Dietary protein deprivation upregulates insulin signaling and inhibits gluconeogenesis in rat liver

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Abstract

This study was undertaken to elucidate the effects of dietary protein deprivation on glucose metabolism and hepatic insulin signaling in rats. The results of glucose and pyruvate tolerance tests in rats fed with a 12% casein diet (12C) and a protein-free diet (PF) indicated that protein deprivation enhanced clearance of blood glucose and suppressed gluconeogenesis. Correspondingly, the mRNA level of hepatic phosphoenolpyruvate carboxykinase, a key gluconeogenic enzyme, was suppressed by dietary protein deprivation. In PF-fed rats, total tyrosine phosphorylation of insulin receptor (IR) in the liver induced by insulin injection was enhanced compared with 12C pair-fed rats due to an increase in IR protein level. In addition, protein deprivation caused an increase in protein levels of IR substrate 1 (IRS1) and IRS2, leading to the marked enhancement of insulin-induced tyrosine phosphorylation of IRS2 and its binding to the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). Based on these results, we conclude that protein deprivation suppresses gluconeogenesis by a mechanism primarily mediated by the enhancement of the insulin signals through the IR/IRS/PI3K/mammalian target of rapamycin complex 1 pathway in the liver. Taken together with our previous report, these findings suggest that tissue-specific potentiation of insulin action in the liver and the skeletal muscle plays important roles in maintaining glucose homeostasis even when energy usage is reduced by dietary protein deprivation.


Introduction

It is well known that when the nutritional state deteriorates, for example, during fasting or protein malnutrition, insulin and insulin-like growth factor 1 (IGF1) secretion decrease and plasma anti-insulin/IGF1 factors increase, leading to a catabolic state (Garber et al. 1976, DeFronzo et al. 1978, Takahashi et al. 1990). This regulation is important in an energy and protein crisis to attenuate the bioactivity of anabolic hormones that stimulate the synthesis of glycogen, protein, and lipids. Thus, the deterioration of nutritional state could result in the inhibition of insulin-like signaling. On the other hand, some reports have shown that malnutrition enhances insulin sensitivity in various human and animal tissues (Reis et al. 1997, Rigalleau et al. 1998, Aparicio et al. 2001, Rojas et al. 2001, Toyoshima et al. 2004). However, it is not yet clear how protein malnutrition increases insulin sensitivity.

Recent studies which have focused on the intracellular signaling pathway of the insulin receptor (IR) have expanded our understanding of the molecular mechanisms of regulation of glucose and lipid metabolism (Taniguchi et al. 2006). The binding of insulin to the IR initiates a cascade of events including the interaction of multiple molecules and their phosphorylation. The key molecules in this pathway are IR substrate 1 (IRS1) and IRS2 (White 1998, 2002), docking molecules that connect IR to activate the essential downstream kinase cascade. After tyrosine phosphorylation and activation, IRS1 and IRS2 transmit the signals to downstream machinery, including the phosphatidylinositol 3-kinase (PI3K) pathway and the mitogen-activated protein kinase pathway. The PI3K pathway mediates most of the metabolic action of insulin, including the stimulation of glucose transport, glycogen synthesis, and lipid synthesis. In addition, it has been shown that the activation of PI3K is essential for the inhibition of gluconeogenesis by insulin, including the suppression of the genes for key gluconeogenic enzymes (Barthel & Schmoll 2003). Moreover, mammalian target of rapamycin complex 1 (mTORC1) pathway is one of the components of the PI3K pathway (Wullschleger et al. 2006) and has recently been shown to be important to regulate gluconeogenesis in the liver (Houde et al. 2010).
In a previous study, we have shown that the phosphorylation states of IR and IRS1 in rat muscle change greatly depending on protein supply (Toyoshima et al. 2004). Feeding rats a protein-free diet (PF) for 7 days resulted in a decrease in serine phosphorylation of IRS1, while markedly increasing the phosphorylation of its tyrosine residues after insulin injection. In addition, the stimulatory effect of insulin on glucose uptake in the muscle was more prominent in PF-fed rats than in those fed with a 12% casein diet (12C) as a control diet. These results led us to conclude that protein deprivation causes the sensitization of IRS1 to IR tyrosine kinase by reducing serine phosphorylation of IRS1 in rat skeletal muscle.

This study was undertaken to elucidate the effect of protein deprivation on glucose metabolism and on the action and signaling components of insulin, particularly in the liver. For the comparison with the effects in skeletal muscle, severe protein malnutrition was induced by feeding rats a PF for 1 week as in the previous study (Toyoshima et al. 2004). The effects of protein deprivation on glucose and pyruvate tolerances and on the responses of IR/IRS/Pi3K/mTORC1 pathway to insulin in the liver were examined.

