Impaired insulin signaling in the liver of transgenic rats with low circulating growth hormone levels

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Abstract

GH is known to regulate glucose and lipid metabolism as well as body growth. Controversy exists as to whether GH-deficient adults are indeed insulin sensitive or insulin resistant. In GH-deficient animal models, however, no clear observation indicating insulin resistance has been made, while increased insulin sensitivity has been reported in those animals. We have produced human GH (hGH) transgenic rats characterized by low circulating hGH levels and virtually no endogenous rat GH secretion. Although the body length of the transgenic rat is normal, they develop massive obesity and insulin resistance, indicating that the transgenic rat is a good model for the analysis of insulin resistance under GH deficiency. In this study, we have examined how GH deficiency affects the early steps of insulin signaling in the liver of the transgenic rat.

Circulating glucose and insulin concentrations were significantly higher in the transgenic rats than in their littermates. In addition, impaired glucose tolerance was observed in the transgenic rat. The amount of insulin receptor was smaller in the liver of the transgenic rat, resulting in decreased tyrosine phosphorylation in response to insulin stimulation. The amounts of insulin receptor

substrate-1 and -2 (IRS-1 and -2) and insulin-stimulated phosphorylation of IRSs were also smaller in the transgenic rat. Despite the decrease in tyrosine phosphorylation levels of IRSs being mild to moderate (45% for IRS-1 and 16% for IRS-2), associated phosphatidylinositol 3-kinase (PI3-kinase) activity was not increased by insulin stimulation at all in the transgenic rat. To elucidate whether this discrepancy resulted from the alteration in binding of the p85 subunit of PI3-kinase to phosphotyrosine residues of the IRSs, we determined the amount of p85 subunit in the immunocomplexes with anti-phosphotyrosine antibody. Insulin did not affect the amount of p85 subunit associated with phosphotyrosine in the transgenic rats, while it significantly increased in the controls, indicating that alteration may have occurred at the sites of phosphorylated tyrosine residues in IRSs.

These results suggest that GH deficiency in the transgenic rat leads to impairment in at least the early steps of insulin signaling in the liver with a resultant defect in glucose metabolism.

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Introduction

Insulin is a major regulator of glucose homeostasis, and a deficiency in insulin action would cause diabetes mellitus. Insulin signaling is initiated by the activation of insulin receptor tyrosine kinase, leading to the phosphorylation of intracellular receptor substrates, including insulin receptor substrates-1 and -2 (IRS-1 and IRS-2), which contain over 20 potential tyrosine phosphorylation sites (Sun *et al.* 1993, 1995). The possible tyrosine phosphorylation sites are highly conserved between IRS-1 and IRS-2 (Sun *et al.* 1995). The motifs containing phosphorylated tyrosine residues in IRS-1 and IRS-2 serve as docking sites for binding to various proteins having the Src homology-2 (SH2) domain. Among these SH2 proteins, phosphatidylinositol 3-kinase (PI3-kinase) is considered to be particularly important for the insulin-induced glucose

uptake that is observed in muscle and adipose tissue via the translocation of a glucose transporter to the plasma membrane (Kanai *et al.* 1993, Cheatham *et al.* 1994, Okada *et al.* 1994). Furthermore, the activation of PI3-kinase has been reported to play an important role in insulin-induced glycogen synthesis and suppression of phosphoenolpyruvate carboxykinase (PEPCK) expression in hepatocytes (Sakaue *et al.* 1995, Shepherd *et al.* 1995, Sutherland *et al.* 1995, Gabbay *et al.* 1996).

