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Dynamic Functional Relay between Insulin Receptor Substrate 1 and 2 in Hepatic Insulin Signaling during Fasting and Feeding

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DOI 10.1016/j.cmet.2008.05.007

SUMMARY

Insulin receptor substrate (Irs) mediates metabolic actions of insulin. Here, we show that hepatic Irs1 and Irs2 function in a distinct manner in the regulation of glucose homeostasis. The PI3K activity associated with Irs2 began to increase during fasting, reached its peak immediately after refeeding, and decreased rapidly thereafter. By contrast, the PI3K activity associated with Irs1 began to increase a few hours after refeeding and reached its peak thereafter. The data indicate that Irs2 mainly functions during fasting and immediately after refeeding, and Irs1 functions primarily after refeeding. In fact, liver-specific Irs1-knockout mice failed to exhibit insulin resistance during fasting, but showed insulin resistance after refeeding; conversely, liver-specific Irs2-knockout mice displayed insulin resistance during fasting but not after refeeding. We propose the concept of the existence of a dynamic relay between Irs1 and Irs2 in hepatic insulin signaling during fasting and feeding.

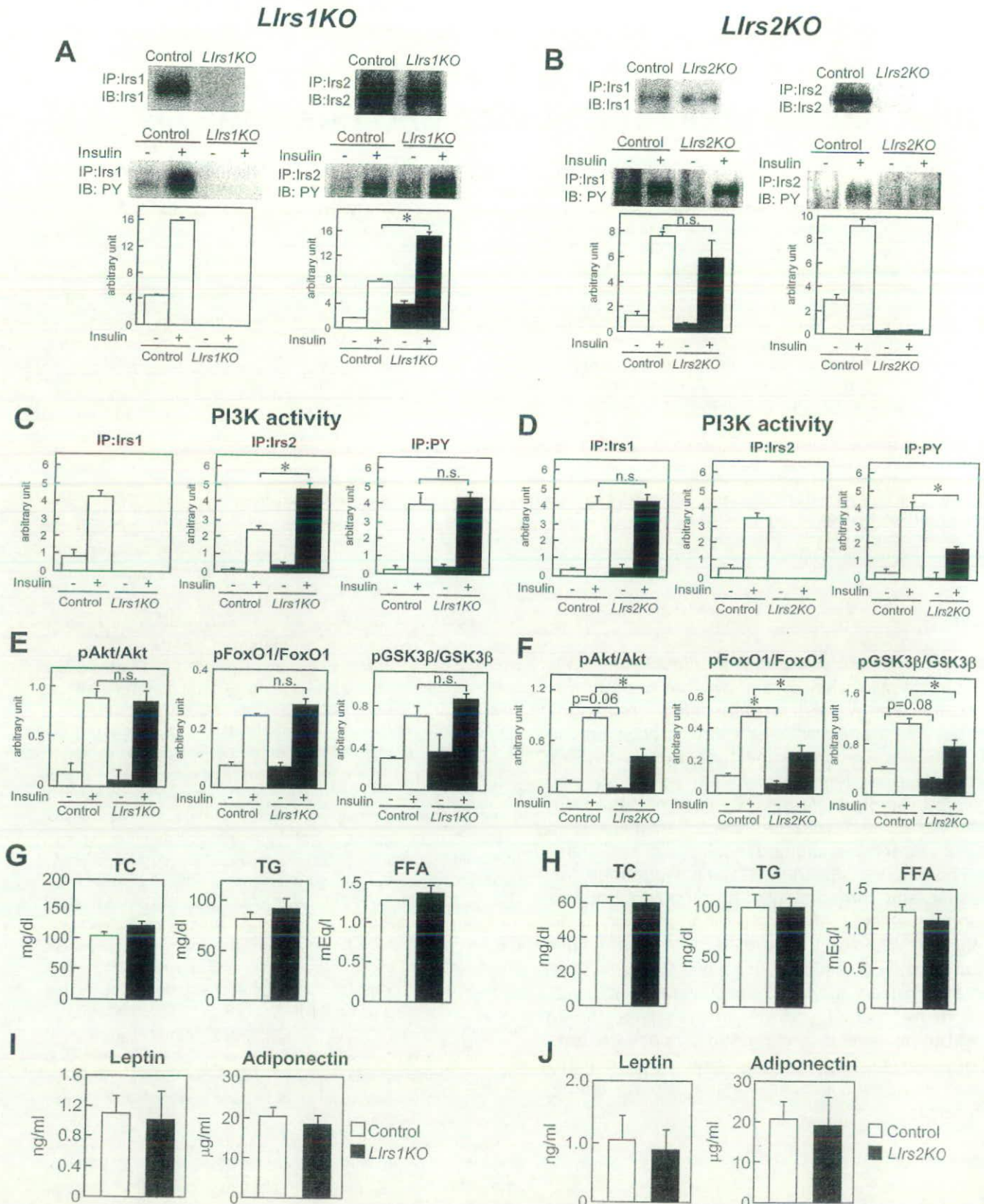
INTRODUCTION

The liver is an essential organ for glucose homeostasis; it stores excess glucose after food intake in the form of glycogen and releases glucose by glycogenolysis or gluconeogenesis during fasting (Gribble, 2005). Insulin promotes glycogen synthesis

and inhibits glycogenolysis and gluconeogenesis by exerting direct and indirect effects on the enzyme activities and gene expressions in the liver (Saltiel and Kahn, 2001). Dysregulation of the hepatic insulin actions is a critical component in the pathogenesis of type 2 diabetes (Saltiel and Kahn, 2001).

Recently, it was reported that circulating insulin not only directly inhibits glucose production in the liver, but also acts at the level of the brain to suppress gluconeogenesis (Wada et al., 2005; Kasuga, 2006; Plum et al., 2006). Downregulation of the hypothalamic insulin receptor caused hepatic insulin resistance (Obici et al., 2002a), and intracerebroventricular injection of insulin suppressed hepatic glucose production (Obici et al., 2002b), suggesting that insulin inhibits hepatic glucose production via both the classical direct hepatic pathway and a newly identified central indirect pathway. Considering this indirect central action of insulin, it was considered that genetically engineered animals lacking in insulin signaling exclusively in the liver may be useful models for clarifying the physiological roles of the direct effects of insulin in the liver. In fact, mice with genetic deletion of the hepatic insulin receptors (*LIRKO* mice) were found to exhibit severe insulin resistance and hyperglycemia after feeding, indicating that hepatic insulin-receptor signaling plays a pivotal role in the regulation of hepatic and systemic glucose homeostasis (Michael et al., 2000).

Insulin receptor substrate (Irs) 1 and Irs2 exhibit high structural homology, are abundantly expressed in the liver, and are thought to be responsible for transmitting insulin signaling from the insulin receptor to the intracellular effectors in the regulation of glucose and lipid homeostasis (Saltiel and Kahn, 2001; Nandi et al., 2004; Taniguchi et al., 2006; Thirone et al., 2006). However, while mice lacking in systemic Irs1 failed to show any impairment



of hepatic insulin signaling (Tamemoto et al., 1994; Araki et al., 1994; Tobe et al., 1995; Yamauchi et al., 1996), those lacking in systemic *Irs2* exhibited impaired hepatic insulin signaling and impaired suppression of glucose production by insulin (Withers et al., 1998; Kubota et al., 2000). Hyperinsulinemic-euglycemic clamp studies have revealed that systemic *Irs2*-knockout mice show markedly impaired suppression of hepatic glucose production and reduced stimulation of liver glycogen synthesis, while systemic *Irs1*-knockout mice show insulin resistance predominantly in the skeletal muscle and adipose tissue but not in the liver (Previs et al., 2000). These data point to the significance of liver *Irs2* and/or brain *Irs2* in the hepatic actions of insulin.

Recently, liver-specific *Irs2*-knockout mice were generated, which unexpectedly exhibited little or no disturbance of glucose homeostasis (Dong et al., 2006; Simmgen et al., 2006). Considering the severe insulin resistance and hyperglycemia observed after feeding in the *LIRKO* mice (Michael et al., 2000), these data led us to re-examine the role of *Irs1*, the other major *Irs* protein in the liver. In this study, we generated liver-specific *Irs1*-knockout mice, as well as liver-specific *Irs2*-knockout mice, to investigate the physiological roles of hepatic *Irs1* and *Irs2* in the regulation of glucose metabolism. Furthermore, we also generated liver-specific *Irs1/Irs2* double-knockout mice in an attempt to elucidate whether hepatic insulin signaling is exclusively mediated by *Irs1* and *Irs2*.

RESULTS

Generation of Liver-Specific *Irs1*- and *Irs2*-Knockout Mice

Genomic DNA PCR was performed to detect Cre-mediated recombination at the genomic DNA level. The *Irs1* and *Irs2* alleles were found to be deleted in the livers of the liver-specific *Irs1*-knockout (*Llrs1KO*) and liver-specific *Irs2*-knockout (*Llrs2KO*) mice, respectively, but not in any of the tissues in the control mice (Figure S1A). While the *Irs1* mRNA was almost completely abrogated, the *Irs2* mRNA levels remained unchanged in the *Llrs1KO* mice; conversely, while the *Irs1* mRNA levels remained unchanged, the *Irs2* mRNA was almost completely abrogated in the *Llrs2KO* mice (Figure S1B). Western blotting of the *Irs1* or *Irs2* immunoprecipitates from the livers of the control and *Llrs1KO* mice under the fasting condition revealed that while expression of the *Irs1* protein was almost completely abrogated in the *Llrs1KO* mice, the expression level of the *Irs2* protein was indistinguishable from that in the control mice (Figure 1A, upper panels). When insulin was administered via the inferior vena

cava under the fasting condition, tyrosine phosphorylation of *Irs1* (Figure 1A, lower-left panels) and insulin-stimulated recruitment of the p85-adaptor subunit of phosphatidylinositol 3-kinase (PI3K) to the *Irs1* immunoprecipitates (Figure S2A, left panels) were also abrogated in the *Llrs1KO* mice. On the other hand, insulin-stimulated tyrosine phosphorylation of *Irs2* (Figure 1A, lower-right panels) and insulin-stimulated recruitment of the p85-adaptor subunit of PI3K to the *Irs2* immunoprecipitates (Figure S2A, right panels) were significantly increased in these *Llrs1KO* mice, similar to the findings in systemic, *Irs1*-deficient mice (Tobe et al., 1995; Yamauchi et al., 1996). On the other hand, in the *Llrs2KO* mice, the *Irs2* protein was almost completely abrogated, while the expression level of the *Irs1* protein was indistinguishable from that in the control mice (Figure 1B, upper panels). While insulin-stimulated tyrosine phosphorylation of *Irs2* (Figure 1B, lower-right panels) and insulin-stimulated recruitment of the p85-adaptor subunit of PI3K to the *Irs2* immunoprecipitates (Figure S2B, right panels) were abrogated in these *Llrs2KO* mice, insulin-stimulated tyrosine phosphorylation of *Irs1* (Figure 1B, lower-left panels) and insulin-stimulated recruitment of the p85-adaptor subunit of PI3K to *Irs1* immunoprecipitates (Figure S2B, left panels) under the fasting condition were similar between the control and *Llrs2KO* mice, similar to the findings in systemic, *Irs2*-deficient mice (Kubota et al., 2000). When lysates from the livers of fasted *Llrs1KO* mice were immunoprecipitated with anti-*Irs1* antibody, the insulin-stimulated PI3K activity associated with *Irs1* was completely abrogated (Figures 1C, left panel, and S2C, left panel), while that associated with *Irs2* was ~2-fold as high as that in the control mice (Figures 1C, middle panel, and S2C, middle panel). Thus, insulin-stimulated PI3K activity associated with tyrosine-phosphorylated proteins was similar between the two genotypes under the fasting condition (Figures 1C, right panel, and S2C, right panel). On the other hand, in fasted *Llrs2KO* mice, insulin-stimulated PI3K activity associated with *Irs2* was completely abrogated (Figures 1D, middle panel, and S2D, middle panel), while that associated with *Irs1* was similar to that in the control mice (Figures 1D, left panel, and S2D, left panel). Consequently, insulin-stimulated PI3K activity associated with tyrosine-phosphorylated proteins was reduced by half in the *Llrs2KO* mice as compared with that in the control mice (Figures 1D, right panel, and S2D, right panel). Basal and insulin-stimulated phosphorylation of Akt, forkhead transcription factor O1 (FoxO1), and glycogen synthase kinase (GSK)3 β , which are all among the major substrates of Akt, were similar in the liver of the control and *Llrs1KO* mice under the fasting condition (Figures 1E and S2E). In contrast, basal phosphorylation of Akt and GSK3 β tended to be

