroxisome proliferator-activated receptor γ (PPARγ) promoted hepatic triglyceride (TG) accumulation and the fatty liver development in ob/ob mice, a well-characterized leptin-deficient mouse and a model for type 2 diabetes, obesity, and fatty liver. Fsp27 was identified as a PPARγ target gene in the liver responsible in part for the fatty liver phenotype in obese mice. Thus, Fsp27 is directly associated with hepatic TG accumulation, and fatty liver generation is dependent on hepatic PPARγ and Fsp27 expression.

Fsp27 was recently reported to be induced in the fasting livers of normal mice. Fsp27 has two alternative isoforms, Fsp27α and Fsp27β. In the present study, fasting-induced Fsp27 expression in the liver was dramatically repressed by refeeding a high sucrose diet. In addition, the depletion of insulin by streptozotocin treatment induced Fsp27 expression. Further, fasting-induced Fsp27 was repressed by direct injection of insulin or glucose into fasting mice. Thus, our study demonstrated that Fsp27 is transcriptionally regulated in the liver in a PPARγ-dependent manner and that insulin is a negative regulator of hepatic Fsp27 expression.

MATERIALS AND METHODS

Animal Studies All animal protocols and studies were performed according to guidelines from the Center for Experimental Animals at Fukuoka University. Liver-specific PPARγ knockout mice (Pparγfl/fl) were generated by breeding the Pparγ-floxed mice (Pparγγγγ) with mice expressing Cre recombinase under the control of the albumin promoter, as previously described. For the fasting and refeeding study, C57BL/6Jc1 mice (male, 10 weeks) were fed a regular chow diet (MF, Oriental Yeast, Japan) ad libitum until the experimental treatment commenced. Mice in the fasting group (n=4) were fasted 24 h and then killed. Mice in the refeeding group (n=4) were fasted 24 h and then refed with a 50% (w/w) sucrose/MF diet for 24 h and then killed. Mice in the control group (n=4) were fed ad libitum with a regular chow diet and euthanized at the same time as the refeeding group. Total RNA was extracted from liver and white adipose tissue. Measurement of hepatic TG level was performed as described previously.

For the streptozotocin (STZ) study, C57BL/6Jc1 mice (male, 10 weeks) were fasted for 4 h before injection of STZ (n=4). STZ (50 mg/kg) was intraperitoneally injected for 5 d. Control mice were injected with a citrate solution (pH 4.5) used as a solvent for STZ (n=4). Five days after STZ treatment, plasma glucose levels were evaluated and diabetes was confirmed (blood glucose level >250 mg/dL).

For the direct injection of human insulin or glucose, mice

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were fasted for 24 h before treatment. Insulin (Thermo Fisher Scientific, Japan) and glucose were intraperitoneally injected at concentrations of 8 mU/g and 5 mg/g, respectively. Ten min, 30 min, 1 h, and 24 h after injection (n=3, at each time point), the mice were killed and total RNA was extracted from the livers.

RNA Extraction and Quantitative Real-Time PCR
RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Japan), and quantitative polymerase chain reaction (QPCR) performed using cDNA generated from 1 µg of total RNA with an AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies, Japan). The primer sequences used

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Fig. 1. Induction of Fsp27 mRNA in Fasting Liver Is Significantly Repressed by Refeeding
QPCR analyses of Fsp27, Pparγ, Fas and Pck1 mRNAs in liver (A–D) or white adipose tissue (WAT: E–G) performed using each total RNA from ad libitum fed-mice (Control), 24 h-fasted mice (Fasting), and 24 h-fasted/refed mice (Refeeding). Gene expression was normalized to 36b4 mRNA; each bar represents an average±S.E.M. of four separate experiments. Significant differences compared with Control, †p<0.05, ††p<0.01, †††p<0.001. Significant differences compared with Fasting, *p<0.01, **p<0.001.
RESULTS