Growth hormone (GH) is known to modulate insulin actions (Davidson 1987). For example, GH-deficient (GHD) subjects have long been recognized as exhibiting increased insulin sensitivity. In fact, the occurrence of fasting hypoglycemia has been demonstrated in GHD children (Wolfsdorf *et al.* 1983) and adults (Merimee *et al.* 1971). This notion is supported by studies using animal models such as dwarf rats (Daugaard *et al.* 1999) and GH

receptor-knockout mice (Dominici *et al.* 2000). In contrast, several researchers have recently reported observing insulin resistance in GHD subjects. Cuneo *et al.* (1992) have reported increased fasting insulin levels in GHD adults. Decreased insulin sensitivity was also shown in GHD adults by the euglycemic-hyperinsulinemic clamp method (Johansson *et al.* 1995, Hew *et al.* 1996). In addition, the studies on GHD subjects have shown that GH deficiency induces fat accumulation, which is one of the major risk factors for developing non-insulin-dependent diabetes mellitus (NIDDM) with insulin resistance (Kopelman 2000). These recent findings indicate that insulin resistance can occur under the state of GH deficiency.

In contrast with clinical studies, there have been few reports of studies investigating the relationship between insulin resistance and GH deficiency in animal models. One reason may be that, until recently, no good animal models that exhibit both insulin resistance and GH deficiency were available. We have previously reported the production of a strain of transgenic rats carrying a chimeric gene of mouse whey acidic protein (WAP) promoter and human GH (hGH) which exhibit unique phenotypes (Ikeda *et al.* 1994, 1998). Although hGH is mainly produced in the mammary gland like other transgenic animals having the WAP-hGH gene (Günzburg *et al.* 1991, Tojo *et al.* 1993), serum hGH level in this transgenic rat was relatively low. Moreover, the secretion of endogenous rat GH (rGH) was almost totally suppressed. Despite the low circulating levels of both hGH and rGH, their insulin-like growth factor-I levels and body length were almost normal, while they developed severe obesity and insulin resistance. In addition, blood glucose, insulin, triglyceride and free fatty acid levels, which are known to be indicators of early diabetes, were elevated, and glucose intolerance after a bolus injection of glucose was observed in the transgenic rats. Thus, we believe our transgenic rats are a useful model for investigating the relationship between insulin resistance and impaired GH status.

In the present study, we analyzed the early steps of insulin signaling in the liver of the transgenic rat. Namely, we evaluated the insulin-stimulated tyrosine phosphorylation levels of insulin receptor and IRS-1 and -2, and the PI3-kinase activity associated with IRS-1 and -2 and the amount of insulin receptor and IRS-1 and -2 in the liver of the transgenic rat. In addition, we determined the association of the p85 subunit of PI3-kinase with phosphotyrosine-containing proteins.

Materials and Methods

Antibodies

The anti-insulin receptor β -subunit (anti-IR- β) antibody, the anti-IRS-1 antibody, the anti-IRS-2 antibody, the

anti-PI3-kinase p85 subunit (anti-PI3Kp85) antibody and the monoclonal anti-phosphotyrosine (anti-pY) antibody were purchased from Upstate Biotechnology (Lake Placid, NY, USA).

Animals

Generation of the hGH transgenic rats has been described previously (Ikeda *et al.* 1994). In this experiment, we used the male transgenic rats (heterozygotes) and their male littermates at 17 weeks of age. After being weaned from the mother at 3 weeks of age, the rats were individually housed with continuous free access to laboratory chow and water. They were kept under a constant room temperature ($23 \pm 1^\circ\text{C}$) and lighting schedule (12 h light:12 h darkness; lights on at 0700 h).

Glucose tolerance test

The animals were fasted overnight and received an i.p. injection of 0.5 g/kg body weight D-glucose (20% solution). Blood samples were obtained from the tail vein without anesthesia, and glucose concentrations were determined before (0 min) and 15, 30, 60, 90, 120 and 150 min after glucose injection. Plasma glucose was measured using a glucose analyzer (Antsense; Miles-Sankyo Co. Ltd, Tokyo, Japan) by the immobilized enzyme membrane/ H_2O_2 method (Shirai *et al.* 1991), and the serum concentration of insulin was assessed by the enzyme-linked immunosorbent assay kit (Amersham Pharmacia Biotech, Amersham, Bucks, UK).