Figure 1. Generation of the *Llrs1KO* and *Llrs2KO* Mice

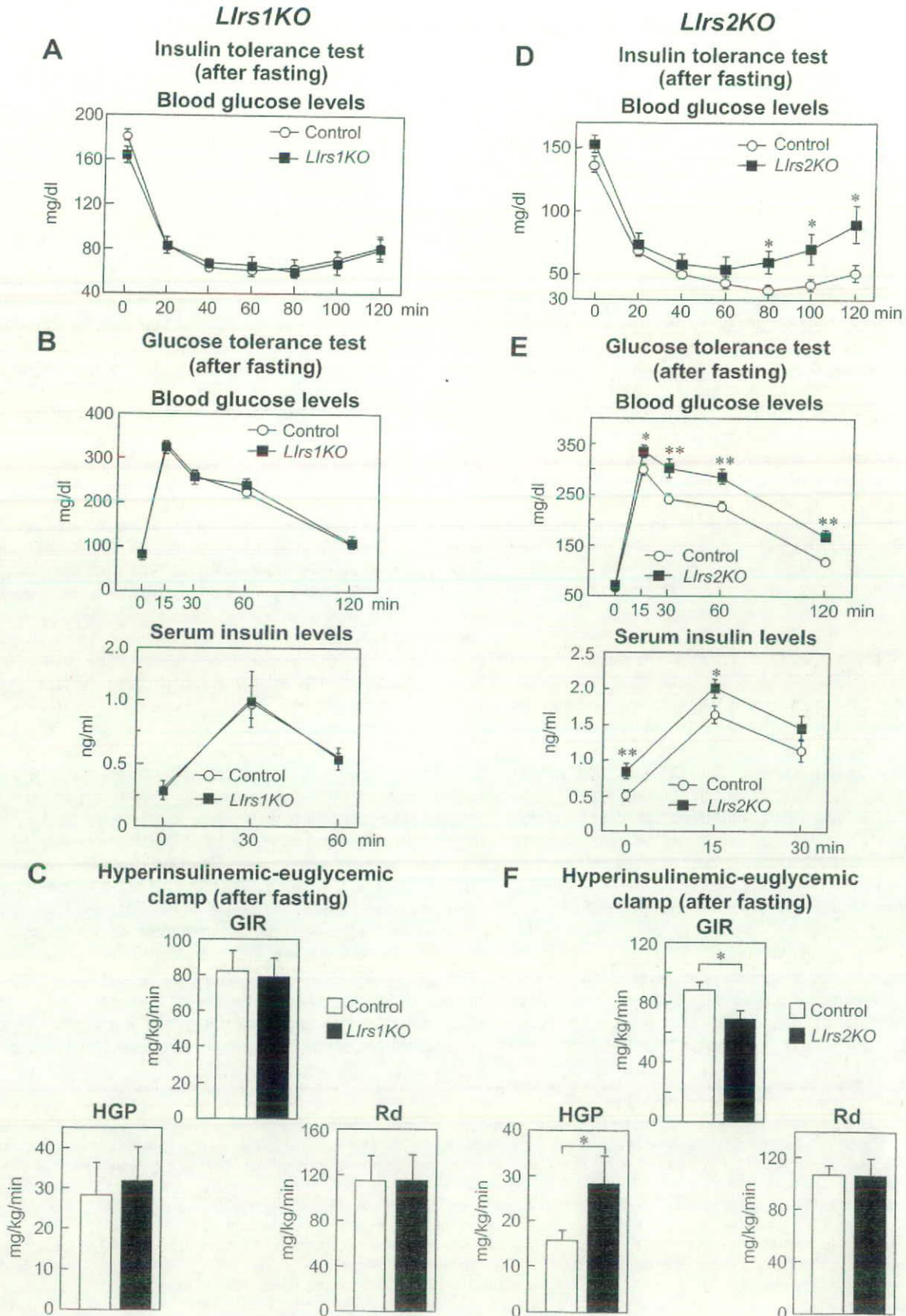
(A and B) Upper panels show liver lysates from *Llrs1KO* (A) and *Llrs2KO* (B) mice were immunoprecipitated with anti-*Irs1* or anti-*Irs2* antibody and subsequently immunoblotted with anti-*Irs1* or anti-*Irs2* antibody after overnight (about 16 hr) fasting. Lower panels show insulin-stimulated tyrosine phosphorylation of *Irs1* or *Irs2* at 70 s after administration of 10 U of insulin in the liver of the *Llrs1KO* (A) and *Llrs2KO* (B) mice after overnight (about 16 hr) fasting. Results are representative of three independent experiments.

(C and D) Insulin-stimulated PI3K activity associated with *Irs1*-, *Irs2*-, and tyrosine-phosphorylated proteins at 70 s after administration of 10 U of insulin in the liver of the *Llrs1KO* (C) and *Llrs2KO* (D) mice after overnight (about 16 hr) fasting. Results are representative of three independent experiments.

(E and F) Insulin-stimulated phosphorylation of Akt, FoxO1, and GSK3 β at 70 s after administration of 10 U of insulin in the liver of the *Llrs1KO* (E) and *Llrs2KO* (F) mice after overnight (about 16 hr) fasting. Results are representative of three independent experiments.

(G and H) Serum levels of TC, TG, and FFA in 8-week-old *Llrs1KO* (G) and *Llrs2KO* (H) mice after overnight (about 16 hr) fasting (n = 11–17).

(I and J) Serum leptin and adiponectin levels in 8-week-old *Llrs1KO* (I) and *Llrs2KO* (J) mice after overnight (about 16 hr) fasting (n = 6–11). All the experiments illustrated in this figure were performed using 8- to 10-week-old male mice, unless otherwise specified. Results are represented as mean \pm SEM *p < 0.05.



reduced, and that of FoxO1 was significantly reduced in the livers of the *Lirs2KO* mice (Figures 1F and S2F). Insulin-stimulated phosphorylation of Akt, FoxO1, and GSK3 β was also significantly reduced in the livers of the *Lirs2KO* mice (Figures 1F and S2F). We next measured insulin-stimulated phosphorylation of Irs1, Irs2, Akt, and FoxO1 at 30 min after administration of 0.1 U of insulin in the livers of the *Lirs1KO* and *Lirs2KO* mice to investigate the events as they might occur under more physiological conditions. The insulin-stimulated phosphorylation levels of Irs1, Irs2, Akt, and FoxO1 remained essentially the same in the *Lirs1KO* (Figure S2G) and *Lirs2KO* (Figure S2H) mice, regardless of the dose of insulin. These data indicate that the potentially reduced insulin-stimulated PI3K activity following acute or sub-acute administration of insulin under the fasting condition is compensated for in the *LirsKO* mice by increased tyrosine phosphorylation of Irs2, whereas that in the *Lirs2KO* mice is not compensated for by increased tyrosine phosphorylation of Irs1. The serum levels of total cholesterol (TC), triglyceride (TG), and free fatty acid (FFA) were similar between the control and *LirsKO* mice (Figure 1G) or between the control and *Lirs2KO* mice (Figure 1H) under the fasting condition. In addition, the serum leptin and adiponectin levels were also not significantly different between the control and *LirsKO* mice (Figure 1I) or between the control and *Lirs2KO* mice (Figure 1J), under the fasting condition.

***Lirs2KO* (but Not *Lirs1KO*) Mice Showed Insulin Resistance under the Fasting Condition**

LirsKO mice showed similar body-weight gain to the control mice (data not shown), unlike the finding reported previously in systemic Irs1-deficient mice (Tamemoto et al., 1994; Araki et al., 1994). An insulin-tolerance test (ITT) conducted after fasting revealed that the glucose-lowering effect of insulin was similar between the control and *Lirs1KO* mice (Figure 2A). In the oral-glucose-tolerance test (OGTT) conducted after fasting, the blood glucose and serum insulin levels before and after glucose loading were not significantly different between the two mouse genotypes (Figure 2B). We next carried out a hyperinsulinemic-euglycemic clamp study in the animals under the fasting condition. None of the parameters (i.e., the glucose infusion rate [GIR]), hepatic glucose production (HGP), or the rate of glucose disappearance (R_d) differed significantly between the control and *Lirs1KO* mice (Figure 2C), indicating that *Lirs1KO* mice do not exhibit insulin resistance or glucose intolerance under the fasting condition.

Lirs2KO mice also showed similar body-weight gain to the control mice (data not shown), unlike the finding reported previously in the systemic Irs2-deficient mice (Tobe et al., 2001). An ITT conducted after fasting revealed insulin resistance in the *Lirs2KO* mice (Figure 2D). In the OGTT conducted after fasting, the blood glucose levels after glucose loading (Figure 2E, upper

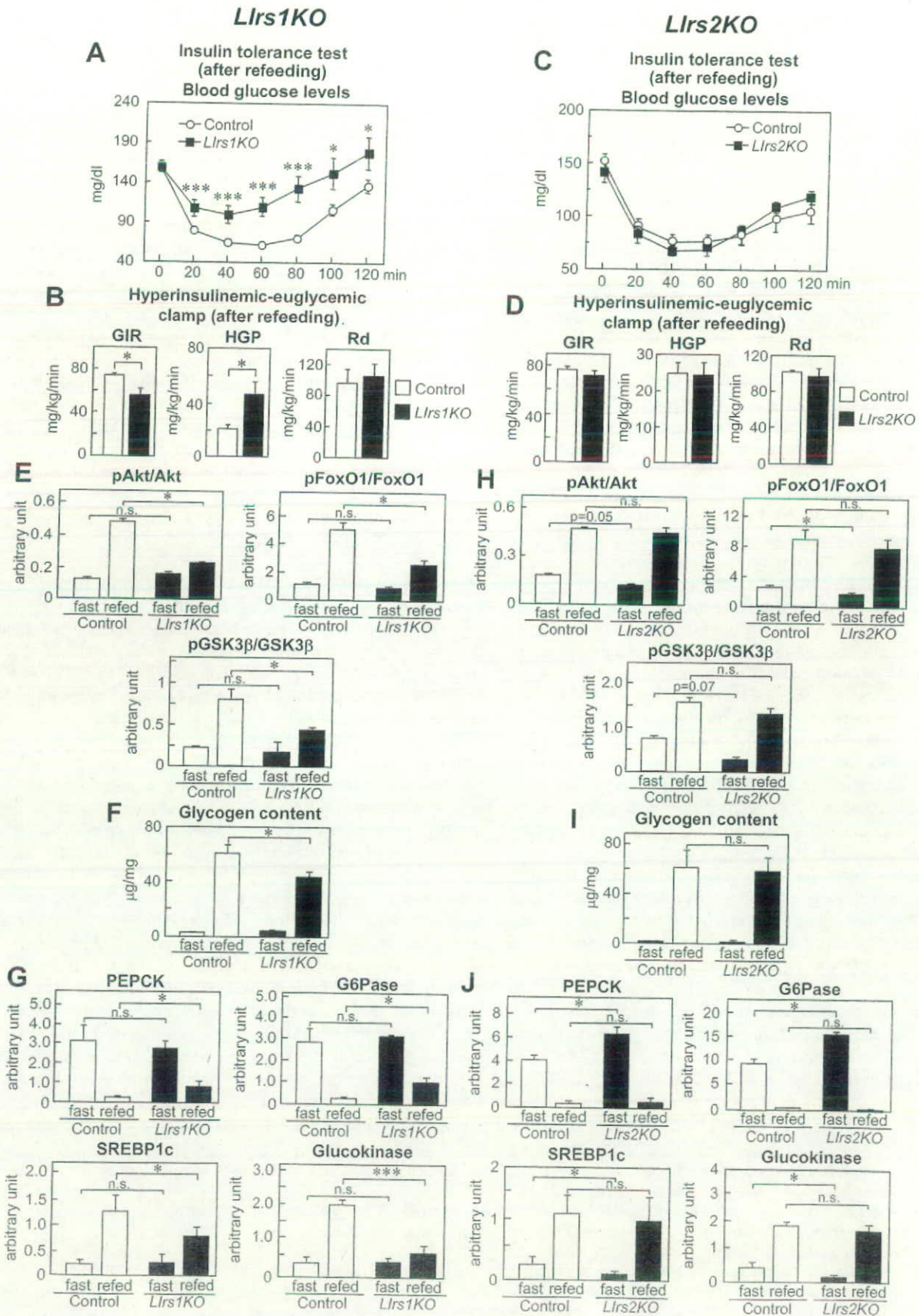
panel), as well as the serum insulin levels (Figure 2E, lower panel) before and after glucose loading, were significantly elevated in these mice. In the hyperinsulinemic-euglycemic clamp study conducted under the fasting condition, the GIR was significantly lower and the HGP significantly higher in the *Lirs2KO* mice than in the control mice, whereas no significant difference in the R_d was noted between the two mouse genotypes (Figure 2F). These findings indicate that the *Lirs2KO* mice show hepatic insulin resistance under the fasting condition. In the skeletal muscle (Figures S3A and S3B) and white adipose tissue (Figures S3C and S3D), basal- and insulin-stimulated phosphorylation of Akt was similar between the control and *Lirs1KO* mice or between the control and *Lirs2KO* mice, indicating that insulin signaling was not impaired in either the skeletal muscle or white adipose tissue of the *Lirs1KO* and *Lirs2KO* mice under the fasting condition. There were no significant histological changes in the livers of either the *Lirs1KO* or *Lirs2KO* mice (Figure S4A), unlike the observations in the *LirsKO* mice (Michael et al., 2000). The fasting TG content in the liver (Figure S4B), the serum aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (Figure S4C) levels also showed no significant differences in the *Lirs1KO* and *Lirs2KO* mice as compared with the findings in the control mice, suggesting that disruption of Irs1 or Irs2 does not cause any significant hepatic disorder.

