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

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## Abstract

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 $\gamma$ (PPAR$\gamma$) 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 PPAR$\gamma$. 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.

### Keywords

insulin; peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$); 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[1,2] and belongs to the cell death-inducing DNA fragmentation factor 45-like effector (CIDE) family based on protein sequence homology.[3] The CIDE family consists of three proteins, CIDEA, CIDEB and Fsp27/CIDEC. The human homolog of mouse Fsp27 was reported as CIDEC.[4] Fsp27 was found to be highly expressed in white and brown adipose tissue and localized to lipid droplets (LDs) in adipocytes[5] through amino acids 173–220 of the Fsp27 protein.[6] In earlier adipocyte studies, Fsp27

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promoted the formation of LD–LD fusion and enlarged unilocular LDs in cooperation with perilipin 1, another LD-associated protein.[7] *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.[8,9] Adipocyte-specific *Fsp27*-null mice also had a small white adipose tissue mass and hepatosteatosis.[10]

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,[11] 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.[12] Thus, Fsp27 is directly associated with hepatic TG accumulation, and fatty liver generation is dependent on hepatic PPARγ and Fsp27 expression.[12,13]

*Fsp27* was recently reported to be induced in the fasting livers of normal mice.[14–16] *Fsp27* has two alternative isoforms, *Fsp27a* and *Fsp27b*.[17] 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-independent 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 ($Pparγ^{Δhep}$) were generated by breeding the *Pparγ*-floxed mice ($Pparγ^{fl/fl}$) with mice expressing Cre recombinase under the control of the albumin promoter, as previously described.[11] For the fasting and refeeding study, C57BL/6JJc1 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.[11]

For the streptozotocin (STZ) study, C57BL/6JJc1 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 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 $\mu$g of total RNA with an AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies, Japan). The primer sequences used were as follows: *Fsp27*: forward, 5′-ATG AAG TCT CTC AGC CTC CTG-3′ and reverse, 5′-AAG CTG TGA GCC ATG ATG C-3′; *Pparγ*: forward, 5′-CAT GGC CAT TGA GTG CCG AGT-3′ and reverse, 5′-ACA TCC CCA CAG CAA GGC AC-3′; *Fatty acid synthase* (*Fas*): forward, 5′-GGA GGT GGT GAT AGC CGG TAT-3′ and reverse, 5′-TGG GTA ATC CAT AGA GCC CAG-3′; *phosphoenolpyruvate carboxykinase 1* (*Pck1*): forward, 5′-CAT ATG CTG ATC CTG GGC ATA AC-3′ and reverse, 5′-CAA ACT TCA TCC AGG CAA TGT C-3′; *acidic ribosomal phosphoprotein P0* (*36b4*): forward, 5′-AAA CTG CTG CCT CAC ATC CG-3′ and reverse, 5′-TGG TGC CTC TGG AGA TTT TCG-3′. *36b4* gene was selected as an internal control of QPCR because *glyceraldehyde 3-phosphate dehydrogenase* or *beta-actin* gene as the general internal control is increased by insulin.[18,19] QPCR reactions were performed by using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) in an Mx3005P Real-Time PCR System (Agilent Technologies). Values for sample mRNAs were normalized to expression of 36b4 mRNA.

### 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.

## 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.[14–16] 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.[17] 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 Ppar$\gamma$ 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,[20] while Pck1 mRNA, known as fasting-inducible gene[21,22] revealed the opposite expression pattern of *Fas* gene (Figs. 1C, D). Fsp27 mRNA analysis revealed constitutively abundant expression in white adipose tissue.[5,12]

Interestingly, and contrary to the liver, *Fsp27* expression in white adipose tissue (WAT) was repressed by approximately 25% upon fasting and recovered with refeeding to the control level (Fig. 1E). Ppar$\gamma$ 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 PPAR$\gamma$