Preparation of liver extracts

The transgenic and control rats were fasted overnight. They were anesthetized with pentobarbital sodium (45 mg/kg body weight, i.p.). After anesthesia, the portal vein was exposed and 1 IU insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) in normal saline in a final volume of 0.1 ml was injected via the vein. Saline was injected to evaluate the basal condition. Approximately 1 min after the injection, the liver was removed, minced coarsely and homogenized in 10 volumes of homogenization buffer A containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM Na_2VO_4 , 1 mM NaF and a protease inhibitor cocktail tablet (Complete mini; Boehringer Mannheim GmbH, Mannheim, Germany) at 4°C . The extracts from the liver were centrifuged briefly to eliminate insoluble material. Protein concentration was determined using a commercial kit (DC protein assay kit; Bio-Rad, Hercules, CA, USA).

Immunoprecipitation

Liver extracts containing 5 mg total protein were incubated at 4°C overnight with 5 μg anti-IR- β , anti-IRS-1

Table 1 Characteristics of rats used. Data are expressed as means $\pm$ S.E.M. Numbers in parentheses represent the number of animals in each group

Line ^a	Body weight (g)	Plasma glucose (mmol/l)	Serum insulin (ng/ml)
Control rats	520 $\pm$ 20.5 (6)	6.56 $\pm$ 0.18 (6)	6.6 $\pm$ 0.9 (6)
Transgenic rats	670 $\pm$ 26.8 (4)*	7.67 $\pm$ 0.25 (7)*	22.3 $\pm$ 4.2 (8)*

^aAnimals were fasted overnight. * $P < 0.01$ vs control rats.

and -2 and anti-pY antibodies. After incubation, 50 μ l protein A-Sepharose (Amersham Pharmacia Biotech; 50% v/v) was added to the mixture. The preparation was further incubated with constant rocking for 2 h and centrifuged at 3000 g for 10 s at 4 °C. The supernatant was discarded, and the precipitate was washed five times with homogenization buffer A. The final pellet was resuspended in 15 μ l of reducing sample buffer containing 50 mM Tris, 2% SDS, 10% glycerol, 6% 2-mercaptoethanol and 0.02% bromophenol blue, boiled for 5 min and stored at -70 °C until electrophoresis.

Immunoblotting

Samples were subjected to SDS-PAGE on a 6% polyacrylamide gel with a Bio-Rad Mini protein apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Electro-transfer of proteins from the gel to Immobilon P membranes (Millipore, Bedford, MA, USA) was performed overnight at 30 V (constant) with the Bio-Rad miniature transfer apparatus (Bio-Rad Laboratories) in 25 mM Tris, 192 mM glycine, 20% methanol and 0.05% SDS. To reduce non-specific antibody binding, the membranes were incubated at 4 °C overnight in Blockace (a blocking buffer; Yukijirushi, Sapporo, Japan). The membranes were then incubated for 1 h at room temperature with anti-pY (1 μ g/ml), anti-IR- β (1 μ g/ml), anti-IRS-1 (1 μ g/ml), anti-IRS-2 (1 μ g/ml) or anti-PI3Kp85 (1 μ g/ml) antibody diluted in the corresponding blocking buffer. The membranes were washed three times for 10 min in Tris-buffered saline-Tween 20 buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl and 0.02% Tween 20 and were then incubated with optimal second antibodies conjugated with horseradish peroxidase (1:1000) for 1 h at room temperature. After the membranes had been washed three times for 10 min, they were exposed to X-ray film (Eastman Kodak, Rochester, NY, USA). The pictures were scanned with a scanner (GT-6500; Epson, Tokyo, Japan), and the relative intensity of each band was analyzed using NIH imaging software (NIH, Bethesda, MD, USA).