***Lirs1KO* (but Not *Lirs2KO*) Mice Exhibited Insulin Resistance under the Refeeding Condition**

Since insulin regulates glucose homeostasis in the liver not only during fasting but also after refeeding, the insulin sensitivity and insulin signaling under the refeeding condition were also examined in the knockout mice. Unexpectedly, the ITT revealed insulin resistance in the *Lirs1KO* mice at 6 hr after the start of refeeding following 24 hr of fasting (Figure 3A), unlike under the fasting condition (Figure 2A). The GIR was significantly lower (and the HGP significantly higher) in the *Lirs1KO* mice as compared with the values in the control mice under the refeeding condition (Figure 3B), unlike under the fasting condition (Figure 2C). In contrast to these findings in the *Lirs1KO* mice, the ITT revealed insulin resistance under the fasting condition (Figure 2D) but not under the refeeding condition (Figure 3C) in the *Lirs2KO* mice. Unlike the observations under the fasting condition in these animals (Figure 2F), no significant differences in the GIR, HGP, or R_d were found between the control and *Lirs2KO* mice under the refeeding condition (Figure 3D). Significantly reduced phosphorylation of Akt, FoxO1, and GSK3 β was observed after refeeding, but not under the fasting condition, in the livers of the *Lirs1KO* mice (Figures 3E and S5A). The hepatic glycogen content was also decreased significantly in these mice after refeeding, but not under the fasting condition (Figure 3F). While the expression levels of PEPCK and G6Pase showed no significant differences

Figure 2. *Lirs2KO* (but Not *Lirs1KO*) Mice Showed Insulin Resistance and Glucose Intolerance under the Fasting Condition

(A) ITT in 8-week-old control and *Lirs1KO* mice after 3 hr fasting ($n = 25-27$).
 (B) Blood glucose and serum insulin levels in 8-week-old control and *Lirs1KO* mice during an OGTT conducted after 24 hr fasting ($n = 22$).
 (C) GIR, HGP, and R_d in control and *Lirs1KO* mice in the hyperinsulinemic-euglycemic clamp study conducted after 6 hr fasting ($n = 5$).
 (D) ITT in 8-week-old control and *Lirs2KO* mice after 3 hr fasting ($n = 17-19$).
 (E) Blood glucose and serum insulin levels in 8-week-old control and *Lirs2KO* mice during an OGTT conducted after 24 hr fasting ($n = 17-19$).
 (F) GIR, HGP, and R_d in control and *Lirs2KO* mice in the hyperinsulinemic-euglycemic clamp study conducted after 6 hr fasting ($n = 6-10$). All the experiments illustrated in this figure were performed using 8- to 10-week-old male mice, unless otherwise specified. Results are represented as mean \pm SEM * $p < 0.05$, ** $p < 0.01$.



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as compared with those in the control mice under the fasting condition, suppression of these expressions after refeeding was significantly impaired in the *Llrs1KO* mice as compared with that in the control mice (Figure 3G, upper panels). While the expression levels of SREBP1c and glucokinase were indistinguishable between the control and *Llrs1KO* mice under the fasting condition, these expressions were significantly lower in the *Llrs1KO* mice as compared with the levels in the control mice under the refeeding condition (Figure 3G, lower panels). While reduced phosphorylation of Akt, FoxO1, and GSK3 β was observed in the *Llrs2KO* mice under the fasting condition, no such reduction of phosphorylation was observed under the refeeding condition (Figures 3H and S5B). The hepatic glycogen content was also similar between the control and *Llrs2KO* mice under the refeeding condition (Figure 3I). Although the expression levels of PEPCK and G6Pase were significantly higher in the *Llrs2KO* mice than in the control mice under the fasting condition, suppression of these expressions under the refeeding condition was similar in degree to that noted in the control mice under the refeeding condition (Figure 3J, upper panels). The expression levels of SREBP1c and glucokinase were significantly lower in the *Llrs2KO* mice under the fasting condition; however, these expressions were restored to levels similar to those in the control mice under the refeeding condition (Figure 3J, lower panels). These findings suggest that in the liver, lack of Irs1 affects insulin signaling under the refeeding condition, and lack of Irs2 affects insulin signaling under the fasting condition. In the skeletal muscle (Figure S5C) and white adipose tissue (Figure S5D), the phosphorylation level of Akt was similar between the control and *Llrs1KO* mice or between the control and *Llrs2KO* mice under both the fasting and refeeding conditions. This indicates that insulin signaling was not impaired in the skeletal muscle and white adipose tissue of the *Llrs1KO* and *Llrs2KO* mice under either the fasting or the refeeding condition.

Distinct Roles of Irs1 and Irs2 in Mediating the Actions of Insulin in the Liver

What are the reasons for these differences between the *Llrs1KO* and *Llrs2KO* mice? We examined the expressions of Irs1, Irs2, and the PI3K activity associated with Irs1 and Irs2 under the fasting and refeeding conditions. The Irs1 mRNA and protein levels remained unchanged at 6 hr after the start of refeeding following the 24 hr fasting, which is associated with an ~5-fold increase of the Irs1-associated PI3K activity under the refeeding condition (Figure 4A). In contrast, the Irs2 mRNA and protein levels were significantly decreased after refeeding, associated with Irs2-associated PI3K activity that was not increased even under the

refeeding condition (Figure 4B). We next examined in greater detail the diurnal changes in the glucose and insulin levels, the expressions of Irs1 and Irs2, and their downstream molecules under the fasting and refeeding conditions in the control, *Llrs1KO*, and *Llrs2KO* mice. The blood glucose levels were almost indistinguishable between the control and *Llrs1KO* mice or between the control and *Llrs2KO* mice throughout the experiment (Figure 5A, upper panels). In contrast, the *Llrs1KO* mice exhibited higher serum insulin levels at 4 and 6 hr after the start of refeeding than the control mice (Figure 5A, lower-left panel), the *Llrs2KO* mice exhibited higher serum insulin levels after the 24 hr fasting and at 30 and 60 min after the start of refeeding than the control mice (Figure 5A, lower-right panel). The mRNA levels of *Irs1* were slightly increased during the second half of the 24 hr fasting and remained essentially unaltered after refeeding in both the control and *Llrs2KO* mice (Figure 5B, upper panel). The mRNA levels of *Irs2* were markedly increased during the second half of the 24 hr fasting, and then fell immediately after the start of refeeding in both the control and *Llrs1KO* mice (Figure 5B, lower panel). Although the *Irs2* mRNA expression after refeeding remained suppressed in the control mice, the *Irs2* expression in the *Llrs1KO* mice began to increase by 2 hr after the start of refeeding (Figure 5B, lower panel). The Irs1 protein levels remained essentially unaffected by food intake throughout the experiment, which is associated with a mobility shift from 30 min until 4 hr after refeeding in the control mice (Figure 5C). The Irs2 protein levels increased during the 24 hr fasting in the control mice (Figure 5C). The Irs2 protein underwent a prominent mobility shift immediately after the start of refeeding, presumably due to insulin-stimulated tyrosine phosphorylation (Figure 5C). The Irs2 protein levels rapidly decreased after refeeding (Figure 5C). Recruitment of the p85-adaptor subunit of PI3K to Irs1 immunoprecipitates (Figure S6A, left panels) and the PI3K activity associated with Irs1 (Figures 5D, upper-left panel, and S6B, upper panel) began to increase by 2 hr after the start of refeeding. In contrast, recruitment of the p85-adaptor subunit of PI3K to Irs2 immunoprecipitates (Figure S6A, right panels) and the PI3K activity associated with Irs2 (Figures 5D, lower-left panel, and S6B, lower panel) increased during the second half of the 24 hr fasting, despite the decrease in the serum insulin levels, and increased further up to 30 min after the start of refeeding, before decreasing rapidly thereafter. These data suggest that Irs2 mainly acts during fasting and immediately after the start of refeeding, and Irs1 takes over the lead role thereafter. In fact, during fasting or immediately after the start of refeeding, while the PI3K activity associated with Irs2 (Figures 5D, lower-right panel, and S6C, upper panel) and with

Figure 3. *Llrs1KO* (but Not *Llrs2KO*) Mice Exhibited Insulin Resistance under the Refeeding Condition

- (A) ITT in the control and *Llrs1KO* mice at 6 hr after the start of refeeding following 24 hr fasting (n = 17–23).
 (B) Hyperinsulinemic-euglycemic clamp study in the control and *Llrs1KO* mice at 6 hr after the start of refeeding following 24 hr fasting (n = 5–6).
 (C) ITT in the control and *Llrs2KO* mice at 6 hr after the start of refeeding following 24 hr fasting (n = 10–20).
 (D) Hyperinsulinemic-euglycemic clamp study in the control and *Llrs2KO* mice at 6 hr after the start of refeeding following 24 hr fasting (n = 6–10).
 (E–G) Phosphorylation of Akt, FoxO1, and GSK3 β in the liver (E), the hepatic glycogen content (F), and the expression levels of PEPCK, G6Pase, SREBP1c, and glucokinase in the liver (G) of the control and *Llrs1KO* mice after 24 hr fasting or at 6 hr after the start of refeeding following 24 hr fasting (F and G, n = 5–6). In (E), results are representative of three independent experiments.
 (H–J) Insulin-stimulated phosphorylation of Akt, FoxO1, and GSK3 β in the liver (H), the hepatic glycogen content (I), and the expression levels of PEPCK, G6Pase, SREBP1c, and glucokinase in the liver (J) of the control and *Llrs2KO* mice after 24 hr fasting or at 6 hr after the start of refeeding following 24 hr fasting (I and J, n = 6–10). In (H), results are representative of three independent experiments. All the experiments illustrated in this figure were performed using 8- to 10-week-old male mice. Results are represented as mean \pm SEM *p < 0.05, ***p < 0.001.