*Fsp27* is highly expressed in fatty livers of *ob*/*ob* mice. However, the expression was markedly decreased by the hepatic *Ppar*$\gamma$-null mice.[12] To examine whether PPAR$\gamma$ is associated with the induction of *Fsp27* by fasting, *Ppar*$\gamma^{\Delta hep}$ were used for a fasting study. Although the *Ppar*$\gamma$ gene was expressed at markedly lower levels in the *Ppar*$\gamma^{\Delta hep}$ liver (Fig. 2A), *Fsp27* was induced by fasting in the *Ppar*$\gamma^{\Delta hep}$ liver (Fig. 2B). There was no significant difference between the Fsp27 mRNA levels of *Ppar*$\gamma^{\Delta hep}$ and *Ppar*$\gamma^{fl/fl}$ mice under fasting conditions (Fig. 2B). Pck1 mRNA was also unchanged between *Ppar*$\gamma^{\Delta hep}$ and *Ppar*$\gamma^{fl/fl}$ mouse livers (Fig. 2C). Hepatic PPAR$\gamma$ 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 *Ppar*$\gamma^{\Delta hep}$ and *Ppar*$\gamma^{fl/fl}$ mouse livers (Fig. 2D). These results indicate that the induction of *Fsp27* by fasting was independent of hepatic PPAR$\gamma$.

### 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 destruction of $\beta$ cells by STZ administration. After STZ injection for 5 d, blood glucose levels were above 250 mg/dL (data not shown). Although Ppar$\gamma$ 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 as a positive control for STZ administration[23] (Fig. 3C).

Results of the refeeding and STZ studies strongly suggested the involvement of insulin for refeeding-associated repression of the *Fsp27*. Thus, insulin or glucose was directly injected into fasting mice. Insulin and glucose repressed fasting-induced Fsp27 mRNA expression by approximately 70 and 50% at 30 min post-injection, respectively. This degree of Fsp27 mRNA repression achieved by insulin recovered to the same level by 0 min at 24 h post-injection (Fig. 4A), but the repression achieved by glucose continued to decrease up to 24 h (Fig. 4B). These results suggested that insulin is a negative regulator of hepatic *Fsp27*.

## DISCUSSION

PPAR$\gamma$ is induced in the leptin-deficient *ob*/*ob* mouse liver and was critical for the development of a fatty liver.[11] Elevated PPAR$\gamma$ 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 Ppar$\gamma$ mRNAs clearly differed in each fatty liver, suggesting that the mechanism of transcriptional regulation of *Fsp27* is dependent on the etiology of fatty 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.[12] It was recently demonstrated that fasting leads to the marked induction of Fsp27 mRNA in the normal mouse liver.[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$\alpha$,[15] cAMP-responsive element binding protein (CREB)[16] or cAMP-responsive element binding protein H (CREBH).[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).[25] Conversely, insulin signaling under the refeeding conditions downregulated CREB activity by disruption of CREB, CRTC2 and CBP/p300 complexes through ubiquitin-dependent degradation mediated by salt-inducible kinase 2 (SIK2) activated by insulin.[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.[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.[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[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,[28] and is an insulin-inducible repressor.[22] Therefore, besides the SIK2-CRTC2 pathway, SMILE-mediated inhibition of CREBH may also be critical for insulin-dependent repression of *Fsp27*. The PPAR$\gamma$ in *ob*/*ob* liver promoted the TG accumulation and formed fatty liver through Fsp27.[11] The induction of *Fsp27* by CREBH[17] or PPAR$\alpha$[15] in fasting liver appears to associate with the formation of fasting fatty liver.[15] In the present study, the hepatic TG of *Ppar*$\gamma^{\Delta hep}$ mice increased in fasting liver. This data supports the formation of fasting fatty liver is mediated by the other factors except for PPAR$\gamma$.

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.[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*.