P13-kinase assay

The P13-kinase assay was performed essentially as described by Carvalho *et al.* (1996). Liver extracts

containing 5 mg total protein were incubated at 4 °C overnight with anti-IRS-1 or anti-IRS-2 antibody. Immunocomplexes were collected by centrifugation following the addition of 20 μ l of a 50% (v/v) slurry of protein A-Sepharose in homogenization buffer and further incubation for 2 h at 4 °C. The immunoprecipitates were washed with ice-cold homogenization buffer A, 100 mM ice-cold Tris (pH 7.4) containing 100 mM LiCl and 100 μ M Na₃VO₄, deionized water and 10 mM ice-cold Tris (pH 7.4) containing 100 mM NaCl, 1 mM EDTA and 100 μ M Na₃VO₄. The precipitates were then resuspended in 40 μ l reaction buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 0.5 mM EGTA and 10 μ g phosphatidylinositol.

The reaction was started by the addition of 10 μ l 150 μ M ATP containing 20 μ Ci [γ -³²P]ATP (6000 Ci/mmol), 5 mM Na₃VO₄, 100 mM dithiothreitol and 100 mM MgCl₂. After 60 min at 25 °C, the reaction was terminated by the addition of 100 μ l methanol-chloroform-HCl (10:20:1). Mixtures were centrifuged, and the lower organic phase was applied to silica thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) coated with 1% (w/v) potassium oxalate. The TLC plate was developed in CH₃Cl-CH₃OH-H₂O-NH₄OH (43:38:6:6), visualized by autoradiography and quantitated by optimal densitometry as described above.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. For comparisons, the data were analyzed by ANOVA followed by Student's *t*-test and the difference was considered significant at $P < 0.05$.

Results

Data on body weight, blood glucose concentration and serum insulin concentration of the animals studied are summarized in Table 1. The transgenic rats had significantly increased body weight, plasma glucose and serum insulin levels. In addition, the results of a glucose tolerance test are presented in Fig. 1. After a bolus injection of glucose, blood glucose levels were immediately increased in both transgenic and control rats. Then, however, plasma glucose concentrations of the transgenic rats decreased

Figure 1 Glucose intolerance in the transgenic rat. Glucose tolerance tests were performed on transgenic and control rats which had been fasted overnight. Animals were injected i.p. with 0.5 g/kg body weight D-glucose (20% solution). Plasma glucose was measured immediately before injection (0) and 15, 30, 60, 120 and 150 min after injection. Results are expressed as the means $\pm$ S.E.M. from three or four animals. * $P < 0.01$ vs control.

more modestly compared with those of control rats. The area under the glucose response curve in transgenic rats was 23% greater than that in control rats ($P < 0.05$). These results are indicative of the development of insulin resistance in the transgenic rat.

The amount of insulin receptors, as assessed by Western blotting (WB) of IR- β , was 49% decreased in the liver of transgenic rats compared with that of control rats (Fig. 2A). Moreover, autophosphorylation of the insulin receptors in response to exogenous insulin (1 IU/rat) was decreased by 22% in the liver from transgenic rats (Fig. 2B). To further investigate whether the alteration of the insulin receptor function in the transgenic rats affected downstream elements of the insulin signal transduction cascade, the amount and phosphorylation of IRS-1 and IRS-2 were evaluated. The amount of IRS-1 and IRS-2 in the liver of the transgenic rat was decreased by 31% and 32% respectively (Figs 3A and 4A). The basal level of IRS-1 tyrosine phosphorylation in transgenic rats was lower than that of controls (Fig. 3B). As shown in Fig. 3B, i.v. administration of insulin increased IRS-1 phosphorylation in the liver of both control and transgenic rats, but the level of IRS-1 phosphorylation in transgenic rats was 45% lower than that of control rats. Although basal IRS-2 tyrosine phosphorylation was not different between transgenic and control animals, insulin-stimulated IRS-2 tyrosine phosphorylation in transgenic rats was 16% lower than that of control rats (Fig. 4B).