Fasting-Induced Fsp27 Expression Is Repressed by Refeeding

It was recently reported that the Fsp27 was induced in the fasting livers of wild-type mice. In the present study, 24 h fasting also caused a marked induction (approximately 22-fold vs. control) of hepatic Fsp27 expression. However, Fsp27 expression was decreased to the same level as the control by refeeding (Fig. 1A). Fsp27 has two alternative isoforms, Fsp27a and Fsp27b which consist of a different exon 1. In the present study, Fsp27 mRNA was measured as the sum of Fsp27a and Fsp27b mRNAs since primer pairs for the QPCR amplify common region (junction of exon 2 and exon 3) both isoforms. No marked difference in Pparγ mRNA was observed between control and the fasted/refed mice (Fig. 1B). Fas mRNA was decreased in fasting liver, but induced in refeeding liver as a positive control of refeeding-inducible gene, while Pck1 mRNA, known as fasting-inducible gene revealed the opposite expression pattern of Fas gene (Figs. 1C, D). Fsp27 mRNA analysis revealed constitutively abundant expression in white adipose tissue. Interestingly, and contrary to the liver, Fsp27 expression in white adipose tissue (WAT) was repressed by approximately 25% upon fasting and recovered with refeding to the control level (Fig. 1E).

Statistical Analysis

Quantitative values are presented as the mean±standard error of the mean (S.E.M.). Differences between mouse groups were confirmed for statistical significance with 2-tailed Student’s t-test, with p<0.05 considered statistically significant.

Fig. 2. Induction of Hepatic Fsp27 mRNA in Fasting Mice Is PPARγ-Independent

QPCR analyses of Pparγ (A), Fsp27 (B) and Pck1 (C) mRNAs performed using total RNA from each genotyped mouse liver. Each liver was also used for the measurement of hepatic triglyceride (TG) contents (D). Fasting conditions were the same as in the Fig. 1 legend. Gene expression was normalized to 36b4 mRNA; each bar represents the average±S.E.M. of four separate experiments. PPARγfl/fl, wild-type mouse liver; PPARγΔhep, liver-specific PPARγ knockout mouse liver. Significant differences compared with PPARγfl/fl, *p<0.05, **p<0.001.
Ppar and Fas mRNAs in WAT showed a similar tendency as Fsp27 mRNA (Figs. 1F, G). These results suggest that refeeding represses the induction of fasting-induced hepatic Fsp27 mRNA but not WAT Fsp27.

**Fsp27 Induction by Fasting Is Independent of Hepatic PPARγ**  
Fsp27 is highly expressed in fatty livers of ob/ob mice. However, the expression was markedly decreased by the hepatic Pparγ-null mice. 12) To examine whether PPARγ is associated with the induction of Fsp27 by fasting, PparγΔhep mice were used for a fasting study. Although the Pparγ gene was expressed at markedly lower levels in the PparγΔhep liver (Fig. 2A), Fsp27 was induced by fasting in the PparγΔhep liver (Fig. 2B). There was no significant difference between the Fsp27 mRNA levels of PparγΔhep and Pparγfl/fl mice under fasting conditions (Fig. 2B). Pck1 mRNA was also unchanged between PparγΔhep and Pparγfl/fl mouse livers (Fig. 2C). Hepatic PPARγ promoted the accumulation of a hepatic TG in ob/ob mice through the Fsp27. 12) Although the fasting liver also causes the accumulation of TG, 14,15) no difference in hepatic TG levels was observed between PparγΔhep and Pparγfl/fl mouse livers (Fig. 2D). These results indicate that the induction of Fsp27 by fasting was independent of hepatic PPARγ.

**Insulin Represses the Expression of Fasting-Induced Fsp27**  
Repression of Fsp27 mRNA by refeeding was predicted to be associated with insulin regulation. Therefore, to evaluate the potential association between Fsp27 repression and insulin, mouse insulin was depleted by induction of β cells by STZ administration. After STZ injection for 5 d, blood glucose levels were above 250 mg/dL (data not shown). Although Pparγ mRNA was unchanged between control and STZ-treated mouse livers (Fig. 3A), the Fsp27 mRNA was approximately 6-fold higher than that in control livers (Fig. 3B). Pck1 mRNA was significantly increased in STZ-treated mouse livers (Fig. 3C). These results suggested that insulin is a negative regulator of hepatic Fsp27.

**RESULTS**

**Expression of Hepatic Fsp27 Is Induced by STZ Administration**

QPCR analyses of Pparγ (A), Fsp27 (B) and Pck1 (C) mRNAs performed using total RNA from livers of control (Control) and streptozotocin-injected mice (STZ). Control mice were injected with a citrate solution used as a vehicle for STZ. Gene expression was normalized to 36b4 mRNA; each bar represents the average±S.E.M. of four separate experiments. Significant differences compared with Control, *p<0.05, **p<0.001.

