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The Novel Roles of Liver for Compensation of Insulin Resistance in Human Growth Hormone Transgenic Rats

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Chronic excess of GH is known to cause hyperinsulinemia and insulin resistance. We developed human GH transgenic (TG) rats, which were characterized by high plasma levels of human GH and IGF-I. These TG rats showed higher levels of plasma insulin, compared with control littermates, whereas plasma glucose concentrations were normal. Insulin-dependent glucose uptake into adipocytes and muscle was impaired, suggesting that these rats developed insulin resistance. In contrast, insulin-independent glucose uptake into hepatocytes from TG rats was significantly increased, and glycogen and lipid levels in livers of TG rats were remarkably high. Because the role of liver in GH-induced insulin resistance is poorly understood, we studied insulin signaling at early stages and insulin action in liver and primary cultures of hepatocytes prepared from TG rats. There was no difference in insulin receptor kinase activity induced by insulin between TG and control rats; however, insulin-dependent insulin receptor substrate-2 tyrosine phosphorylation, glycogen synthase activation, and expression of enzymes that induce lipid synthesis were potentiated in hepatocytes of TG rats. These results suggest that impairment of insulin-dependent glucose uptake by GH excess in adipose tissue and muscle is compensated by up-regulation of glucose uptake in liver and that potentiation of insulin signaling through insulin receptor substrate-2 in liver experiencing GH excess causes an increase in glycogen and lipid synthesis from incorporated glucose, resulting in accumulation of glycogen and lipids in liver. This novel mechanism explains normalization of plasma glucose levels at least in part in a GH excess model. (*Endocrinology* 147: 5374–5384, 2006)

GH POSSESSES BIOLOGICAL properties for regulating growth and metabolism. GH is known to mediate antiinsulin effects on glucose and lipid metabolism in various tissues (1). Insulin resistance caused by GH is often observed in acromegalic patients, and about half of these patients are diagnosed with diabetes mellitus (2). In addition, chronic GH treatment of GH-deficient patients in physiological or pharmacological doses is also reported to cause diabetes mellitus (3). These results suggest that GH affects the insulin signaling pathway in target tissues, resulting in impairment of insulin action.

Insulin signaling is initiated by the binding of insulin to the α-subunit of insulin receptor (4). This binding leads to tyrosine kinase activation of the insulin receptor β-subunit, followed by its autophosphorylation (5). The activated receptor kinase in turn phosphorylates receptor kinase substrates, including insulin receptor substrate (IRS)-1 (6) and IRS-2 (4, 7, 8). Tyrosine-phosphorylated IRSs are recognized by various signaling molecules that contain a Src-homology region-2 domain, such as the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). This interaction induces PI 3-kinase activation (9), and it has recently been shown that activation of the PI 3-kinase cascade is essential to mediate a variety of insulin actions, especially regulation of glucose and lipid metabolism (9, 10).

To understand the growth-promoting and metabolic effects of GH, several investigators used GH-overexpressing animals (11–16). Parlmiter and colleagues (17) first developed metallothionein promoter-GH transgenic mice that exhibited hyperinsulinemia. Dominici *et al.* (15) reported that transgenic mice overexpressing bovine GH in liver displayed an acromegalic phenotype and loss of sensitivity to early events in the insulin signaling pathway in liver. Furthermore, in skeletal muscle of bovine GH-transgenic mice, insulin-dependent insulin receptor tyrosine kinase activation was reduced, as was the efficiency of IRS-1 tyrosine phosphorylation, resulting in defective activation of PI 3-kinase induced by insulin. Thus, GH seems to have negative effects on insulin signaling at the receptor substrate level, leading to down-regulation of insulin action.

We developed a line of transgenic (TG) rats carrying a human GH gene (18). At 10–14 wk of age, hyperinsulinemia is observed, whereas plasma glucose levels are normal in these TG rats. The same phenotypes have been reported in some acromegalic patients and GH excess animal models (12, 13, 19). Using these animals, we first confirmed that insulin

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Abbreviations: ACC, Acetyl-CoA carboxylase; BW, body weight; DEX, dexamethasone; FAS, fatty acid synthase; GK, glucokinase; G6P, glucose 6-phosphate; GSI, glycogen synthase activity index; hGH, human GH; IR, insulin receptor; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; TG, transgenic rats; WE, Williams' E medium; WGA, wheat germ agglutinin.