For the measurement of PI3-kinase activities, extracts of liver were prepared and immunoprecipitated with anti-IRS-1 and anti-IRS-2 antibodies after the rats were

Figure 2 Amounts of insulin receptor β -subunit protein and tyrosine phosphorylation of insulin receptor in the liver of transgenic (TG) and control rats. Animals were anesthetized, and normal saline (–) or insulin (1 IU) (+) was injected into the portal vein. One minute after the injection, the liver was excised and protein was isolated. Equal aliquots of protein were immunoprecipitated (IP) with anti-insulin receptor β -subunit antibodies (α IR- β), and subsequently with protein A-Sepharose. Immunoprecipitated proteins were separated by SDS-PAGE and subjected to immunoblot analysis with the same antibody (A, upper). Quantitation of insulin receptor amount in the liver was performed by scanning densitometry, and expressed as relative to the control value that was set at 1 (A, lower). Data are means $\pm$ S.E.M. of three separate experiments. The same samples were immunoprecipitated with α IR- β and immunoblotted with anti-phosphotyrosine antibodies (α pY) (B, upper). The phosphorylation level of insulin receptor was quantified by scanning densitometry and expressed as relative values (insulin-stimulated control = 1) (B, lower). Data are means $\pm$ S.E.M. of three separate experiments. Values with different superscripts are significantly different ($P < 0.05$).

Figure 3 Amounts of insulin receptor substrate (IRS-1) protein and tyrosine phosphorylation of IRS-1 in the liver of transgenic (TG) and control rats. Animals were anesthetized, and normal saline (–) or insulin (1 IU) (+) was injected into the portal vein. One minute after the injection, the liver was excised and protein was isolated from the liver. Equal aliquots of protein were immunoprecipitated (IP) with anti-IRS-1 antibodies (α IRS-1) and subsequently with protein A–Sepharose. Immunoprecipitated proteins were separated by SDS–PAGE and subjected to immunoblot analysis with the same antibody (A, upper). Quantification of IRS-1 amount in the liver was performed by scanning densitometry and expressed as relative to the control value that was set at 1 (A, lower). Data are means $\pm$ S.E.M. of three separate experiments. The same samples were immunoprecipitated with α IRS-1 and immunoblotted with anti-phosphotyrosine antibodies (α pY) (B, upper). Phosphorylation levels of IRS-1 were quantified by scanning densitometry and expressed as relative values (insulin-stimulated control=1) (B, lower). Data are means $\pm$ S.E.M. of three separate experiments. Values with different superscripts are significantly different ($P<0.01$).

Figure 4 Amounts of IRS-2 protein and tyrosine phosphorylation of IRS-2 in the liver of transgenic (TG) and control rats. Animals were anesthetized, and normal saline (–) or insulin (1 IU) (+) was injected into the portal vein. One minute after the injection, the liver was excised and protein was isolated from the liver. Equal aliquots of protein were immunoprecipitated (IP) with anti-IRS-2 antibodies (α IRS-2) and subsequently with protein A–Sepharose. Immunoprecipitated proteins were separated by SDS–PAGE and subjected to immunoblot analysis with the same antibody (A, upper). Quantification of IRS-2 abundance in the liver was performed by scanning densitometry and expressed as relative to the control value that was set at 1 (A, lower). Data are means $\pm$ S.E.M. of three separate experiments. The same samples were immunoprecipitated with α IRS-2 and immunoblotted with anti-phosphotyrosine antibodies (α pY) (B, upper). Phosphorylation levels of IRS-2 were quantified by scanning densitometry and expressed as relative values (insulin-stimulated control=1) (B, lower). Data are means $\pm$ S.E.M. of three separate experiments. Values with different superscripts are significantly different ($P<0.01$).