Materials and methods

Materials

Anti-IR β subunit (IRβ; C-19), IRS1 (C-20), and IRS2 (H-205) antibodies were purchased from Santa Cruz Biochemistry, Inc. (Santa Cruz, CA, USA). Anti-phospho-tyrosine (4G10), phospho-IRS1 (Ser307), IRS2, and Pi3K p85 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Abcam plc (Cambridge, UK). Anti-phospho-Thr389 p70 ribosomal S6 kinase (S6K), S6K, phospho-Thr37/46 eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and 4E-BP1 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Protein G-Sepharose 4FF and HRP-conjugated secondary antibodies were purchased from GE Healthcare (Piscataway, NJ, USA). All other chemicals were of reagent grade and were obtained commercially.

Animals

Male Wistar rats weighing 130–150 g were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The rats were kept in a room maintained at 22 ± 1°C with a relative humidity of 60%, and with a 12 h light (0800–2000 h) and 12 h darkness (2000–0800 h) cycle. They were allowed free access to water throughout the experiment. All rats were trained to eat a 12% casein diet (12C) as a control diet between 1000 and 1800 h for 4 days, after which they were divided into three groups and then given experimental diets. For the next 7 days (days 1–7), one group was then fed 12C and the other group was fed an isocaloric PF between 1000 and 1800 h. They are called as the 12C ad libitum and PF groups. On day 8, the rats were used for the experiments. The daily food intake during the experimental diet period of the 12C ad libitum and PF groups was 17.0 ± 0.7 and 9.8 ± 0.4 g/day (n = 15) respectively. To exclude the effect of the difference in calorie intake, another group of rats given 12C was pair-fed with the PF group. This group is called as the 12C pair-fed group. The amount of food consumed by each rat in the PF group was monitored each day, and the mean of those amounts was given to the 12C pair-fed group on the next day. Accordingly, the tissue sampling of the 12C pair-fed group was performed 1 day after those of the 12C ad libitum and PF groups. During experiments, body weight was measured at 1000 h every day.

To measure the plasma insulin, glucagon, and corticosterone, and serum IGF1, we bled the animals from the carotid artery before killing them under anesthesia with pentobarbital (50 mg/kg body weight). Insulin levels were measured using a rat Insulin ELISA kit (Shibayagi, Gunma, Japan). Glucagon levels were measured using a Rat Glucagon ELISA kit Wako (Wako Pure Chemical Industries, Osaka, Japan). Corticosterone levels were measured using the AssayMax Corticosterone ELISA kit (AssayPure, St Charles, MO, USA). IGF1 levels were measured using the Quantikine mouse IGF1 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA).

The experimental procedures used in this study were in accordance with the guidelines of the Animal Usage Committee of the Faculty of Agriculture, The University of Tokyo and verified by the committee (permission number 1316T0015).

Glucose and pyruvate tolerance tests

Glucose and pyruvate tolerance tests (PTT) were performed with 20 h fasting rats on days 5 and 7. The solutions of glucose (1 g/kg body weight) or sodium pyruvate (1 g/kg body weight) were injected i.p. Glucose levels were determined in blood obtained from the tail vein at 0, 15, 30, 60, and 120 min after the injection. Glucose levels were measured using Ascensia BREEZE (Bayer Medical Ltd).

In vivo insulin stimulation and preparation of protein extract from liver

At 1000 h on day 8, rats were anesthetized with pentobarbital (50 mg/kg body weight). Then, the abdominal cavity was opened, and 1 ml of different
concentrations of insulin (0, 0·014, 0·07, 0·14, and 1·4 U) was injected into the inferior vena cava. The liver was removed 1 and 15 min post injection, frozen immediately in liquid nitrogen, and stored at −80°C until analysis. The frozen tissues were ground into a fine powder using a mortar and pestle, and homogenized in a polytron homogenizer at 4°C with 10 times the liver weight of homogenizing buffer (50 mmol/l HEPES–NaOH, pH 7·6, 10 mmol/l sodium orthovanadate, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 2 mmol/l phenylmethylsulfonyl fluoride, 100 KIU/ml aprotinin, 2 mmol/l EDTA, and 2% v/v Triton X-100). The extracts were centrifuged at 100 000 g for 1 h at 4°C, and the supernatants were collected for further analysis. The protein concentration was determined with a Bio-Rad Protein Assay kit (Bio-Rad).