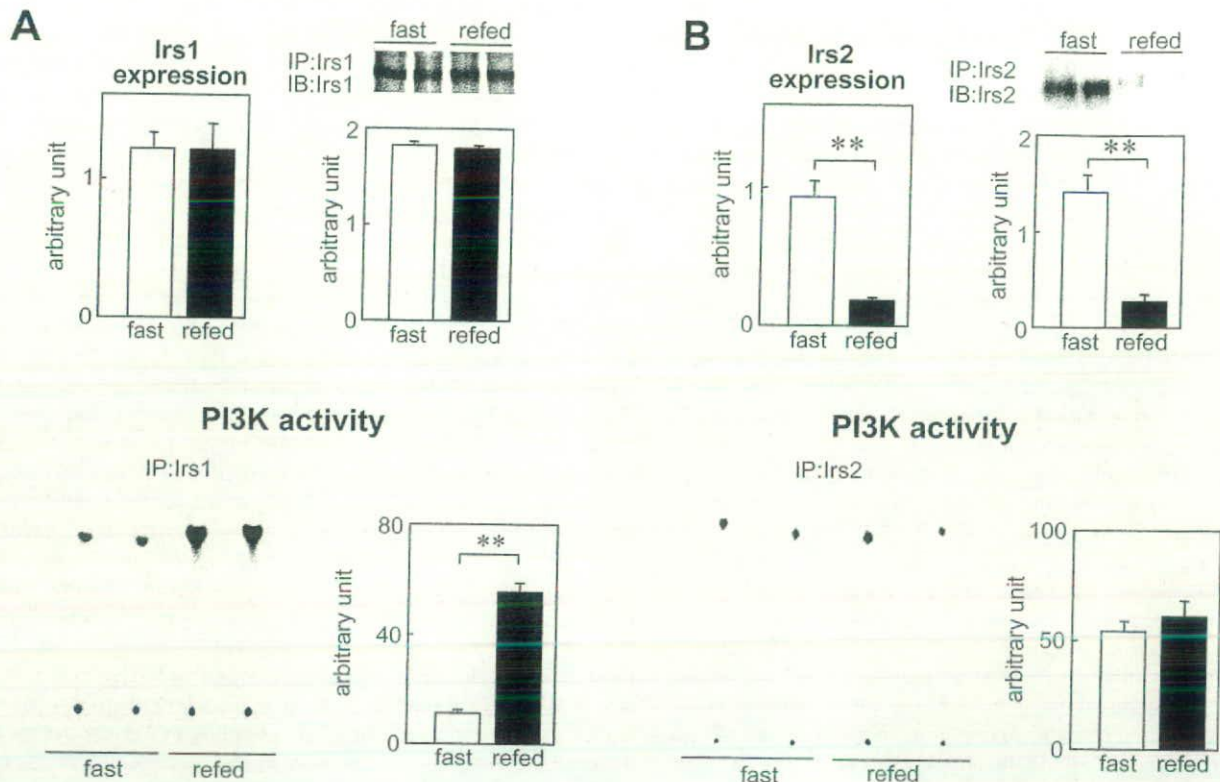


Figure 4. Expressions of Irs1 and Irs2, and the PI3K Activity Associated with Irs1 and Irs2 under the Fasting and Refeeding Conditions (A and B) Irs1 mRNA and protein levels and Irs1-associated PI3K activity (A), and Irs2 mRNA and protein levels and Irs2-associated PI3K activity (B) in the liver of the control mice after 24 hr fasting or at 6 hr after the start of refeeding following 24 hr fasting ($n = 6$). The results of western blotting and the PI3K activity are representative of three independent experiments.

All the experiments illustrated in this figure were performed using 8- to 10-week-old male mice. Results are represented as mean \pm SEM. ** $p < 0.01$.

tyrosine-phosphorylated proteins (Figure S6C, lower panels) increased in the *Lirs1KO* mice, the PI3K activity associated with Irs1 (Figures 5D, upper-right panel and S6D, upper panel) and with tyrosine-phosphorylated proteins (Figure S6D, lower panels) did not increase in the *Lirs2KO* mice. However, the PI3K activity associated with Irs1 (Figures 5D, upper-right panel, and S6D, upper panel) and with tyrosine-phosphorylated proteins (Figure S6D, lower panels) in the *Lirs2KO* mice began to increase by 2 hr after the start of refeeding, while the PI3K activity associated with Irs2 (Figures 5D, lower-right panel, and S6C, upper panel) and with tyrosine-phosphorylated proteins (Figure S6C, lower panels) in the *Lirs1KO* mice rather decreased after refeeding. Consistent with this, Akt phosphorylation was induced immediately after the start of refeeding in the *Lirs1KO* mice (Figure S6E), but not in the *Lirs2KO* mice (Figure S6F). Akt phosphorylation in the *Lirs2KO* mice began to increase by 2 hr after the start of refeeding (Figure S6F), but this Akt phosphorylation in the *Lirs1KO* mice rather decreased after refeeding (Figure S6E). Thus, Akt phosphorylation decreased during fasting and was induced immediately after the start of refeeding, and this induction was sustained for at least 6 hr after the start of refeeding in the control mice (Figures 5E and S6G). These data and the phenotypes observed in the *Lirs1KO* and *Lirs2KO* mice suggest that Irs2 mainly functions under the fasting condition and for a short period immediately after the start of refeeding; Irs1 primarily acts thereafter, under the refeeding condition,

suggesting the existence of a functional relay between Irs1 and Irs2 in insulin signaling during fasting and after refeeding.

***Lirs1/2DKO* Mice Developed Diabetes and Exhibited Insulin Resistance**

In order to determine whether abrogation of both Irs1 and Irs2 in the liver may cause insulin resistance under both the fasting and refeeding conditions, we generated *Lirs1/2DKO* mice. While the *Irs1* and *Irs2* alleles were not detected in the livers, both continued to be expressed in all of the other tissues than the livers of the *Lirs1/2DKO* mice (Figure S7A). The expressions of both *Irs1* and *Irs2* mRNA (Figure S7B) and protein (Figure 6A, upper panels) were almost completely abrogated in the livers of the *Lirs1/2DKO* mice. Insulin-stimulated tyrosine phosphorylation of Irs1 and Irs2 (Figure 6A, lower panels) and recruitment of the p85-adaptor subunit of PI3K to the Irs1 or Irs2 immunoprecipitates (Figure S7C) were also abrogated in the fasted *Lirs1/2DKO* mice. Insulin-stimulated PI3K activity associated with Irs1 (Figure 6B, left panel), Irs2 (Figure 6B, middle panel), and tyrosine-phosphorylated proteins (Figure 6B, right panel) was almost completely abrogated in the liver of the fasted *Lirs1/2DKO* mice. Insulin-stimulated phosphorylation of Akt, FoxO1, and GSK3 β was also almost completely abrogated in these fasted mice (Figures 6C and S7D), suggesting that insulin signaling is almost completely mediated by Irs1 and Irs2 in the liver under the fasting condition. The *Lirs1/2DKO* mice showed

similar body-weight gain to the control mice (data not shown). An ITT after fasting in 8-week-old *Lirs1/2DKO* mice revealed severe insulin resistance (Figure 6D), and an OGTT conducted after fasting in these mice revealed severe glucose intolerance with marked hyperinsulinemia (Figure 6E), indicating that *Lirs1/2DKO* mice developed diabetes. These phenotypes were similar to those of the *LIRKO* mice (Michael et al., 2000), suggesting that insulin-receptor signaling may be almost exclusively mediated by Irs1 and Irs2 in the liver. However, while the glucose tolerance gradually improved by the age of 24 weeks in the *LIRKO* mice (Michael et al., 2000), the severe insulin resistance (Figure 6F) and glucose intolerance (Figure 6G) persisted in the *Lirs1/2DKO* mice until 24 weeks of age. Consistent with the results of the ITT, the GIR and R_d were significantly lower (and the HGP significantly higher) in the *Lirs1/2DKO* mice under the fasting condition (Figure 6H). The serum levels of TC, TG, and FFA were not significantly different between the control and *Lirs1/2DKO* mice under the fasting condition (Figure 6I). The serum leptin and adiponectin levels, however, were significantly higher in the *Lirs1/2DKO* mice than in the control mice under the fasting condition (Figure 6J). The TG content in the liver was not significantly different between the control and *Lirs1/2DKO* mice under the nonfasted condition (Figure 6K).

Unlike the *Lirs1KO* or *Lirs2KO* mice, the degree of insulin resistance remained unchanged even after refeeding in the *Lirs1/2DKO* mice (Figure 7A). Consistent with the results of the ITT, the GIR and R_d were significantly lower (and the HGP significantly higher) in the *Lirs1/2DKO* mice under the refeeding condition (Figure 7B). Moreover, phosphorylation of Akt, FoxO1, and GSK3 β remained almost completely abrogated in the *Lirs1/2DKO* mice under the refeeding condition (Figure 7C). The hepatic glycogen content in the *Lirs1/2DKO* mice was significantly lower than that in the control mice under the refeeding condition—although it was increased as compared with that noted under the fasting condition (Figure 7D)—despite the absence of GSK3 β phosphorylation (Figure 7C). While the PEPCK and G6Pase expressions were increased in the *Lirs2KO* mice under the fasting condition (Figure 3J), unexpectedly, their expression levels were similar in the control and *Lirs1/2DKO* mice (Figure 7E). This may be explained by the suppression of hepatic gluconeogenesis via the hypothalamus induced by the marked hyperinsulinemia (Obici et al., 2002b). Suppression of PEPCK and G6Pase expressions under the refeeding condition was significantly impaired in the *Lirs1/2DKO* mice (Figure 7E). The expression level of SREBP1c was similar between the control and *Lirs1/2DKO* mice under the fasting condition, but was significantly decreased under the refeeding condition in the *Lirs1/2DKO* mice as compared with that in the control mice (Figure 7E). The expression of glucokinase was almost completely abrogated in the *Lirs1/2DKO* mice under both the fasting and refeeding conditions (Figure 7E). These findings indicate that the *Lirs1/2DKO* mice showed insulin resistance under both the fasting and refeeding conditions.