## Acknowledgments

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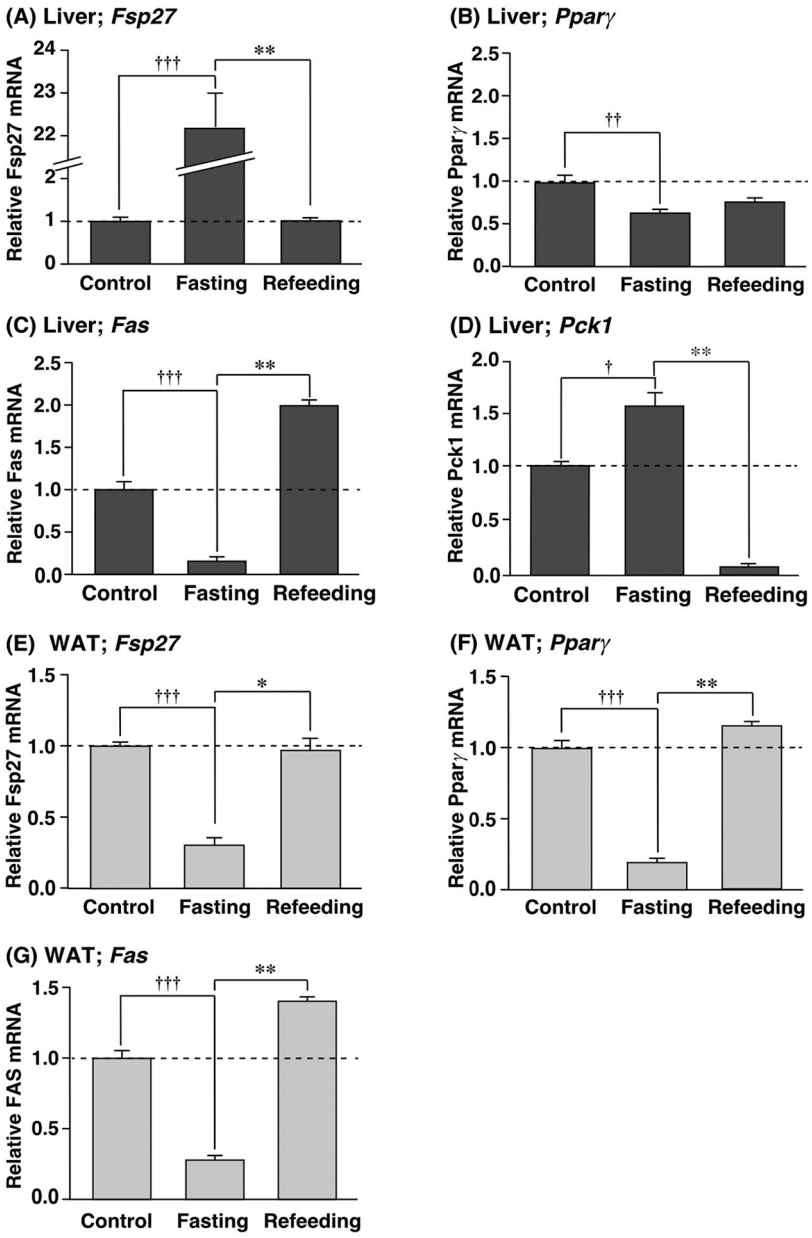


**Fig. 1. Induction of Fsp27 mRNA in Fasting Liver Is Significantly Repressed by Refeeding**

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, * $p<0.01$, ** $p<0.001$.