**Immunoprecipitation and immunoblotting analysis**

The extracted protein (10 mg) was incubated with anti-IRβ, anti-IRS1, or anti-IRS2 antibody at 4°C overnight, followed by the addition of 30 µl protein G-Sepharose 4FF (50% v/v) for 1·5 h. The immunocomplexes were washed three times with the homogenizing buffer at 4°C, suspended in Laemmli’s sample buffer, and boiled for 5 min. To analyze the amount of IR, IRS1, IRS2, p85 regulatory subunit of PI3K, 4E-BP1 and S6K in rat liver, and Ser307 phosphorylation of IRS1 in rat liver and skeletal muscle, the same livers and skeletal muscles from saline-injected rats were used. Each sample was subjected to SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Co., Billerica, MA, USA). After transblotting, the membranes were blocked with a blocking buffer (3% w/v BSA in TBS-T (10 mmol/l Tris–HCl, pH 7·5, 100 mmol/l NaCl, and 0·1% v/v Tween 20)) at room temperature for 1 h. The membranes were then incubated with appropriate antibodies diluted in the blocking buffer at 4°C overnight, and washed five times with TBS-T. The membranes were then incubated with HRP-conjugated secondary antibodies diluted in TBS-T at room temperature for 1 h, and washed again as described above. The bands of immunoreactive proteins were detected with an ECL kit (PerkinElmer Life Sciences, Inc., Boston, MA, USA) and quantified using a cooled CCD camera system, LAS-3000 mini (Fuji Film Co., Kanagawa, Japan).

**Real-time RT-PCR analysis**

At 1000 h on day 8, rats of each group were anesthetized with pentobarbital (50 mg/kg body weight) and their livers were excised. Total RNA was prepared from the liver using TRIzol reagent according to the manufacturer’s protocol (Invitrogen). The concentration of RNA was determined by absorbance at 260 nm and the purity was indicated by 260/280 nm ratio with values consistently between 1·8 and 2·0. The integrity and quantification of RNA were confirmed by visualization of rRNAs after electrophoresis on denaturing agarose gel. To remove potential genomic DNA contamination, the RNA samples were treated with RNase-free DNase I. The cDNA was then synthesized from 2 µg of DNase-treated total RNA using SuperScript II (Invitrogen). Then cDNA was diluted 1:25 with distilled water, and 2·5 µl from each sample was used for PCR at a total volume of 25 µl. The SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and Thermal Cycler Dice Real Time System TP800 (TAKARA Bio, Inc., Shiga, Japan) were used. The abundance of specific mRNAs was determined by comparison with a standard curve constructed by serial dilution of the sample and normalized to Gapdh mRNA (Endo et al. 2002). Primer sequences used were as follows: 5′-ATGCGGCCCTTCTTTGCTCACAC-3′ and 5′-CTTCGATGGTCGCAACATCACACT-3′ for phosphoenolpyruvate carboxykinase (Pepck); 5′-GTACCCCTGCGCTACTTTC-3′ and 5′-ATCAGTGAGCAAACAC-3′ for glucose-6-phosphate (G6pase); and 5′-GAACCTCCTAACGTTGAGCA-3′ and 5′-GGCATGGACTGTGGTCATGA-3′ for Gapdh.

**Statistical analysis**

Values for immunoblotting are expressed as means ± s.e.m. for six rats. Values for glucose tolerance test (GTT), PTT, and gene expression levels are given as means ± s.e.m. for five rats. When we compared the two groups, the results were analyzed statistically using unpaired Student’s t-test. Where multiple comparisons were made, the results were analyzed statistically using ANOVA and Tukey–Kramer test. Differences were considered to be statistically significant at P<0·05.

**Results**

**Characterization of animals**

The body weight and endocrine parameters in the fasted state are summarized in Table 1. The body weight of 12C pair-fed rats at the time of killing was lower than that of 12C ad libitum-fed rats, and the body weight of PF-fed rats was lowest. The 7-day PF intake significantly reduced serum IGF1 levels compared with 12C ad libitum-fed rats and 12C pair-fed rats. As reported before (Takahashi et al. 1990), serum IGF1 levels are well correlated with growth. The liver weight of 12C pair-fed
and PF-fed rats was significantly lower than that of 12C
*ad libitum*-fed rats.

Plasma insulin was not significantly different among three groups. Plasma corticosterone in 12C pair-fed and PF-fed rats was significantly higher compared with 12C *ad libitum*-fed rats. Plasma glucagon in 12C *ad libitum*-fed and PF-fed rats was significantly higher compared with 12C pair-fed rats.

### Dietary protein deprivation enhances glucose clearance and inhibits hepatic gluconeogenesis

Our previous studies indicated that dietary protein deprivation enhances glucose uptake in the skeletal muscle, suggesting that whole body glucose metabolism could be activated (Toyoshima et al. 2004). To evaluate the effect of dietary protein deprivation on whole body glucose metabolism, we performed a GTT to observe glucose clearance and PTT to observe gluconeogenesis because pyruvate is a substrate for glucose production in the liver. In GTT, the PF feeding potentiated glucose tolerance compared with both 12C *ad libitum*-fed and 12C pair-fed groups (Fig. 1A). In PTT, all groups showed peak blood glucose levels at 15 min after the i.p. pyruvate injection (Fig. 1B). The peak was significantly lower in PF-fed rats than in both 12C *ad libitum*-fed and 12C pair-fed rats, indicating that PF feeding suppressed gluconeogenesis in the liver. Furthermore, GTT and PTT were not different in the 12C *ad libitum*-fed compared with the 12C pair-fed rats, indicating that the reduction in calorie intake under our conditions did not change glucose tolerance or gluconeogenesis.