Hepatic Overexpression of Dominant-Negative FoxO1-Ameliorated Diabetes in the *Lirs1/2DKO* Mice

FoxO1 is a forkhead winged/helix transcription factor that mediates many effects of insulin downstream of the Irs-PI3K-Akt cascade (Nakae et al., 1999; Kops et al., 1999; Brunet et al.,

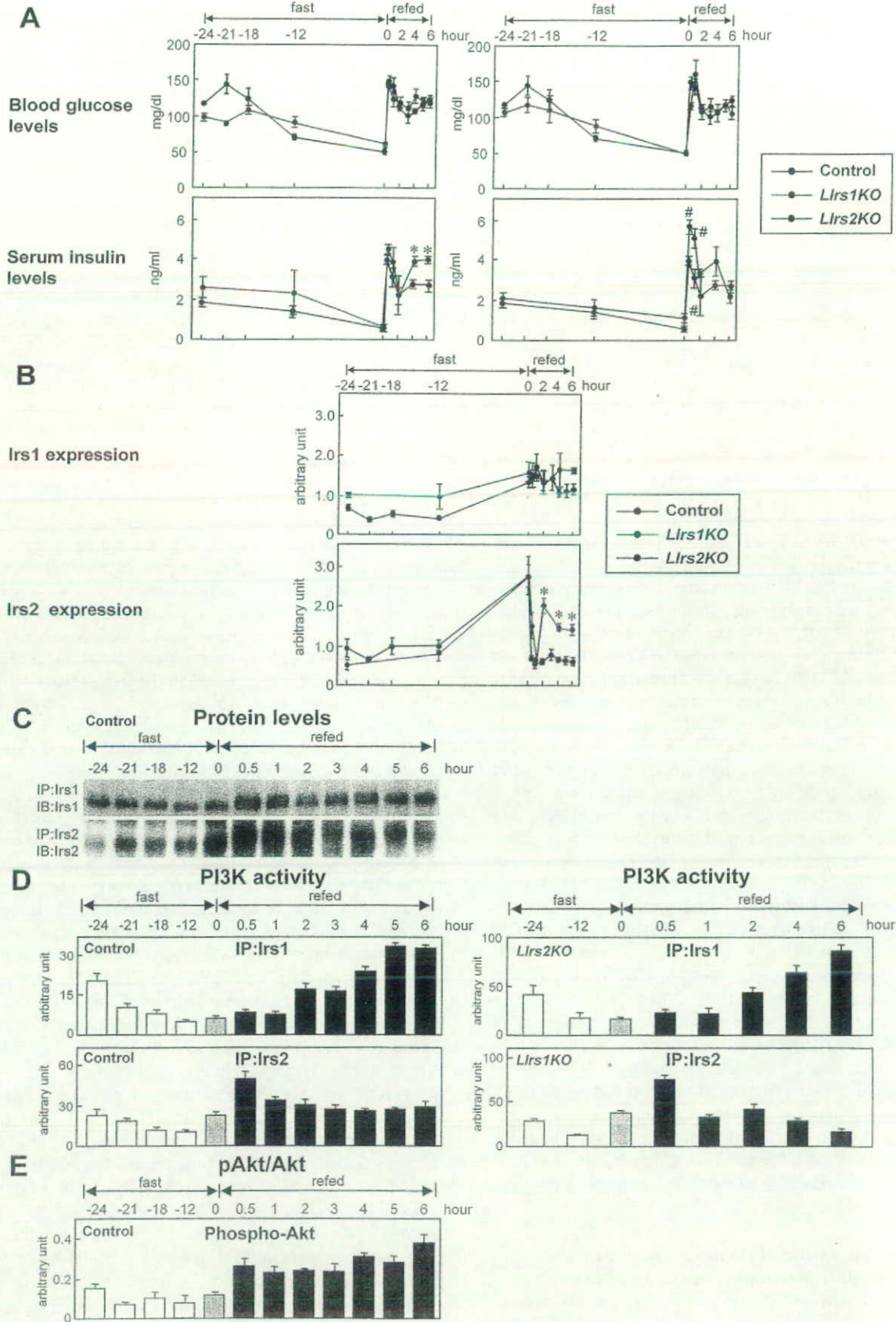
1999). In mammals, nuclear FoxO1, which accumulates in the presence of insulin resistance, dysregulates nutrient homeostasis by promoting hepatic gluconeogenesis, while the suppression of FoxO1 expression has been demonstrated to reverse the effects of insulin resistance and restore glucose tolerance in diabetic mice (Altomonte et al., 2003; Taniguchi et al., 2006). Thus, in order to investigate whether FoxO1 mediates insulin-induced regulation of glucose homeostasis downstream of Irs1 and Irs2 in the liver, expression of dominant-negative FoxO1 (DN-FoxO1) (Nakae et al., 2001) was induced in the livers of the *Lirs1/2DKO* mice. The blood glucose and serum insulin levels before and after glucose loading were partially, but significantly, decreased during an OGTT conducted after fasting in the *Lirs1/2DKO* mice treated with DN-FoxO1, as compared with the levels in the *Lirs1/2DKO* mice treated with lacZ (Figure 7F). These findings suggest that the Irs1/Irs2-FoxO1 pathway may play an important role in the regulation of glucose homeostasis in the liver under the fasting condition.

DISCUSSION

The liver maintains blood glucose levels within a normal range: while it ensures a sufficient supply of glucose in the fasting state, it takes up ingested carbohydrate to store it as glycogen and synthesizes lipids in the fed state, although this dual nature has not been fully accounted for in terms of insulin signaling. In this study, the *Lirs1KO* mice failed to exhibit insulin resistance during fasting, but showed insulin resistance after refeeding, associated with decreased expressions of glucokinase and SREBP1c. Conversely, the *Lirs2KO* mice exhibited insulin resistance during fasting but not after refeeding, associated with increased expression of PEPCK and G6Pase. These data suggest that both Irs1 and Irs2 are physiologically required for diurnal regulation of glucose metabolism by insulin in the liver.

Our close monitoring of the diurnal changes of glucose and insulin levels revealed that although the blood glucose levels were almost indistinguishable between the *Lirs1KO* and *Lirs2KO* mice (Figure 5A, upper panels), the *Lirs1KO* mice exhibited higher serum insulin levels at 4 and 6 hr after the start of refeeding. The *Lirs2KO* mice exhibited higher serum insulin levels after the 24 hr fasting and at 30 and 60 min after the start of refeeding than the control mice (Figure 5A, lower panels). These results indicate the presence of insulin resistance at these time points, which was confirmed by the results of the ITT and hyperinsulinemic-euglycemic clamp studies in the *Lirs1KO* mice under the refeeding condition (Figures 3A and 3B) and the *Lirs2KO* mice under the fasting condition (Figures 2D and 2F).

Insulin has been shown to suppress the expression of Irs2 both in vitro and in vivo, by inhibiting the synthesis of Irs2 mRNA at the transcriptional level (Zhang et al., 2001; Hirashima et al., 2003; Taniguchi et al., 2006). Indeed, the mRNA levels of Irs2 in the control mice increased during the second half of the 24 hr fasting—when the serum insulin levels were quite low—and immediately decreased within 30 min after the start of refeeding, which is associated with increase of the serum insulin levels induced by the food intake (Figures 5A and 5B). Our results strongly suggest that suppression of Irs2 immediately after the start of refeeding, presumably due to the increased serum insulin levels, may be mediated by Irs2 itself, because PI3K activity



associated with Irs2— but not Irs1—increased immediately after the start of refeeding not only in the control mice, but also in the *Lirs1KO* mice (Figures 5C, S6B, S6C, and S6D). Downregulation of *Irs2* expression by *Irs2* itself implies the existence of a feedback mechanism to prevent excessive insulin-signal transmission by *Irs2* in response to food intake. Whereas the sharp fall of the *Irs2* mRNA levels in the control mice persisted immediately after the start of refeeding, the levels in the *Lirs1KO* mice began to increase again at 2 hr after the start of refeeding (Figure 5B). This suggests that *Irs1*, as well as *Irs2*, is required for the suppression of *Irs2* mRNA after the start of refeeding.

Expression of a sufficient amount of hepatic *Irs2* during fasting appears to be pivotal for normal glucose homeostasis. If *Irs2* protein is abundantly expressed during fasting, elevated glucose levels promptly decrease after refeeding. However, in the presence of hyperinsulinemia with insulin resistance (when the fasting *Irs2* level is scarce), insulin signaling is impaired, and the elevated glucose levels fail to decrease immediately after the start of refeeding. In fact, *Lirs2KO* mice showed glucose intolerance (Figure 2E), while the *Lirs1KO* mice exhibited no such glucose intolerance (Figure 2B).

Based on our findings, we propose that glucose homeostasis is regulated by both hepatic *Irs1* and *Irs2*; *Irs1* and *Irs2* exert distinct actions under the fasting and refeeding conditions. The serum insulin levels decrease gradually during fasting and increase immediately after the start of refeeding. *Irs2* protein levels increase during the second half of a fasting period and decrease immediately after the start of refeeding, while the *Irs1* protein levels remain essentially unaffected by food intake. The PI3K activity associated with *Irs2* begins to increase during the second half of a fasting period despite the decrease in serum insulin levels and reaches its peak immediately after the start of refeeding, before rapidly decreasing thereafter. On the other hand, the PI3K activity associated with *Irs1* begins to increase by a few hours after the start of refeeding and reaches its peak thereafter, suggesting a dynamic switch of the principal effector in the hepatic insulin signaling during fasting and after refeeding. Thus, we propose the concept of the existence of a functional relay between *Irs1* and *Irs2* in hepatic insulin signaling during fasting and after refeeding; *Irs1* functions primarily after refeeding and *Irs2* functions mainly during fasting and immediately after the start of refeeding. This also indicates that *Irs2* may be the main contributor to the regulation of gluconeogenesis, i.e., PEPCK and G6Pase, while *Irs1* may predominantly regulate the expression of SREBP1c and glucokinase.

This concept could explain most (if not all) of the findings of previous studies on the roles of *Irs1* and *Irs2* in the regulation of glucose and lipid metabolism by insulin both in vitro and in vivo. With respect to *Irs1*-dependent signals, Kasuga and colleagues argued that the *Irs1*-PI3K pathway is essential for the

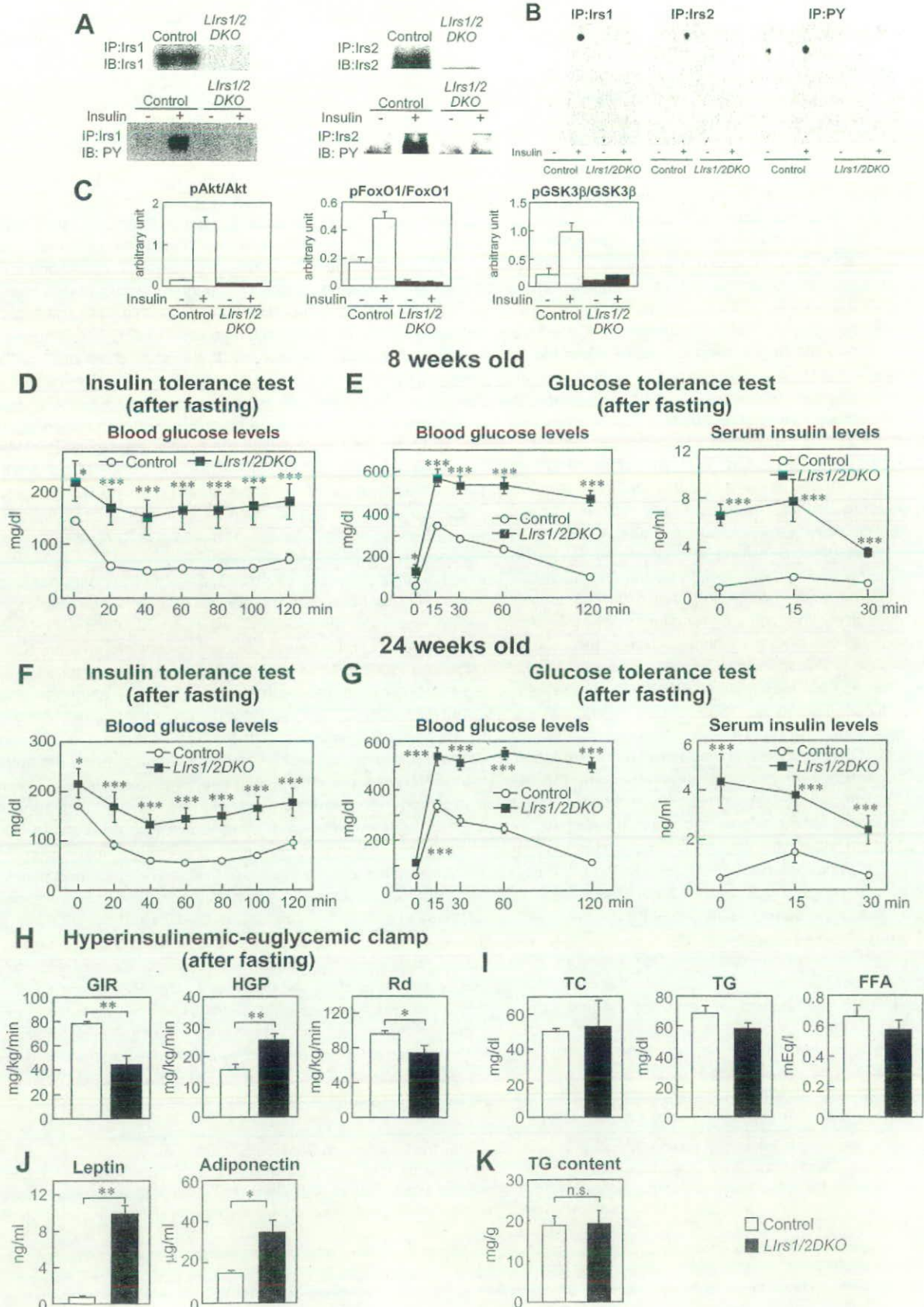
insulin-induced expression of the SREBP1c and glucokinase genes in cultured-rat hepatocytes (Matsumoto et al., 2002). The group led by Kahn has proposed that acute suppression of hepatic *Irs1* by adenovirus-mediated short hairpin RNA (shRNA) results in decreased expression of glucokinase induced by insulin (Taniguchi et al., 2005). With respect to *Irs2*-dependent pathways, Dong et al. reported that the fasted *Lirs2KO* mice in their study exhibited glucose intolerance and that the expression of PEPCK increased under the fasting condition (Dong et al., 2006).