Figure 5 Phosphatidylinositol 3-kinase (PI3-kinase) activity associated with IRS-1 in the liver of the transgenic (TG) and control rats. Animals were anesthetized, and normal saline (–) or insulin (1 IU) (+) was injected into the portal vein. One minute after the injection, the liver was excised and protein was isolated from the liver. Equal aliquots of protein were immunoprecipitated (IP) with anti-IRS-1 antibodies (α IRS-1) and subsequently with protein A–Sepharose. PI3-kinase was assayed in the immunoprecipitates in the presence of phosphatidylinositol and [γ – 32 P]ATP. The resulting labeled lipids were extracted and separated by TLC (upper). Quantification of PI3-kinase activity associated with IRS-1 in the liver was performed by scanning densitometry and expressed as relative to the control value that was set at 1 (lower). Data are means $\pm$ S.E.M. of three separate experiments. Values with different superscripts are significantly different ($P < 0.01$). PIP, phosphatidylinositol.

injected with insulin or saline. There was no significant difference in basal hepatic PI3-kinase activity associated with IRS-1 and IRS-2 between transgenic and control rats. Although insulin markedly stimulated hepatic PI3-kinase activity in control rats, it did not affect it in the transgenic rats. Insulin-stimulated PI3-kinase activity associated with IRS-1 and IRS-2 in transgenic rats was smaller by 62% and 60% respectively than that in the control rats (Figs 5 and 6). To determine whether the binding levels of the PI3-kinase regulatory subunit, p85 subunit, to the phosphotyrosine in IRSs were altered in the transgenic animals, the amount of p85 subunit in the immunoprecipitates by anti-pY antibody was analyzed by immunoblotting. The levels of p85 subunit associated with phosphotyrosine in the transgenic rats were not significantly different from the levels in control rats. Insulin

Figure 6 PI3-kinase activity associated with IRS-2 in the liver of transgenic (TG) and control rats. Animals were anesthetized, and normal saline (–) or insulin (1 IU) (+) was injected into the portal vein. One minute after the injection, the liver was excised and protein was isolated from the liver. Equal aliquots of protein were immunoprecipitated (IP) with anti-IRS-2 antibodies (α IRS-2) and subsequently with protein A–Sepharose. PI3-kinase was assayed in the immunoprecipitates in the presence of phosphatidylinositol and [γ – 32 P]ATP. The resulting labeled lipids were extracted and separated by TLC (upper). Quantification of PI3-kinase activity associated with IRS-2 in the liver was performed by scanning densitometry and expressed as relative to the control value that was set at 1 (lower). Data are means $\pm$ S.E.M. of three separate experiments. Values with different superscripts are significantly different ($P < 0.01$). PIP, phosphatidylinositol.

significantly increased p85 subunit associated with phosphotyrosine in the controls but not in the transgenic rats (Fig. 7). The amount of p85 subunit in the immunoprecipitates in transgenic rats was 40% smaller than that in control rats.

Discussion

The transgenic rat with low circulating GH levels used in the present study exhibited impaired glucose tolerance as well as increased plasma glucose and insulin levels. These results suggest that insulin resistance took place in the transgenic rat, supporting our previous report (Ikeda *et al.* 1998). Although many researchers have reported that

IP: α pY
WB: α PI3K-p85

Figure 7 Amount of PI3-kinase p85 subunit (phosphotyrosine immunoprecipitable) in the liver of transgenic (TG) and control rats. Animals were anesthetized, and normal saline (–) or insulin (1 IU) (+) was injected into the portal vein. One minute after the injection, the liver was excised and protein was isolated from the liver as described in Materials and Methods. Equal aliquots of protein were immunoprecipitated (IP) with anti-phosphotyrosine antibodies (α pY) and subsequently with protein A–Sepharose. Immunoprecipitated proteins were separated by SDS-PAGE and subjected to immunoblot analysis with PI3-kinase p85 subunit antibody (α PI3K-p85) (upper). Quantification of p85 subunit in the immunoprecipitates in the liver was performed by scanning densitometry, and expressed as relative to the control value that was set at 1 (lower). Data are means $\pm$ S.E.M. of three separate experiments. Values with different superscripts are significantly different ($P < 0.01$).

GHD subjects tend to have increased body fat mass (de Boer *et al.* 1995), controversy exists as to whether GHD adults are indeed insulin sensitive (Merimee *et al.* 1971, Wolfsdorf *et al.* 1983) or insulin resistant (Cuneo *et al.* 1992, Johansson *et al.* 1995, Hew *et al.* 1996). Our transgenic rats indicate that GH deficiency can cause insulin resistance, at least under specific conditions.