### Dietary protein deprivation reduces PEPCK expression in rat liver

To elucidate the possible mechanism of the suppressed gluconeogenesis in the liver by dietary protein deprivation, we measured the hepatic gene expression of *Pepck* and *G6pase*, two key gluconeogenesis enzymes. *Pepck* gene expression was significantly reduced in the PF-fed rats (Fig. 1C) as compared with both 12C
*ad libitum*-fed and 12C pair-fed rats. The levels of *G6pase* mRNA were not significantly different among the three groups although they tended to be reduced in PF-fed rats (Fig. 1D). These results suggest that protein deprivation itself suppressed gluconeogenesis mainly through reducing PEPCK activity in the liver.

#### Table 1 Body weight and endocrine parameters in 12C *ad libitum*, 12C, and PF-fed rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>12C <em>ad libitum</em></th>
<th>12C</th>
<th>PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>207.3 ± 4.4^a</td>
<td>172.1 ± 2.6^b</td>
<td>138.8 ± 2.5^c</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>4.4 ± 0.2^a</td>
<td>3.4 ± 0.1^b</td>
<td>3.5 ± 0.2^b</td>
</tr>
<tr>
<td>Serum IGF1 (ng/ml)</td>
<td>587.5 ± 25.7^a</td>
<td>481.8 ± 16.9^b</td>
<td>238.8 ± 12.2^c</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>2.65 ± 0.22</td>
<td>2.08 ± 0.50</td>
<td>1.53 ± 0.30</td>
</tr>
<tr>
<td>Plasma glucagon (pg/ml)</td>
<td>424.2 ± 11.2^a</td>
<td>359.5 ± 12.6^b</td>
<td>411.9 ± 16.9^a</td>
</tr>
<tr>
<td>Plasma corticosterone (ng/ml)</td>
<td>28.2 ± 3.4^a</td>
<td>71.6 ± 7.4^b</td>
<td>60.0 ± 6.7^b</td>
</tr>
</tbody>
</table>

Body weights were measured and the blood samples were collected at 1000 h on day 8; n = 5. There are significant differences between values with different superscript characters (P<0.05).

#### Figure 1 Effect of dietary protein deprivation on glucose and pyruvate tolerance and on gene expression of insulin-sensitive gluconeogenic enzymes in rat liver. Rats were fed a 12% casein diet (12C) or a protein-free diet (PF) from 1000 to 1800 h for 7 days (group name; 12C *ad libitum* or PF). One more group of rats was given 12C according to the mean amount consumed by the PF rats on the previous day to equal the daily food intake (group name; 12C pair-fed). A glucose tolerance test (A) and pyruvate tolerance test (B) were performed with 20 h fasting rats on days 5 and 7. Values shown are the means±S.E.M. for five rats of both the groups. There are significant differences between values with different characters (P<0.05). (C and D) mRNAs were performed as described in ‘Materials and methods’ section. Values shown are the means±S.E.M. for five rats. There are significant differences between values with different characters (P<0.05).
Protein deprivation does not affect the levels of glucagon and glucocorticoid in rat plasma

The PF intake suppressed Pepck expression, which is negatively regulated by insulin and positively regulated by glucagon and glucocorticoids (Barthel & Schmoll 2003, Postic et al. 2004), but the changes of their circulating levels, shown in Table 1, could not explain the reduction in Pepck expression by PF feeding.

Dietary protein deprivation increases the amount of IR, IRS1, and IRS2 in rat liver

Since our previous study showed that the PF intake upregulated insulin signaling and its action through the reduction of serine phosphorylation of IRS1 in rat skeletal muscle, we hypothesized that dietary protein deprivation enhanced insulin signaling in rat liver; 12C pair-fed and PF-fed rats were used to evaluate the effect of protein deprivation on insulin signaling under the condition of the same calorie intake since it has been shown that calorie restriction affects insulin signaling in the liver and the skeletal muscle (Dean & Cartee 2000, Zhu et al. 2005, Bonkowski et al. 2009). We first analyzed the amounts of IRβ, IRS1, IRS2, and p85 regulatory subunit of PI3K in the liver of 12C pair-fed and PF-fed rats. The amount of p85 regulatory subunit of PI3K was not different in the two groups (Fig. 2A and E). In contrast, the amounts of IRβ, IRS1, and IRS2 were significantly increased in the PF-fed group compared with the 12C pair-fed group (Fig. 2A–D), indicating that dietary protein deprivation itself increases the amounts of these insulin signaling molecules in rat liver, which could enhance insulin signal transduction.