On the other hand, it is likely that distinct compensatory mechanisms are induced by genetic ablation or relatively acute shRNA-mediated inactivation, giving rise to the phenotypic differences (Michael et al., 2000; Taniguchi et al., 2005). Indeed, chronic, complete shutdown of insulin signaling in the livers of the *LIRKO* mice results in decreased serum TG levels but no alteration of lipid accumulation in the liver (Michael et al., 2000), whereas double knockdown of *Irs1* and *Irs2* by shRNA treatment results in increased TG levels in the serum and liver (Taniguchi et al., 2005), shown by the same group. Thus, the phenotype in regard to the lipid synthesis in the liver of the *Lirs1/2DKO* mice is indeed more consistent with that of the *LIRKO* mice, and it is possible that incomplete inactivation of *Irs* proteins may enhance some of the insulin actions and/or promote the shift of substrates for energy storage from glucose to lipids, leading to upregulation of SREBP1c.

Although the serum levels of TC, TG, and FFA were not significantly different between the control and *Lirs1/2DKO* mice, the serum leptin and adiponectin levels were significantly higher in the *Lirs1/2DKO* mice under the fasting condition (Figure 6J), as observed in the *LIRKO* (Cohen et al., 2007) and *Irs1/Irs2* double-knockdown mice treated with shRNA (Taniguchi et al., 2005). Recently, it has been reported that the expression levels of the short (Ob-Ra), long (Ob-Rb), and soluble (Ob-Re) forms of the leptin receptor were significantly increased in the liver of the *LIRKO* mice and that insulin suppressed the leptin-receptor expression in isolated hepatocytes from normal mice, suggesting that high levels of circulating leptin receptor bind to leptin and alter its clearance (Cohen et al., 2007). Thus, insulin signaling in the liver plays an important role in leptin homeostasis. Consistent with this, it has been reported that leptin-receptor expression was significantly increased in the livers of the liver-specific *Irs2*:total *Irs1*-knockout mice (Dong et al., 2006) and our *Lirs1/2DKO* mice (data not shown). In contrast, the expressions of adiponectin receptors (AdipoR1 and AdipoR2 [Yamauchi et al., 2003]) were not increased in the livers of the *Lirs1/2DKO* mice (data not shown), suggesting the absence of a significant change in the adiponectin clearance. The molecular mechanism underlying the increase of the serum adiponectin levels in these mice remains unknown.

Figure 5. Distinct Roles of *Irs1* and *Irs2* in Insulin Signaling in the Liver under the Fasting and Refeeding Conditions

(A and B) Blood glucose and serum insulin levels (A) and the mRNA levels of *Irs1* and *Irs2* (B) in the control (black), *Lirs1KO* (red), and *Lirs2KO* (blue) mice under the fasting and refeeding conditions ($n = 4$). Results are represented as mean \pm SEM * $p < 0.05$, *Lirs1KO* versus control mice. # $p < 0.05$, *Lirs2KO* versus control mice. (C) Protein levels of *Irs1* and *Irs2* in the liver of the control mice under the fasting and refeeding conditions. Results are representative of three independent experiments. (D) PI3K activity associated with *Irs1* or *Irs2* in the control, *Lirs1KO*, and *Lirs2KO* mice under the fasting and refeeding conditions. Results are representative of three independent experiments. (E) Phosphorylation of Akt in the liver of the control mice under the fasting and refeeding conditions. Results are representative of three independent experiments. All the experiments illustrated in this figure were performed using 8- to 10-week-old male mice.



Why do hepatic Irs1 and Irs2 have distinct roles? One of the reasons may be the dual aspects of the role played by the liver. While other target tissues of insulin simply take in glucose, the liver produces and consumes glucose to adapt to various physiological conditions. The opposing functions of utilizing glucose, that is, glucose production during fasting and glucose uptake after food intake, may be the reason for the distinctive roles of Irs1 and Irs2. In the long history of evolution, mammals had to survive for certain periods without food. The necessity of development of a mechanism to handle sporadic starvation may have resulted in the refined roles of Irs1 and Irs2 under the fasting and refeeding conditions.

In conclusion, Irs1 and Irs2 exert distinct actions under the fasting and refeeding conditions and are almost exclusively responsible for the mediation of insulin signaling in the liver. Understanding the molecular basis of hepatic insulin signaling in the regulation of glucose metabolism may be expected to provide a basis for a better understanding of the pathogenesis and treatment of type 2 diabetes mellitus.

EXPERIMENTAL PROCEDURES

Construction of the Targeting Vector, ES Cell Culture, and Generation of the Mutant Mice

To construct the targeting vector for the *Irs1* gene, a mouse genomic DNA library packaged in Lambda DASH II (Clontech, Mountain View, CA) was screened using mouse *Irs1* cDNA as the probe. Five clones containing the *Irs1* gene were isolated. We constructed a targeting vector in which a third loxP site was introduced into the 5' side of the *Irs1* gene and the floxed neomycin-resistance gene (*neoR*) was introduced into the 3' side of the *Irs1* gene (Figure S8A). The diphtheria toxin A fragment (DTA) gene under an MC1 promoter was ligated onto the 5' end of the homologous region for negative selection against random integration (Kubota et al., 2004). The targeting vector was electroporated into E14.1 embryonic stem cells (129/Sv), and the cells were screened for homologous recombinant clones by Southern blot analysis using probe A and probe B, as described previously (Kubota et al., 2004) (Figures S8A and S8B). The cells were aggregated with eight-cell embryos from ICR mice and transferred into pseudopregnant ICR females to generate chimeric mice. Male chimeric mice with an agouti coat color were mated with C57Bl/6 female mice to generate heterozygous (*Irs1^{lox/+}*) mice (Kubota et al., 2004).

Animals

The mice were housed under a 12 hr light-dark cycle and given regular chow, CE-2 (CLEA Japan, Inc., Tokyo, Japan), consisting of 25.6% (w/w) protein, 3.8% fiber, 6.9% ash, 50.5% carbohydrates, 4% fat, and 9.2% water. Albu-

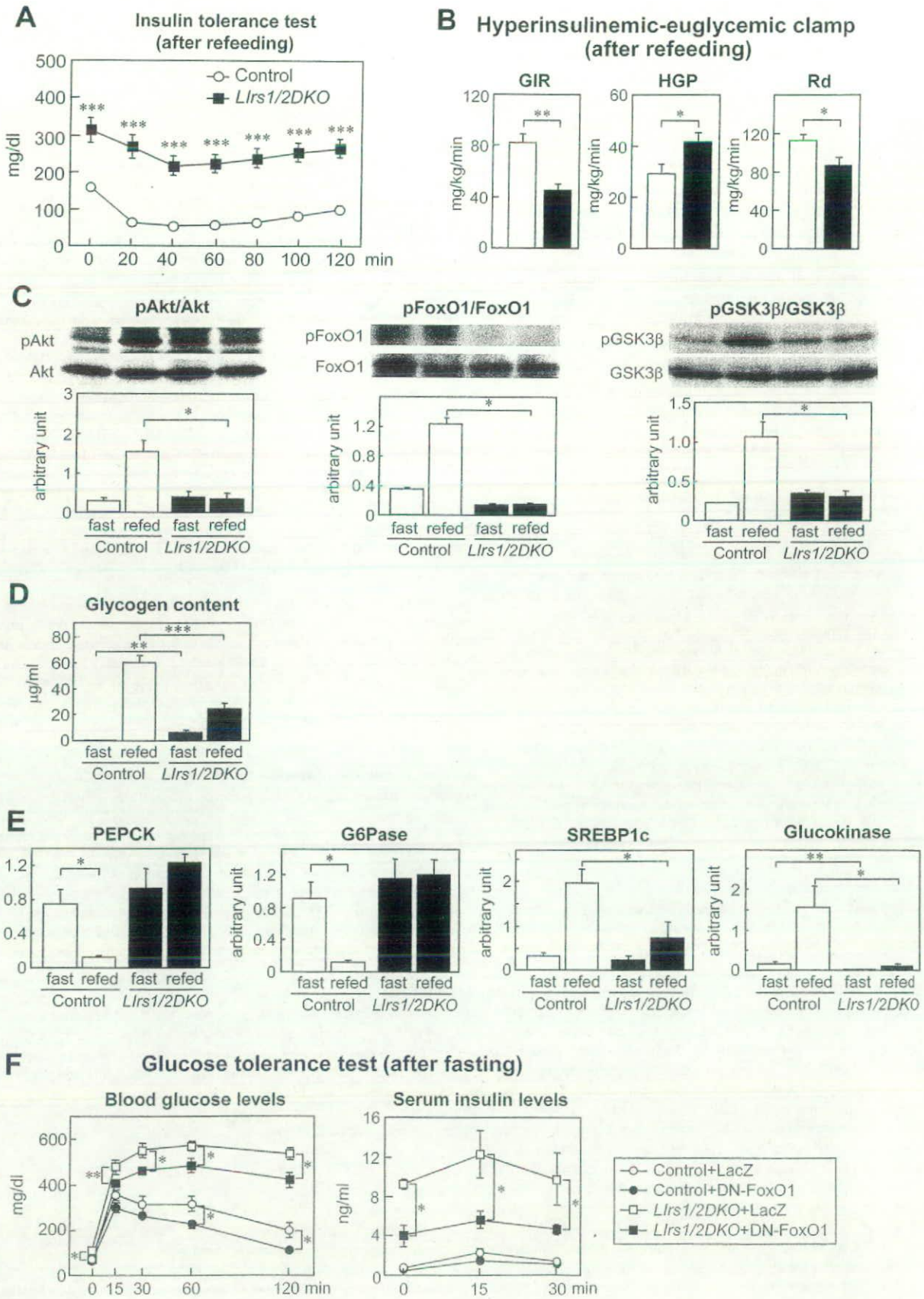
min-Cre-recombinase transgenic mice (*AlbCre* mice) were purchased from Jackson Laboratory. The *Irs1^{lox/+}* mice or mice with a floxed allele of *Irs2* (*Irs2^{lox/+}*) (Kubota et al., 2004) were intercrossed with the *AlbCre* mice to generate *AlbCreIrs1^{lox/+}* or *AlbCreIrs2^{lox/+}* mice. *AlbCreIrs1^{lox/+}* or *AlbCreIrs2^{lox/+}* mice were crossed with *Irs1^{lox/+}* or *Irs2^{lox/+}* mice to obtain wild-type, *Irs1^{lox/+}*, *AlbCre*, and *AlbCreIrs1^{lox/lox}* (*Llrs1KO*) mice; or wild-type, *Irs2^{lox/+}*, *AlbCre*, and *AlbCreIrs2^{lox/lox}* (*Llrs2KO*) mice, respectively. To generate liver-specific *Irs1/Irs2* double-knockout (*Llrs1/2DKO*) mice, *AlbCreIrs1^{lox/+}* or *AlbCreIrs2^{lox/+}* mice were crossed with *Irs2^{lox/+}* or *Irs1^{lox/+}* mice, then the resultant *AlbCreIrs1^{lox/+}/Irs2^{lox/+}* were crossed with *Irs1^{lox/+}/Irs2^{lox/+}* mice. The wild-type, *AlbCre*, *Irs1^{lox/lox}*, *Irs2^{lox/lox}*, and *Irs1^{lox/lox}/Irs2^{lox/lox}* mice were phenotypically indistinguishable; the *Irs1^{lox/lox}*, *Irs2^{lox/lox}*, and *Irs1^{lox/lox}/Irs2^{lox/lox}* mice were used as controls. All the mouse lines were maintained on a mixed background derived from C57Bl/6 and 129/Sv. All experiments in this study were performed using male littermates. For the refeeding condition, the mice were first deprived of food for 24 hr, followed by refeeding for 6 hr before the experiments. Genotyping was performed by PCR amplification of the tail DNA from each mouse at 3 weeks of age. The PCR primers for the Cre recombinase were 5'-ACATGTTTCAGGGATCGCCAGG-3' and 5'-TAACCAGTGAACAGCATTG C-3'. The primers for the *Irs1* and floxed *Irs1* alleles were 5'-TCTGTGAGC CTGTTTTCTGGTGTC-3', 5'-TCTATTGATGAAAGCCCAAGGCAC-3', and 5'-CAGCGCATCGCCTTCTATCGCCTTC-3'. The primers for the *Irs2* and floxed *Irs2* alleles were 5'-CCAGTGGGTGGCAGTGTGGGTAGG-3', 5'-CAGC GCATCGCCTTCTATCGCCTTC-3', and 5'-GCCATGTCCTTACAACCATTAGC GG-3', respectively. To detect Cre-mediated recombination at the genomic DNA level in various tissues by PCR, primers were designed for the upstream portion (primer a) and downstream portion (primer b) of *Irs1* genomic DNA and the upstream portion (primer c) and downstream portion (primer d) of *Irs2* genomic DNA (Figure S1A). Primer a was 5'-TTTCTACATAATGCGAGG TCCCC-3', primer b was 5'-AAATGTTGGAAGCAGATCAGGACC-3', primer c was 5'-CCAGTGGGTGGCAGTGTGGGTAAGA-3', and primer d was 5'-CC CATGCTGCTTGTATGGAGAGCC-3'. Primer pairs a and b yielded PCR products with a length of 0.2 kb after Cre-mediated *Irs1* deletion, and primer pairs c and d yielded PCR products with a length of 0.4 kb after Cre-mediated *Irs2* deletion. The primers used for G3PDH were 5'-TGAAGTCCGGTGTGAACG GATTTGGC-3' and 5'-CATGTAGGCCATGAGGTCCACCAC-3'. The methods used for animal care and the experimental procedures were approved by the Animal Care Committee of the University of Tokyo.