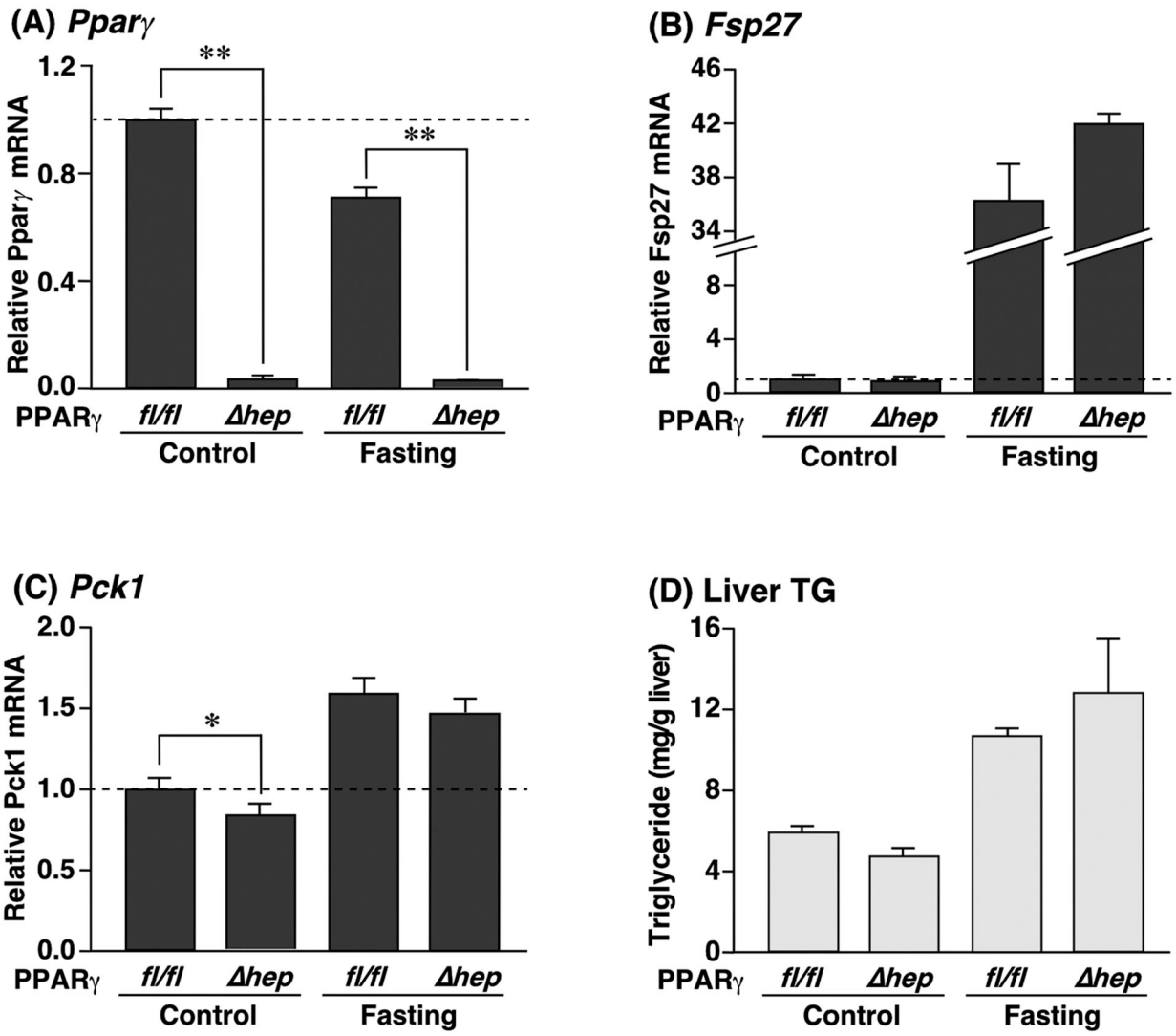


**Fig. 2. Induction of Hepatic Fsp27 mRNA in Fasting Mice Is PPARγ-Independent$ $PARABREAKHERE$$QPCR analyses of Pparγ**

(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. PPARγ$^{fl/fl}$, wild-type mouse liver; PPARγ$^{\Delta hep}$, liver-specific PPARγ knockout mouse liver. Significant differences compared with PPARγ$^{fl/fl}$, * $p$<0.05, ** $p$<0.001.

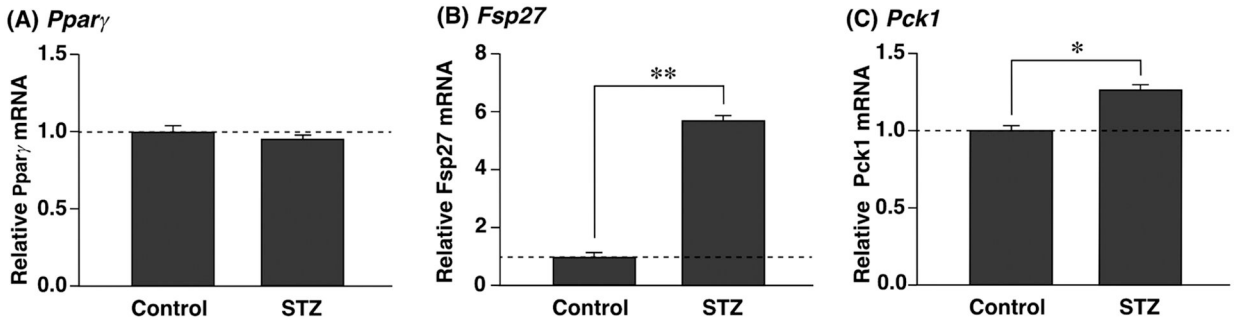


**Fig. 3. Expression of Hepatic *Fsp27* Is Induced by STZ Administration$$PARABREAKHERE$ $QPCR analyses of Pparγ**

(A), Fsp27 (B) and Pck1 (C) mRNAs performed using total RNAs 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, * $p<0.05$, ** $p<0.001$.