Dietary protein deprivation enhances insulin-induced autophosphorylation of IR in rat liver

We then analyzed insulin-induced tyrosine phosphorylation of IRβ in the livers of 12C pair-fed and PF-fed rats, which were injected with various doses of insulin. Tyrosine phosphorylation of IRβ was increased by insulin injection in a dose-dependent manner (Fig. 3A). When autophosphorylation of IRβ was normalized to the amount of immunoprecipitated IRβ protein, the ratios that reflect tyrosine kinase activity per IR molecule were not changed in the two groups (Fig. 3B). In addition, total tyrosine phosphorylation of IRβ was calculated by multiplying tyrosine phosphorylation of IRβ per the immunoprecipitated IRβ level by levels of IRβ in each sample. PF intake increased total tyrosine phosphorylation of IRβ in response to 0.07 U insulin injection compared with 12C pair-fed rats (Fig. 3C), which was due to the increase in the amount of IR (Fig. 2A and B). These results suggest that PF intake enhanced total IR tyrosine kinase activity without affecting its specific activity.

Dietary protein deprivation enhances insulin-induced tyrosine phosphorylation of IRS1 in rat liver

We next examined tyrosine phosphorylation of IRS1 in rat liver. The insulin injection increased tyrosine phosphorylation of IRS1 dose dependently (Fig. 4A).
Effect of dietary protein deprivation on insulin-induced tyrosine phosphorylation of insulin receptor β subunit in rat liver. Rats were fed a protein-free diet (PF) or pair-fed a 12% casein diet (12C) from 1000 to 1800 h for 7 days. At 1000 h on day 8, insulin with different concentrations (0.014, 0.07, 0.14, and 1.4 U) or saline was injected into the inferior vena cava. One minute after injection, the liver was excised and protein extracts were prepared. The immunoprecipitated insulin receptor (IRβ) from the protein extracts of the liver was analyzed by immunoblotting with antibodies against the (β-subunit of the insulin receptor (IRβ) and phosphotyrosine (pY). (A) A representative immunoblot. (B) The ratio of tyrosine phosphorylation to the amount of immunoprecipitated IRβ. The results of densitometric scanning are expressed as arbitrary units. Values of 12C pair-fed rats or PF-fed rats shown are the means ± S.E.M. for six rats. (C) Total tyrosine phosphorylation of IRβ with or without 0.07 U insulin injection. Total tyrosine phosphorylation was calculated by multiplying tyrosine phosphorylation of IRβ per the immunoprecipitated IRβ level by levels of IRβ in each sample. The values are expressed as arbitrary units. Values shown are the means ± S.E.M. for six rats. There are significant differences between values with different characters (P < 0.05).

Figure 3 Dietary protein deprivation markedly enhances insulin-induced tyrosine phosphorylation of IRS2 in rat liver

Tyrosine phosphorylation of IRS2, another major isoform in the liver, and the amount of the p85 regulatory subunit of PI3K associated with IRS2 were markedly higher in the livers of PF-fed rats than in those of 12C pair-fed groups across all insulin doses (Fig. 6A). The ratio of tyrosine phosphorylation of IRS2 to its immunoprecipitated amount after 0.07 U insulin injection was significantly higher in PF-fed rats than in 12C pair-fed rats (Fig. 6B). The ratio of the amount of p85 regulatory subunit of PI3K to the amount of immunoprecipitated IRS2 was not different in the two groups although the ratio at basal state was lower without statistical significance in PF-fed rats compared with 12C pair-fed rats (Fig. 6C). Furthermore, total...
Dietary protein deprivation enhances insulin-dependent activation of the mTORC1 pathway in rat liver

To elucidate whether the enhanced insulin signals through the IR/IRS signaling pathway were transmitted to the downstream signaling pathway, we analyzed the activity of mTORC1 pathway that has very recently been shown to be important for inhibition of gluconeogenesis (Houde et al. 2010). It is well known that the activated mTORC1 phosphorylates its substrates, such as 4E-BP1 and S6K, regulating cell growth and metabolism (Wullschleger et al. 2006). We measured the amounts of 4E-BP1 and S6K and their phosphorylation. The amounts of 4E-BP1 and S6K were significantly increased in the PF-fed rats compared with the 12C pair-fed rats (Fig. 7A–C). Total phosphorylation at Thr37/46 of 4E-BP1 by 0·07 U insulin injection was significantly enhanced in PF-fed rats compared with 12C pair-fed rats (Fig. 7D and E). Moreover, total phosphorylation at Thr389 of S6K by 0·07 U insulin injection was also significantly enhanced in PF-fed rats compared with 12C pair-fed rats (Fig. 7F and G). All of the results demonstrate that dietary protein deprivation itself increases total phosphorylation of 4E-BP1 and S6K in rat liver, clearly indicating that thereby increasing their phosphorylation. The upregulation of insulin signals through IR/IRS could lead to the enhancement of mTORC1 pathway under protein deprivation.