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insensitivity is present in peripheral tissues, such as adipose tissue and muscle. However, we found that insulin signaling is enhanced in TG liver, resulting in enhancement of glycogen and lipid syntheses induced by insulin. The data suggested that this is a novel mechanism in liver that plays an important role in compensating for insulin resistance in adipose tissue and muscle by increasing use of glucose.

Materials and Methods

Chemicals

Insulin was from Novo-Nordisk (Bagsvaerd, Denmark). Anti-IRS-1 antibody and anti-IRS-2 antibody for immunoprecipitation were raised according to reported previously reported methods (20). Antirat IRS-1 antibody and anti-IRS-2 (M-19) antibody for immunoblotting were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Antiinsulin receptor (IR) β-subunit antibody (C-19) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and antiphosphotyrosine antibody, 4G10, was from Sigma (St. Louis, MO). Wheat germ agglutinin (WGA)-agarose was from Seikagaku Co. (Tokyo, Japan). Other chemicals were of reagent grade and were available commercially.

Animals

TG rats in this work were derived from animals that were originally produced by microinjection of the coding region of human GH (hGH) gene fused to the promoter region of the mouse whey acidic protein into pronuclei of fertilized rat eggs harvested from naturally mated female rats (21). These TG male rats and control male littermates were used for each experiment in this study. These rats were killed by decapitation between 10 and 14 wk of age, and plasma insulin levels were then measured with rat insulin enzyme immunoassay system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK). Plasma IGF-I levels were measured as described (22). Plasma hGH levels were measured using a hGH ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany). Blood glucose level was measured with ANTSENSE (DAIKIN, Osaka, Japan). All procedures for animal research in this study were approved by the Committee on Laboratory Animal Care, Graduate School of Agriculture and Life Sciences, The University of Tokyo.

hGH gene expression

Ten-wk-old male TG rats were killed in nonfasting state. Total RNA was isolated from frozen tissues by the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Concentrations of RNA were measured spectrophotometrically at 260 nm. First-strand cDNA was synthesized from 100 ng of total RNA with oligo-dT primers using the SuperScript2 RT-PCR kit (Invitrogen). To determine tissue distribution of hGH transgene mRNA, first-strand cDNA was subjected to PCR (35 cycles of sequential incubations at 94 C for 60 sec, 54 C for 60 sec, and 72 C for 60 sec) and final extension at 72 C for 10 min. A sense primer located in exon 2, bases 355–375 (5′-CCTTCCCAACCATTC-CCTTA-3′), and an antisense primer located in exon 5, bases 1416–1436 (5′-TGTCTCGACCTTGTCCATGT-3′), specific for the hGH gene were used. The expected size of the fragment amplified from spliced mRNA was 626 bp. β-Actin was used as the internal control: 5′-ACCCACACT-GTGCCCATCTA-3′ (sense); 5′-CGGAACCGCTCATTGCC-3′ (antisense), GenBank accession no. NM_031144. The expected size of the amplified fragment was 289 bp. The PCR products were analyzed by electrophoresis in 2% agarose gel.

Glucose and insulin tolerance tests

For glucose tolerance test, TG and control rats at 10 wk of age were fasted overnight and received an ip injection of 1 g/kg body weight (BW) D-glucose (20% solution). Blood samples were obtained from the tail vain without anesthesia, and the glucose and insulin concentrations were determined before (0 min) and 15, 30, 60, 90, 120, and 150 min after ip injection of glucose. Insulin tolerance test was performed using random-fed, 10-wk-old TG or control rats. Animals were injected ip with 0.5 U/kg BW of insulin. Blood glucose was measured before injection (0 time) and 30, 60, 90, and 120 min after ip injection of insulin. Results are expressed as mean percent of initial blood glucose concentration ± SEM from four rats.