Insulin signaling is initiated by the activation of insulin receptor tyrosine kinase, leading to the phosphorylation of intracellular receptor substrates, including IRS-1 and -2 (Sun *et al.* 1993, 1995), and the activation of PI3-kinase associated with IRS-1 and -2. To understand the mechanism of insulin resistance observed in the transgenic rat, it is important to analyze these earlier events in insulin signaling. Thus, in the present study, we first measured the amount of insulin receptor in the liver of transgenic rats by WB and found that transgenic rats had decreased insulin receptor levels. It has been reported that insulin receptor was decreased when circulating insulin levels were increased (Gavin *et al.* 1974, Kahn *et al.* 1978, Vigneri

et al. 1978, Almira & Reddy 1979). In addition, it has been reported that GH does not directly affect insulin receptor levels in both *in vivo* (Dominici *et al.* 1998) and *in vitro* (Lesniak & Roth 1979, Maloff *et al.* 1980) experiments. Since elevated circulating insulin levels were observed after 12 weeks of age in transgenic rats (Ikeda *et al.* 1998), decreased insulin receptor levels may result from hyperinsulinemia rather than from low GH levels. Insulin-stimulated phosphorylation of insulin receptor was also proportionally suppressed in transgenic rats, indicating that insulin resistance in the liver is initiated at the receptor level.

The amount of both IRS-1 and -2 protein in the liver of transgenic rats was decreased compared with that of controls. It has been reported that IRS-1 protein was moderately increased in the liver of streptozotocin-treated rats, in which insulin was virtually absent (Saad *et al.* 1992). Conversely, hyperinsulinemic *ob/ob* mice and Zucker rats showed reduced IRS-1 (Saad *et al.* 1992), and both IRS-1 and -2 (Anai *et al.* 1998) proteins in the liver respectively. These findings suggest that IRS-1 and -2 levels are inversely related to circulating insulin levels. Accordingly, decreased IRS-1 and -2 in the liver may be related to hyperinsulinemia in transgenic rats. However, since transgenic mice overexpressing GH display normal concentrations of IRS-1 in the liver despite hyperinsulinemia (Dominici *et al.* 1999), the possibility that other factors, such as changes in the concentrations of glucose or counter-regulatory hormones (including GH), are involved in the regulation of IRS levels cannot be ruled out.

The tyrosine phosphorylation of IRS-1 in the basal state of the transgenic rat was significantly decreased compared with that of controls. This may be secondary to the decreased IRS-1 protein level. However, although the amount of IRS-2 was also similarly decreased, no significant change in basal tyrosine phosphorylation levels of IRS-2 was observed between transgenic and control rats. This suggests the existence of different regulatory mechanisms in basal tyrosine phosphorylation levels of the IRS-1 and -2 in the liver. GH has been shown to stimulate tyrosine phosphorylation of IRS-1 *in vivo* (Yamauchi *et al.* 1998, Thirone *et al.* 1999). In addition, it has been reported that GH transgenic mice show an increased basal tyrosine phosphorylation of IRS-1, which might be one of the causes of insulin resistance in those mice (Dominici *et al.* 1999). Thus, impaired GH level in the transgenic rats may affect basal tyrosine phosphorylation of IRS-1 in the liver of those rats.

Recent evidence has indicated that PI3-kinase is a key enzyme in the insulin-signaling pathway for glucose metabolism. PI3-kinase activation might be essential for insulin-stimulated glucose transport (Kanai *et al.* 1993, Cheatham *et al.* 1994, Okada *et al.* 1994), and PI3-kinase inhibitor has been shown to block insulin-stimulated glucose transport and GLUT4 translocation in rats and