Tyrosine phosphorylation of IRS2 (Fig. 6D) and the total amount of IRS1-associated p85 regulatory subunit of PI3K (Fig. 6E) by both saline and 0·07 U insulin injection were prominently increased in PF-fed rats compared with 12C pair-fed rats. These results suggest that PF intake markedly enhanced IRS2-mediated insulin signals due to an increase in its protein level.

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Figure 4 Effect of dietary protein deprivation on insulin-induced tyrosine phosphorylation of IRS1 and its association with the p85 regulatory subunit of PI3K in rat liver. Immunoprecipitated IRS1 from the protein extracts of the liver taken 1 min after insulin injection as described in the legend of Fig. 3 was analyzed by immunoblotting with antibodies against phospho-tyrosine (pY), the p85 regulatory subunit of PI3K (p85 of PI3K), and IRS1. (A) A representative immunoblot. (B) The ratio of tyrosine phosphorylation to the amount of immunoprecipitated IRS1 in rat liver. Rats were treated as described in the legend of Fig. 2 and then liver and skeletal muscle were excised. Immunoprecipitated IRS1 from the protein extracts of the liver and muscle was analyzed by immunoblotting with antibodies against phospho Ser307 of IRS1 (pSer307 IRS1) and IRS1. (A and C) A representative immunoblot of Ser307 phosphorylation of immunoprecipitated IRS1 in rat liver, clearly indicating that thereby increasing their phosphorylation. The upregulation of insulin signals through IR/IRS could lead to the enhancement of mTORC1 pathway under protein deprivation.

Figure 5 Effect of dietary protein deprivation on Ser307 phosphorylation of IRS1 in rat liver. Rats were treated as described in the legend of Fig. 2 and then liver and skeletal muscle were excised. Immunoprecipitated IRS1 from the protein extracts of the liver and muscle was analyzed by immunoblotting with antibodies against phospho Ser307 of IRS1 (pSer307 IRS1) and IRS1. (A and C) A representative immunoblot of Ser307 phosphorylation of immunoprecipitated IRS1 in rat liver, clearly indicating that thereby increasing their phosphorylation. The upregulation of insulin signals through IR/IRS could lead to the enhancement of mTORC1 pathway under protein deprivation.

Tyrosine phosphorylation of IRS2 (Fig. 6D) and the total amount of IRS1-associated p85 regulatory subunit of PI3K (Fig. 6E) by both saline and 0·07 U insulin injection were prominently increased in PF-fed rats compared with 12C pair-fed rats. These results suggest that PF intake markedly enhanced IRS2-mediated insulin signals due to an increase in its protein level.
**Discussion**

In this study, we observed significant enhancement of glucose tolerance and inhibition of gluconeogenesis in PF-fed rats compared with 12C pair-fed rats (Fig. 1A and B). However, there were not such differences between 12C pair-fed rats and 12C ad libitum-fed rats, even though food intake of 12C pair-fed group was reduced. Based on these results, we concluded that dietary protein deprivation enhances glucose tolerance and inhibits gluconeogenesis, but calorie restriction does not under our conditions. Since we have reported that PF intake enhances insulin signaling in skeletal muscle, resulting in increased insulin-dependent glucose uptake (Toyoshima et al. 2004), protein deprivation-dependent sensitization to insulin in muscle may contribute to the enhancement of glucose tolerance.

The suppression of gluconeogenesis is known to be partly mediated by the reduction in the transcription of the genes for gluconeogenic enzymes, including *Pepck* and *G6pase* (O’Brien & Granmer 1996, Barthel & Schmoll 2003, Postic et al. 2004). Our results showed that *Pepck* gene expression in the liver of PF-fed rats was lower than in the liver of both 12C pair-fed and 12C ad libitum-fed rats and hepatic *G6pase* gene expression was not significantly different in the three groups while there was a tendency toward reduced expression in PF-fed rats (Fig. 1C and D). Similar to the result of PTT, *Pepck* and *G6pase* gene expression in the liver of 12C pair-fed and 12C ad libitum-fed rats was not different, confirming that calorie restriction did not affect gluconeogenesis under our conditions. *Pepck* gene expression is controlled mainly by insulin, glucagon, and glucocorticoids (Postic et al. 2004). Insulin suppresses, and glucocorticoids and glucagon enhance its gene expression. We found that protein deprivation does not affect the circulating levels of corticosterone and increases the plasma levels of glucagon, suggesting that the signals of these catabolic hormones might not be relevant to the suppression of *Pepck* gene expression by protein deprivation (Table 1). Based on these results, we conclude that the reduction in *Pepck* gene expression by protein deprivation mainly led to the inhibition of gluconeogenesis in rat liver. It is well known that the stimulation of PI3K is essential for the regulation of *Pepck* gene expression (Agati et al. 1998, Miyaka et al. 2002, Barthel & Schmoll 2003). It is reasonable to speculate that the enhancement of insulin signaling, the IR/IRs/PI3K pathway, by protein deprivation contributes to reducing *Pepck* gene expression and gluconeogenesis in rat liver.