Immunoprecipitation, Western Blot Analysis, and PI3K Assay

For insulin stimulation, 10 or 0.1 U of human insulin (Novolin R, Novo Nordisk, Denmark) were injected via the inferior vena cava; the liver, skeletal muscle or white adipose tissue were dissected and immediately frozen in liquid nitrogen 70 s or 30 min after the insulin stimulation, respectively. To prepare tissue lysates, frozen tissue was homogenized in buffer A (25 mM Tris-HCl [pH 7.4], 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). For immunoprecipitation of Irs1, Irs2, and phosphotyrosine, 5 mg of the liver extracts were incubated with specific antibodies against

Figure 6. *Llrs1/2DKO* Mice Showed Severe Insulin Resistance and Developed Diabetes

- (A) In upper panels, liver lysates were immunoprecipitated with anti-Irs1 or anti-Irs2 antibody and subsequently immunoblotted with anti-Irs1 or anti-Irs2 antibody in 8- to 10-week-old control and *Llrs1/2DKO* mice. Lower panels show insulin-stimulated tyrosine phosphorylation of Irs1 or Irs2 at 70 s after administration of 10 U of insulin in the liver of the control and *Llrs1/2DKO* mice after overnight (about 16 hr) fasting. Results are representative of three independent experiments.
- (B) Insulin-stimulated PI3K activity associated with Irs1, Irs2, and tyrosine-phosphorylated proteins at 70 s after administration of 10 U of insulin in the liver of 8- to 10-week-old control and *Llrs1/2DKO* mice after overnight (about 16 hr) fasting. Results are representative of three independent experiments.
- (C) Insulin-stimulated phosphorylation of Akt, FoxO1, and GSK3 β at 70 s after administration of 10 U of insulin in the liver of 8- to 10-week-old control and *Llrs1/2DKO* mice after overnight (about 16 hr) fasting. Results are representative of three independent experiments.
- (D) ITT in 8-week-old control and *Llrs1/2DKO* mice after 3 hr fasting (n = 16–19).
- (E) Blood glucose and serum insulin levels in 8-week-old control and *Llrs1/2DKO* mice during an OGTT conducted after 24 hr fasting (n = 17–18).
- (F) ITT in 24-week-old control and *Llrs1/2DKO* mice after 3 hr fasting (n = 9–17).
- (G) Blood glucose and serum insulin levels in 24-week-old control and *Llrs1/2DKO* mice during an OGTT conducted after 24 hr fasting (n = 10–13).
- (H) GIR, HGP, and R_d in 8-week-old control and *Llrs1/2DKO* mice in the hyperinsulinemic-euglycemic clamp study conducted after 6 hr fasting (n = 6–7).
- (I) Serum levels of TC, TG, and FFA in 8-week-old control and *Llrs1/2DKO* mice after overnight (about 16 hours) fasting (n = 10–14).
- (J) Serum leptin and adiponectin levels in 8-week-old control and *Llrs1/2DKO* mice after overnight (about 16 hr) fasting (n = 6–14).
- (K) The TG content of the liver in 8-week-old nonfasted control and *Llrs1/2DKO* mice (n = 6). Results are represented as mean \pm SEM *p < 0.05. **p < 0.01, ***p < 0.001.



Irs1, Irs2, or phosphotyrosine, respectively, for 1 hr at 4°C. Then, protein G-Sepharose was added, followed by incubation for 2 hr at 4°C. After washing three times with buffer A, the immunocomplexes were resolved on 7% SDS-PAGE. Phosphorylated or total protein was analyzed by immunoblotting with specific antibodies against Irs1, Irs2, phosphotyrosine, and anti-p85^{PAN} antibody. Phosphorylated or total protein of Akt, FoxO1, and Gsk3 β were analyzed by immunoblotting with specific antibodies after the tissue lysates were resolved on SDS-PAGE and transferred to a Hybond-P PVDF transfer membrane (Amersham Biosciences, Buckinghamshire, UK). Bound antibodies were detected with HRP-conjugated secondary antibodies, using ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK). For the PI3K assay, 5 mg of the liver extracts were immunoprecipitated with antibody against Irs1, Irs2, or phosphotyrosine, and the immune complexes were assayed for PI3K activity using phosphatidylinositol as the substrate. The procedure was performed as described previously (Kubota et al., 2000). The activities of PI3K were quantitated with an image analyzer (BAS 2000, Fuji Film, Tokyo, Japan) and expressed as the photostimulated luminescence intensity (PSL).

In Vivo Glucose Homeostasis

For the glucose tolerance test, the mice were loaded with oral glucose at 1.5 mg/g body weight. Blood samples were taken at different time points, and the concentrations of glucose were measured with an automatic glucometer (Glutest Ace, Sanwa Chemical Co., Nagoya, Japan). Whole blood was collected and centrifuged in heparinized tubes, and the separated serum samples were stored at -20°C. Insulin levels were determined using an insulin radioimmunoassay (RIA) kit (BIOTRAK, Amersham, UK), with rat insulin as the standard (Kubota et al., 1999). For the insulin tolerance test, mice were intraperitoneally challenged with 0.75 mU/g (body weight) of human insulin (Novolin R, Novo Nordisk, Denmark). Venous blood samples were then drawn at different time points (Kubota et al., 1999).

Hyperinsulinemic-Euglycemic Clamp Study

Clamp studies were carried out as described previously (Kubota et al., 2006), with slight modifications. In brief, 2–3 days before the study, an infusion catheter was inserted into the right jugular vein of the animals under general anesthesia induced with sodium pentobarbital. The studies on the mice were performed under conscious and unstressed conditions. A continuous infusion of insulin (Novolin R, Novo Nordisk, Denmark) was given (5.0 mU/kg/min for the *Lirs1KO* and *Lirs2KO* mice and 7.5 mU/kg/min for the *Lirs1/2DKO* mice), and the blood glucose concentration (monitored every 5 min) was maintained at ~120 mg/dl by administration of glucose (5 g of glucose per 10 ml enriched to approximately 20% with [6,6-²H₂]glucose [Sigma]) for 120 min. Blood was sampled via tail-tip bleeds at 90, 105, and 120 min, for determination of the rate of glucose disappearance (R_d). R_d was calculated according to non-steady-state equations, and hepatic glucose production (HGP) was calculated as the difference between the R_d and the exogenous glucose infusion rates (GIR) (Kubota et al., 2006).

Histology and the Glycogen and TG Contents of the Liver

To study the liver histology, the livers were dissected and fixed in buffered neutral formalin (10%). The fixed-tissue blocks were embedded in paraffin. The paraffin sections (5 μ m) were stained by the standard H&E staining procedure. The method for measurement of the glycogen content in the liver has been described previously (Lo et al., 1970). In brief, approximately 50 mg of the liver samples were weighed and boiled in 30% KOH saturated with Na₂SO₄ and 95% ethanol was added to precipitate the glycogen from the

alkaline digestate. After cooling on ice, the samples were centrifuged and the glycogen precipitates were dissolved in distilled H₂O. The glycogen concentrations were then read on a spectrophotometer after the addition of 5% phenol and 96%–98% H₂SO₄. For determining the TG content in the liver, the tissue homogenates were extracted with 2:1 (vol/vol) chloroform/methanol. Chloroform/methanol was added to the homogenate, and the mixture was shaken for 15 min. After centrifugation at 14,000 rpm for 10 min, the organic layer was collected. This extraction was repeated three times, and the collected sample was dried and resuspended in 1% Triton X-100/ethanol. The measurement was conducted using Triglyceride E-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

SUPPLEMENTAL DATA

Supplemental Data include eight figures and Supplemental Experimental Procedures and can be found at <http://www.cellmetabolism.org/cgi/content/full/8/1/49/DC1/>.

ACKNOWLEDGMENTS

We thank Katsuyoshi Kumagai, Katsuko Takasawa, Eri Yoshida-Nagata, Namiko Kasuga, Miharu Nakashima, Ayumi Nagano, Sayaka Sasamoto, Yuko Miki, Ritsuko Fujita, Norie Ohtsuka-Kowatari, Eishin Hirata, and Hiroshi Chiyonobu for their excellent technical assistance and assistance with the animal care. This work was supported by a grant for CREST from Japan Science and Technology Corporation; a grant for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research; a grant for TSBMI from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Grant-in-aid for Scientific Research in Priority Areas (A) (16209030) and (A) (18209033) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. K.); and a Grant-in-aid for Scientific Research in Priority Areas (C) (19591037) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to N. K.). We certify that none of the authors of this manuscript have any financial interests to declare in relation to this work.

Received: November 7, 2007

Revised: March 28, 2008

Accepted: May 23, 2008

Published: July 1, 2008

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Figure 7. *Lirs1/2DKO* Mice Exhibited Insulin Resistance Under the Refeeding Condition

(A) ITT in the control and *Lirs1/2DKO* mice at 6 hr after the start of refeeding following 24 hr fasting (n = 14–15).

(B) GIR, HGP, and R_d in control and *Lirs1/2DKO* mice in the hyperinsulinemic-euglycemic clamp study at 6 hr after the start of refeeding following 24 hr fasting (n = 5–6).

(C–E) Phosphorylation of Akt, FoxO1, and GSK3 β in the liver (C), the hepatic glycogen content (D), and the expression levels of PEPCK, G6Pase, SREBP1c, and glucokinase in the liver (E) of 8-week-old control and *Lirs1/2DKO* mice after 24 hr fasting or at 6 hr after the start of refeeding following 24 hr fasting (D and E, n = 6). In (C), results are representative of three independent experiments.