**DISCUSSION**

PPARγ is induced in the leptin-deficient ob/ob mouse liver and was critical for the development of a fatty liver. 11) Elevated PPARγ signaling in the ob/ob fatty liver directly induced Fsp27 expression through a PPAR responsive element in its promoter; the induced Fsp27 then coordinates with lipogenic genes encoding Fas and acetyl-CoA carboxylase to elevate hepatic TG levels. 11) Expression level of hepatic Fsp27 is increased in fatty liver generated by high-fat diet, methionine choline-deficient diet, 13) and alcohol feeding 24) but the expression patterns of Fsp27 or Pparγ mRNAs clearly differed in each fatty liver, suggesting that the mechanism of transcriptional regulation of Fsp27 is dependent on the etiology of fatty liver.
liver. These results raised the possibility that hepatic Fsp27 expression is regulated by multiple factors, including nutritional and hormonal factors. Therefore, this study sought to identify regulators governing the Fsp27 expression. Fsp27 is highly expressed in the fatty liver or adipose tissue of ob/ob mice but is only slightly expressed in the genetically normal mouse liver.\textsuperscript{12} It was recently demonstrated that fasting leads to the marked induction of Fsp27 mRNA in the normal mouse liver.\textsuperscript{14–16} In the present study, insulin was found to repress fasting-induced Fsp27 mRNA in the liver but not WAT.

The precise mechanism of Fsp27 repression by insulin remains unclear. It was demonstrated that Fsp27 mRNA in the fasting liver of genetically normal mice is positively regulated by PPAR\(\gamma\),\textsuperscript{15} cAMP-responsive element binding protein (CREB)\textsuperscript{16} or cAMP-responsive element binding protein H (CREBH).\textsuperscript{14,17} Under fasting conditions, the transcriptional activity of CREB is upregulated by interaction of CREB-regulated transcription coactivator 2 (CRTC2) and CREB-binding protein and p300 (CBP/p300).\textsuperscript{25} Conversely, insulin signaling under the refeeding conditions downregulated CREB activity by disputation of CREB, CRTC2 and CBP/p300 complexes through ubiquitin-dependent degradation mediated by salt-inducible kinase 2 (SIK2) activated by insulin.\textsuperscript{25} It was also reported that CREBH in fasting liver activates the expression of Pck1 via a CRTC2-dependent mechanism and directly interacts with CRTC2.\textsuperscript{26} Thus, the repression of Fsp27 expression by insulin is likely mediated by downregulation of CREB and CREBH via the SIK2-CRTC2 pathway.

The expression of fasting-induced Fsp27 in the liver was completely abolished in CREBH knockout mice.\textsuperscript{14,17} Thus, CREBH appears to play a major role in hepatic Fsp27 induction by fasting rather than by CREB. In the present study, repression of Fsp27 mRNA by insulin was observed in liver but not in WAT. Thus, liver-specific expression of CREBH\textsuperscript{27} is likely to explain the liver-specific repression of Fsp27 by insulin. It was recently reported that the transcriptional corepressor small heterodimer partner-interacting leucine zipper protein (SMILE) inhibits CREBH,\textsuperscript{28} and is an insulin-inducible repressor.\textsuperscript{29} Therefore, besides the SIK2-CRTC2 pathway, SMILE-mediated inhibition of CREBH may also be critical for insulin-dependent repression of Fsp27. The PPARY in ob/ob liver promoted the TG accumulation and formed fatty liver through Fsp27.\textsuperscript{11} The induction of Fsp27 by CREBH\textsuperscript{17} or PPARY\textsuperscript{15} in fasting liver appears to associate with the formation of fasting fatty liver.\textsuperscript{15} In the present study, the hepatic TG of Ppary\textsuperscript{shp} mice increased in fasting liver. This data supports the formation of fasting fatty liver is mediated by the other factors except for PPARY.

Taken together, the current study demonstrated that insulin represses fasting-induced Fsp27 expression in the liver. The Fsp27 decreased by insulin is likely to cause the lower fat accumulation in liver. Insulin potently stimulates lipogenesis,\textsuperscript{29} Thus, insulin appears to function to switch from fat accumulation to fat synthesis through hepatic Fsp27 regulation. More mechanistic studies are required to clarify how the insulin pathway represses fasting-induced Fsp27 and whether other nutritional and hormonal factors, such as glucagon could also be involved in the transcriptional regulation of hepatic Fsp27.

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Conflict of Interest The authors declare no conflict of interest.

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