To elucidate whether protein deprivation itself enhance insulin signaling, we examined insulin signaling using 12C pair-fed and PF-fed rats injected several different doses of insulin. Our finding that insulin-dependent tyrosine phosphorylation of IRβ per immunoprecipitated IRβ protein was not different in 12C pair-fed and PF-fed rats (Fig. 3B) indicates that the specific activity of IR tyrosine kinase is not affected by protein deprivation. Protein deprivation, however, significantly increased the amounts of IRβ (Fig. 2A and B), indicating that total IR autophosphorylation that is reflected by its kinase activity is enhanced (Fig. 3C). By contrast, a number of reports have shown that starvation upregulates the amount of IR even though the receptor tyrosine kinase activity is impaired (Balage et al. 1990, Karasik et al. 1990), and these results are distinct from the effects of protein deprivation on IR.

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**Figure 6** Effect of dietary protein deprivation on insulin-induced tyrosine phosphorylation of IRS2 and its association with the p85 regulatory subunit of PI3K in rat liver. Immunoprecipitated IRS2 from the protein extracts of the liver taken 1 min after insulin injection as described in the legend of Fig. 3 was analyzed by immunoblotting with antibodies against phosphotyrosine (pY), the p85 regulatory subunit of PI3K (p85 of PI3K), and IRS2. (A) A representative immunoblot. (B) The ratio of tyrosine phosphorylation to the amount of immunoprecipitated IRS2. Values of 12% casein diet (12C) pair-fed rats or protein-free diet (PF)-fed rats shown are the means ± S.E.M. for six rats. (C) The ratio of the amount of IRS2-associated p85 divided by the amount of immunoprecipitated IRS2. Values of 12C pair-fed rats or PF-fed rats shown are the means ± S.E.M. for six rats. (D) Total tyrosine phosphorylation of IRS1 with or without 0-07 U insulin injection. (E) Total amount of IRS1-associated p85 with or without 0-07 U insulin injection. Values shown are the means ± S.E.M. for six rats. There are significant differences between values with different characters ($P < 0.05$).
Figure 7 Effect of dietary protein deprivation on the amounts and phosphorylation of 4E-BP1 and S6K in rat liver. At 1000 h on day 8, insulin (0.07 U) or saline was injected into the inferior vena cava of protein-free diet (PF)-fed or 12% casein diet (12C) pair-fed rats for 7 days. Fifteen minutes after injection, the liver was excised and protein extracts were prepared. (A) Whole lysate was analyzed by immunoblotting with antibodies against the 4E-BP1, S6K, or GAPDH as an internal control. A representative immunoblot data of pThr37/46 4E-BP1 (D) and pThr389 S6K (G) is shown. Immunoreactivity was detected by ECL and quantified by densitometric scanning. The results of densitometric scanning are expressed as arbitrary units. Values shown are the means ± S.E.M. for five to six rats. *P < 0.05 vs 12C pair-fed rats. (D and F) Whole lysate was analyzed by immunoblotting with antibodies against the phospho-Thr37/46 4E-BP1 (pThr37/46 4E-BP1) or phosphor-Thr389 S6K (pThr389 S6K). A representative immunoblot data of pThr37/46 4E-BP1 (D) and pThr389 S6K (F). (E and G) Total phosphorylation of 4E-BP1 (E) or S6K (G) is shown. Values shown are the means ± S.E.M. for six rats. There are significant differences between values with different characters (P < 0.05).

Similar to IRβ, the ratios of tyrosine phosphorylation of IRS1 as well as IRS1-association of p85 relative to immunoprecipitated IRS1 protein levels were not different in 12C pair-fed and PF-fed rats (Fig. 4B and C). These results suggest that protein deprivation did not affect the availability of IRS1 to IR kinase (Fig. 3B). It is well known that increase in the phosphorylation of serine residues of IRS reduces the availability of IRS1 to IR kinase as well as the response of downstream signaling (Feinstein et al. 1993, Kanety et al. 1995, Rui et al. 2001, Sykiotis & Papavassiliou 2001). Our previous study showed that dietary protein deprivation reduced Ser307 and Ser612 phosphorylation of IRS1, and these alterations were associated with increased sensitivity of IRS1 to IR kinase and with the enhanced insulin-dependent glucose uptake in rat skeletal muscle (Toyoshima et al. 2004). On the other hand, in this study, we could not detect a significant difference in the degree of Ser307 phosphorylation of IRS1 in the liver of 12C pair-fed and PF-fed rats (Fig. 5), confirming the unchanged availability of IRS1 to IR kinase in the liver of PF-fed rats. The mechanisms by which dietary protein regulates the sensitivity of IRS1 to IR may be tissue specific. However, in the liver of PF-fed rats the amount of IRS1 has increased (Fig. 2A and C). Taken together, our results demonstrate that insulin signals through IRS1 increase significantly under protein deprivation (Fig. 4D and E).