(F) Blood glucose and serum insulin levels in the control and *Lirs1/2DKO* mice treated with lacZ or DN-FoxO1 mice during an OGTT conducted after 24 hr fasting (n = 7–10). All the experiments illustrated in this figure were performed using 8- to 10-week-old male mice, unless otherwise specified. Results are represented as mean \pm SEM *p < 0.05, **p < 0.01, ***p < 0.001.

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NOTE

Impact of Increased PPAR γ Activity in Adipocytes *in vivo* on Adiposity, Insulin Sensitivity and the Effects of Rosiglitazone Treatment

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Abstract. Peroxisome proliferator-activated receptor (PPAR) γ , a transcription factor belonging to the nuclear receptor superfamily, is essential for adipogenesis. PPAR γ is recognized as a major target for the insulin-sensitizing effects of the thiazolidinediones. Previous studies have demonstrated that heterozygous PPAR γ -deficient mice are protected from high-fat diet (HFD)-induced adipocyte hypertrophy, obesity and insulin resistance, which suggests that PPAR γ may have a pivotal role in adipocyte hypertrophy, obesity and insulin resistance. In this study, we generated transgenic mice with the gain-of-function PPAR γ Ser112Ala mutation (S112A mice) using the α P2 promoter, to elucidate the impact of increased PPAR γ activity in mature adipocytes. Despite a 2–3-fold increase in the adipocyte PPAR γ 2 gene expression and PPAR γ activity, the S112A mice showed comparable adiposity and insulin sensitivity to wild-type mice under both normal and HFD conditions. Although the expression levels of the PPAR γ target genes involved in lipid metabolism, such as α P2 and stearoyl-CoA desaturase 1, were upregulated in the white adipose tissue of the S112A mice, the serum levels of free fatty acid, triglyceride, adiponectin and leptin, as well as the oxygen consumption, were comparable between the wild-type and S112A mice under the HFD condition. Moreover, treatment with rosiglitazone ameliorated insulin resistance and glucose intolerance to a similar degree in the two genotypes under the HFD condition. In conclusion, whereas the 50% decrease in PPAR γ activity showed protection from HFD-induced obesity and insulin resistance, in the present study, the 2–3-fold increase in PPAR γ 2 expression and PPAR γ activity failed to show obesity and insulin resistance even under the HFD condition.

Key words: Peroxisome proliferator-activated receptor γ , Rosiglitazone

(Endocrine Journal 55: 767–776, 2008)

PEROXISOME proliferator-activated receptor (PPAR) γ , a member of the nuclear hormone receptor family of transcription factors, plays a central role in adipogenesis and lipid storage [1]. PPAR γ exists as two isoforms, PPAR γ 1 and PPAR γ 2 [2]. While PPAR γ 1 is known to be expressed in several tissues, PPAR γ 2 expression is mainly restricted to the adipose tissue [1].

Previous studies have demonstrated that heterozygous PPAR γ -deficient mice are protected from high-

fat diet (HFD)-induced adipocyte hypertrophy, obesity and insulin resistance [3, 4], which suggests that PPAR γ may have a pivotal role in adipocyte hypertrophy and insulin resistance. Consistent with this, a polymorphism of the human PPAR γ 2 gene, Pro12Ala, which decreases PPAR γ activity, was shown to be associated with enhanced insulin sensitivity and a lower body mass index [5, 6]. In addition, partial PPAR γ antagonists decreased the triglyceride (TG) content in white adipose tissue (WAT), skeletal muscle and liver, and ameliorated HFD-induced obesity and insulin resistance [7]. Thus, moderately reduced PPAR γ activity was shown to be associated with improvement of insulin sensitivity.

The thiazolidinediones (TZDs), synthetic PPAR γ

Received: January 17, 2008

Accepted: January 21, 2008

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ligands, are widely used in the treatment of type 2 diabetes [1]. TZD-induced PPAR γ activation increases the plasma levels of the insulin-sensitizing adipokine, adiponectin [8], and simultaneously decreases the plasma levels of free fatty acid (FFA) and insulin-resistance-causing adipokines such as TNF α and resistin [8]. Thus, PPAR γ activation by TZD ameliorates insulin resistance, whereas heterozygous PPAR γ -deficient mice are protected from HFD-induced adipocyte hypertrophy, obesity and insulin resistance [3, 4].

PPAR γ activity is regulated by mitogen-activated protein (MAP) kinase phosphorylation of the serine 112 residue, which reduces its transcriptional activity [9]. The PPAR γ Ser112Ala (S112A) mutation has been shown to be non-phosphorylatable and indeed more active than wild-type PPAR γ [9]. S112A knock-in mice showed improved insulin sensitivity without body weight gain [10]. A gain-of-function PPAR γ mutation in humans had no impact on the insulin resistance, but induced obesity [11]. These phenotypes can probably be explained by the induction of PPAR γ expression in the early stage of differentiation of preadipocytes [12]. In contrast, overexpression of wild-type PPAR γ in mature 3T3L1 adipocytes increased both the cell size and intracellular TG content [13]. Although expression of PPAR γ 2 is maintained at a high level even in mature adipocytes [14], the physiological role of increased PPAR γ activity in mature adipocytes *in vivo* remains unknown.

To understand the role of increased PPAR γ activity in mature adipocytes *in vivo*, we produced transgenic mice with the PPAR γ 2 S112A mutation to activate PPAR γ under the α P2 promoter. Moreover, we examined the effect of increased PPAR γ activity in mature adipocytes on TZD-induced amelioration of insulin sensitivity.

Materials and Methods

Generation of transgenic PPAR γ 2-S112A mutant mice

The mouse PPAR γ 2 cDNA (1.8 kb) with the S112A mutation, generated by PCR mutagenesis, was subcloned into the *KpnI-SacI* site of pBluescript SK(-) (Stratagene, La Jolla, CA, USA). PPAR γ 2-S112A cDNA was ligated into the unique *EcoRI* site between the rabbit β -globin intron and the polyadenylation signal. The resultant fragment was ligated into the *SmaI*

site of a plasmid containing the α P2 promoter in pBluescript SK(-). The DNA fragment was excised from its plasmid by digestion with *ClaI-NotI*, then purified and microinjected into the pronuclei of fertilized eggs obtained from C57Bl/6N mice (Nippon CREA Co. Ltd., Tokyo, Japan). Transgenic founder mice were identified by Southern blot analysis of tail DNAs. F1 offspring were then crossed with C57Bl/6N mice to establish a transgenic lineage. Male mice with access to food *ad libitum* and reared under a light-dark cycle of 12 h were used for the experiments at 8–10 weeks of age. All experimental procedures conformed to the guidelines of the Animal Care Committee of the University of Tokyo.

Genotyping by PCR

Genotype was determined by PCR analysis of genomic DNA obtained from tail snips. To detect the S112A transgene, the sense primer corresponded to sequences in the rabbit β -globin intron (5'-TTATTG GTAGAAACAACACTACATCCT-3'), and the antisense primer corresponded to sequences in the coding region of PPAR γ (5'-ATATTTGTAATCAGCAACCATTG GG-3'). To detect the wild-type allele, the sense primer corresponding to sequences in the coding region of PPAR γ (5'-AACTTCGGAATCAGCTCTGTGGACC-3') and the same antisense primer as that indicated above for detection of the S112A transgene, were used. The three primers and a genomic DNA template were mixed in a tube. The thermal reaction cycles consisted of an initial step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 seconds, 55°C for 1 min and 72°C for 1 min, with a final step at 72°C for 5 min. The wild-type allele yielded a 393 base-pair (bp) product, and the transgene, a 529 bp product.

In vivo glucose homeostasis

Glucose tolerance test: The mice were fasted for more than 16 h before the study. They were then given 1.0 mg/gram (body weight) of glucose orally. Blood samples were collected at different time-points and the blood level of glucose was measured immediately with an automatic blood glucose meter (Glutest Pro: Sanwa Chemical, Nagoya, Japan). Whole blood specimens were collected and centrifuged, and the serum samples obtained after separation were stored at -20°C. Insulin levels were determined with an insulin

radioimmunoassay (RIA) kit (BIOTRAK: Amersham Biosciences, Buckinghamshire, UK) using rat insulin as the standard.

Insulin tolerance test: Mice were given free access to chow before the study. They were administered an intraperitoneal injection of 0.75 mU/gram (body weight) of human insulin (Humulin R: Lilly, Indianapolis, IN, USA). Blood samples were taken at different time-points from the tail vein and the blood levels of glucose were measured with an automatic blood glucose meter.

RNA preparation, Northern blot analysis and real-time quantitative PCR

Total RNA was prepared from epididymal WAT with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Northern blot analysis was performed using the standard protocol. A 20 μ g sample of total RNA was electrophoresed through denaturing formaldehyde-agarose (1%) gel and then transferred to a Hybond N⁺ nylon membrane (Amersham Biosciences). Real-time quantitative PCR was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan PCR Master Mix Reagent Kit (Applied Biosystems), with cyclophilin as the internal control. The primers and probe used for cyclophilin were as follows: forward, GGTCCTGGCATCTTGTC CAT; reverse, CAGTCTTGGCAGTGCAGATAAAA; probe, CTGGACCAAACACAAACGGTCCCA. The primers and probes for the other genes were purchased from Applied Biosystems.

Generation of probe and RNase protection assay (RPA)

A partial murine PPAR γ cDNA probe was generated by reverse transcriptase-PCR using total RNA and primers designed to amplify a region including 90 bp of the PPAR γ 2 transcript and a 185 bp region common to both PPAR γ 1 and PPAR γ 2, as previously described [15]. The PPAR γ cDNA PCR product was subcloned into the *Pst*I-*Eco*RI site of the pBluescript SK(-) (Stratagene). The antisense cRNA was transcribed with [α -32P]UTP (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) using the T7 RNA polymerase (MAXIscript: Ambion, Inc., Austin, TX, USA). A 10 μ g sample of total RNA was subjected to

RPA using an RPAIII kit (Ambion, Inc.) and hybridized overnight with the cRNA probe in Hybridization III Buffer (Ambion, Inc.) at 42°C. After digestion with RNaseA/RNaseT1 diluted 1:100, the protected fragments were separated on 8 M urea/5% polyacrylamide gels and analyzed quantitatively using an imaging plate and BAS2000 (Fuji Film, Tokyo, Japan).

Diets and rosiglitazone treatment

The normal diet (MF) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The diet was composed of 5.3% (wt/wt) fat, 23.6% protein, 54.4% carbohydrate, 2.9% dietary fiber, and 6.1% minerals. The HFD, containing 32% safflower oil, 33.1% casein, 17.6% sucrose and 5.6% cellulose, was prepared as described previously [3]. Rosiglitazone (BRL49653), procured from GlaxoSmithKline (Brentford Middlesex, UK), was given at a 0.01% (wt/wt) mixture with the HFD.

Histochemistry

Epididymal adipose tissue was removed from each animal, fixed in 10% formaldehyde/PBS, and stored at 4°C until use. The tissue specimens were routinely processed for paraffin embedding, and 5- μ m sections were cut and mounted on silanized slides. The adipose tissue was stained with hematoxylin and eosin, and the total adipocyte area was analyzed with Win ROOF software (Mitani Co., Ltd., Chiba, Japan). The white adipocyte area was measured in 400 or more cells per mouse in each group as described previously [3].

Blood sample assays

The serum levels of FFA and TG were determined by a nonesterified fatty acid-C test and triglyceride L-type test (Wako Pure Chemical Industries, Japan), respectively. The serum levels of adiponectin and leptin were assayed with a mouse adiponectin immunoassay kit (Otsuka Pharmaceutical, Tokushima, Japan) and Quantikine M kit (R&D Systems, Minneapolis, MN), respectively.

Energy expenditure

Oxygen consumption was measured every 3 minutes for 24 h in fasting mice using an O₂/CO₂ metabolism