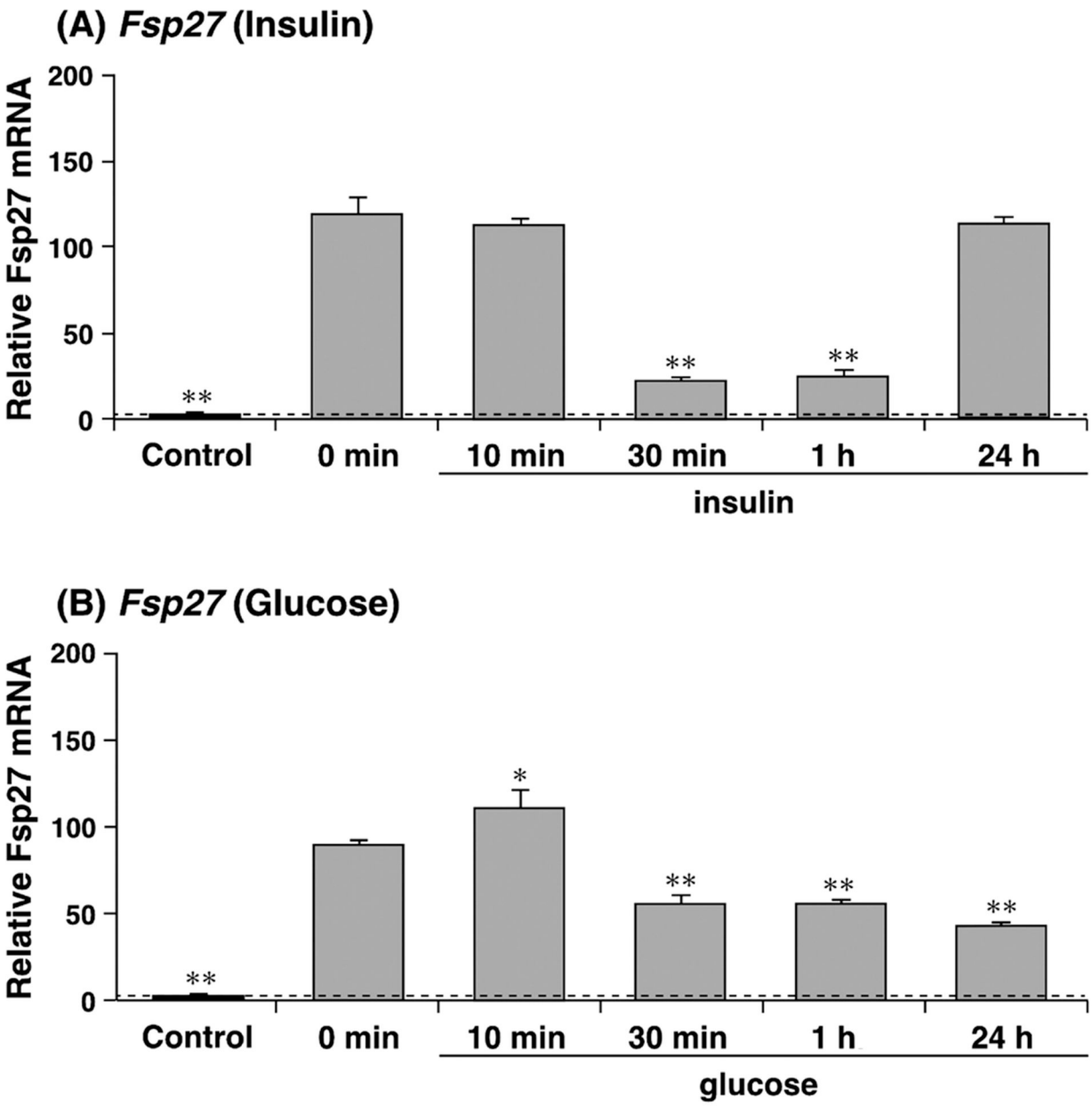


**Fig. 4. Insulin or Glucose Significantly Represses Fasting-Induced Hepatic *Fsp27* Expression**

QPCR analysis of Fsp27 mRNA performed using total RNA from the livers of insulin-injected mice (A) or glucose-injected mice (B). Mice were fasted for 24 h before insulin or glucose injection (except for the control group). After intraperitoneal injection with insulin (8 mU/g of body weight) or glucose (5 mg/g of body weight), mice were killed at 0, 10, 30 min, 1, and 24 h. Control mice (Control) were fed *ad libitum* with a regular chow diet. Gene expression was normalized to 36b4 mRNA; each bar represents the average±S.E.M. of three separate experiments. Significant differences compared with 0 min, * $p<0.01$, ** $p<0.001$.