3T3-L1 adipocytes (Clarke *et al.* 1994, Sanchez-Margalet *et al.* 1994). In hepatocytes, PI3-kinase also plays an important role in glycogen synthesis via c-akt activation (Sakaue *et al.* 1995, Shepherd *et al.* 1995). In addition, insulin suppresses the gene expression of PEPCK, the rate-limiting enzyme of gluconeogenesis in the liver, via PI3-kinase activation (Sutherland *et al.* 1995, Gabbay *et al.* 1996). These findings suggest that PI3-kinase is essential for insulin-stimulated glucose metabolism in the liver. Impaired PI3-kinase activation has been demonstrated in various insulin-resistant diabetic animal models, such as *ob/ob* mice (Folli *et al.* 1993) and Zucker rats (Anai *et al.* 1998). In humans, it has been reported that the activation of PI3-kinase in the skeletal muscle of obese subjects is markedly suppressed as compared with that of control subjects (Goodyear *et al.* 1995). In the present study, although insulin-stimulated tyrosine phosphorylation of IRS-1 and -2 in transgenic rats was only partially impaired as compared with controls, the insulin effect on PI3-kinase activity associated with IRS-1 and -2 was not discernible in transgenic rats. The discrepancy between tyrosine phosphorylation of IRSs and PI3-kinase activation associated with IRSs was also reported in Zucker rats (Anai *et al.* 1998). This phenomenon observed in both our transgenic and Zucker rats might be common among obese early stage NIDDM models.

The association of IRS-1 and -2 with PI3-kinase occurs through the phosphorylated tyrosine residue on IRS-1 and -2 and SH2 domains on the regulatory subunit of PI3-kinase (Backer *et al.* 1992). To elucidate whether this discrepancy results from alterations in the binding of IRSs and the regulatory subunit of PI3-kinase, we detected the p85 subunit of PI3-kinase in the immunoprecipitates with anti-phosphotyrosine antibody by WB. We found that insulin did not stimulate the binding of p85 subunit to tyrosine-phosphorylated proteins in transgenic rats at all. This suggests that, although the insulin-signaling pathway is impaired at multiple steps in transgenic rats, insulin resistance mainly takes place at the level of binding of the p85 subunit to the phosphotyrosine of IRSs. For PI3-kinase activation, binding of the SH2 domain of the p85 subunit of PI3-kinase to phosphorylated YMXM or YXXM motifs is necessary (Backer *et al.* 1992). Although IRS-1 and -2 contain over 20 potential tyrosine phosphorylation sites (Sun *et al.* 1993, 1995), phosphorylation of tyrosine residues different from the YMXM and YXXM motifs would not contribute to the activation of PI3-kinase. If this is the case, a selective decrease in phosphorylation of the tyrosine residues in the YMXM or YXXM motifs of IRS-1 and -2 may occur in the liver of transgenic rats.

Serine/threonine phosphorylations in IRS proteins may also be involved in impaired insulin signaling in transgenic rats. It has been demonstrated that IRS proteins contain more than 70 potential serine/threonine phosphorylation sites (Sun *et al.* 1993, 1995), and that serine/threonine

phosphorylation of IRS-1 impairs the ability of IRS-1 to associate with the insulin receptor, which inhibits subsequent insulin-stimulated tyrosine phosphorylation (Paz *et al.* 1997). In addition, it has also been reported that serine/threonine phosphorylation in IRS-1 is increased in subjects with insulin resistance and NIDDM (Virkamaki *et al.* 1999), and that cytokines, which induce insulin resistance, promote serine phosphorylation of IRS-1 (Kanety *et al.* 1995, Hotamisligil *et al.* 1996, Peraldi *et al.* 1996). Taken together, serine/threonine phosphorylation in IRS proteins seems to play an important role in the development of insulin resistance.

In conclusion, insulin resistance in transgenic rats with low circulating GH levels is characterized by impaired PI3-kinase activation by insulin in the liver due to decreased association of the p85 subunit with phosphotyrosine residues, although decreases in insulin-stimulated tyrosine phosphorylation of insulin receptor and IRS-1 and -2, as well as decreases in the amount of these proteins also take place. How GH deficiency induces the blockade of the p85 subunit binding to IRSs remains to be elucidated.

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