On the contrary, in a previous study, Reis et al. (1997) showed that insulin-induced tyrosine phosphorylation of IR and IRS1 increased without any change in these protein levels in the liver of rats fed with a low-protein diet. The differences between their findings and the results of this study might be due to differences in the duration and severity of protein malnutrition.

In the case of IRS2, the ratio of insulin-dependent tyrosine phosphorylation of IRS2 to immunoprecipitated IRS2 protein molecules in PF-fed rats was significantly higher than that in 12C pair-fed rats treated with 0-07 U insulin (Fig. 6B). The results suggest that protein deprivation increases the availability of IRS2 to IR kinase or reduces dephosphorylation of IRS2. On the other hand, the proportion of IRS2 associated with the p85 regulatory subunit of PI3K was lower but not significantly in PF-fed rats than that in 12C pair-fed rats in the basal state and was not different between the groups after insulin injection (Fig. 6C), suggesting that phosphorylation of tyrosine residues of IRS2 in PI3K-binding motifs is not increased by protein deprivation. As shown in Fig. 2A and D, PF feeding markedly increased IRS2 protein. Consequently, protein deprivation prominently enhances insulin signals through IRS2 (Fig. 6D and E). It is noteworthy that total tyrosine phosphorylation of IRS2 in the basal state was significantly increased in the liver of PF-fed rats compared with 12C pair-fed rats, suggesting that the availability of IRS2 to IR kinase in response to endogenous insulin was markedly enhanced by PF feeding.

It has been shown that regulation of IRS1 and IRS2 in response to the metabolic state is distinct. For example, in the states of hyperinsulinemia and insulin resistance, hepatic IRS2 protein level decreased more severely than IRS1 (Kerouz et al. 1997, Shimomura et al. 2000). The enhancement of insulin sensitivity in
The present data could be useful in understanding and treating diabetes and other metabolic disorders.

roles of IRS1 and IRS2 in hepatic insulin action under protein malnutrition.

Recently, Houde et al. (2010) showed that chronic treatment of rats with rapamycin, which is a specific inhibitor of mTORC1 (Wullschleger et al. 2006), increases gluconeogenesis through the elevated Pedef and G6Pase expression in the liver, indicating that insulin-dependent activation of mTORC1 pathway inhibits gluconeogenesis (Houde et al. 2010). We examined the possibility whether upregulation of the signals through IR/IRS by protein deprivation causes activation of mTORC1, since the mTORC1 pathway is one of the downstream components of the PI3K pathway (Wullschleger et al. 2006). We found that protein deprivation significantly increased total phosphorylation of two substrates of mTORC1 (Wullschleger et al. 2006; Fig. 7). These results strongly suggest that the enhanced insulin signals through the IR/IRS/mTORC1 pathway by dietary protein deprivation cause suppression of gluconeogenesis in rat liver. Further studies using genetically engineered animal models are necessary to elucidate whether the mTORC1 pathway directly regulates hepatic gluconeogenesis under protein malnutrition.

It has been demonstrated that the hypothalamus is also involved in the regulation of hepatic gluconeogenesis. Increased insulin signaling in hypothalamus inhibits gluconeogenesis and glucose production in the liver (Obici et al. 2002a,b). A study to elucidate whether protein malnutrition increases insulin signaling in hypothalamus is in progress in our laboratory.

In general, protein malnutrition is known to decrease protein turnover and energy utilization due to decreases in protein synthesis mediated by reduction in IGF1 at least in a part (Balmagiya & Rozovski 1983, Nam et al. 1990). During the growing phase, this causes growth retardation in spite of the similar energy intake (Table 1). Based on our previous and this studies, using a severe model of protein malnutrition, we conclude that dietary protein deprivation not only induces the stimulation of glucose uptake in the muscle but also enhances insulin-dependent inhibition of glucose release from the liver. The former relates to the increase in insulin sensitivity through the decrease in IRS1 serine phosphorylation and the latter seems to result from the amplification of insulin signals through increases in IR and IRSs. This leads to prevention of increase in plasma glucose by reduction in glucose utilization under protein deprivation (Fig. 8).

In conclusion, this study shows that protein deprivation upregulates the IR/IRS/PI3K/mTORC1 pathway, primarily through increases in the amounts of signaling molecules, leading to suppressed hepatic gluconeogenesis and enhanced insulin responsiveness. The present data could be useful in understanding and treating diabetes and other metabolic disorders.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y T, T N, H K, and S-T designed the research; Y T, R T, Y O, and F H conducted the research and analyzed the data; Y T, S M, H K, and S-T wrote the manuscript. H K had primary responsibility for the final content. All authors read and approved the final manuscript.

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