Isolation and culture of adipocytes and hepatocytes

Adipocytes were obtained from the epididymal fat pad of TG or control rats that were fasted overnight and then incubated at 37 C in HEPES/Krebs-Ringer buffer (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.6 mM $NaHCO_3$, 30 mM HEPES) containing 10 mM D-glucose for about 3 h (23). Parenchymal hepatocytes were isolated from TG or control rats 10–14 wk of age by perfusion of the liver *in situ* with collagenase as previously described (24). Cells were suspended in 10% fetal bovine serum/Williams' E medium (WE) and cultured at a density of 1×10^5 cells/cm^2 in 35-mm collagen-coated plastic dishes at 37 C under 5% CO_2 in air. After about 3 h, the medium was changed to fresh culture medium and cultured for an additional 20 h. After culture for 24 h in 10% fetal bovine serum/WE, cells were washed twice in Hanks' balanced salt solution and starved in 0.1% BSA/WE for about 16 h to be quiescent.

Glucose uptake into adipocytes or hepatocytes

Isolated adipocytes were treated without or with insulin (10^{-7} M) for 20 min at 37 C, and 2-deoxy-D-[^{3}H]glucose (1.5 mCi/ml) was then added to initiate the glucose transport reaction. Three minutes later, glucose uptake was stopped by adding 1 mM cytochalasin B in ice-cold HEPES/Krebs-Ringer buffer, and cells were then washed in ice-cold PBS (−) three times. Cells were digested in 5 ml of liquid scintillation cocktails and counted to determine the level of incorporated 2-deoxy-D-[^{3}H]glucose into adipocytes. Isolated hepatocytes were treated with insulin (10^{-7} M) for 30 min, after which time 2-deoxy-D-[^{3}H]glucose (1.5 mCi/ml) was added. After incubation for 3 min, the reaction was stopped by adding 0.1 mM cytochalasin B on ice and cells were washed in ice-cold PBS (−) three times and digested in 0.05 M NaOH. The radioactivity of incorporation into hepatocytes for 3 min was then determined by liquid scintillation count. In all experiments, each experimental point represents the mean of three replicate dishes.

Glucose uptake into soleus muscles

This assay was performed according to previously reported methods (25).

Histology

Livers excised from TG or control rats were immediately frozen with optimal cutting temperature compound (Sakura, Tokyo, Japan) in ice-cold hexane. Sections (6 μm) of livers were prepared using an AS620 cryotome (Life Sciences International Ltd., Cheshire, UK). After fixation in fresh 4% paraformaldehyde in PBS (pH 7.4), sections were stained for nuclear, glycogen, and lipids with hematoxylin (Mayer's hematoxylin solution; Wako, Tokyo, Japan), periodic acid-Schiff reagent and oil red O, respectively. Each staining was performed using standard protocols.

Administration of insulin to rats

Control and TG rats were starved 24 h before the experiments. Under pentobarbital anesthesia (50 mg pentobarbital per kilogram BW), the abdominal cavity was opened and insulin (2 U/kg BW) or PBS was injected into the portal vein. Liver was excised 2 min after injection, immediately frozen in liquid nitrogen, and stored at −80 C until analysis. Liver samples were homogenized in a 10× volume of lysis buffer [20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM Na_3VO_4, 5 mM EDTA, 1% Triton X-100, 1 μl/ml protease inhibitor cocktail (Sigma), 20 μg/ml phenylmethanesulfonyl fluoride, 10 mg/ml *p*-nitrophenyl phosphate] with polytron operated at maximum speed for 1 min. The extracts were centrifuged at 15,000 × *g* at 4 C for 20 min to remove insoluble material, and the supernatants were used as samples for immunoprecipitation or immunoblotting. The supernatant was diluted with homogenization buffer to 3 mg protein per milliliter as a final concentration. The protein assay was carried out using protein assay kit (Bio-Rad, Hercules, CA).

TABLE 1. Sequence of sense and antisense primers

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	GenBank accession no.
RPS29	TGAAGGCAAGATGGGTCACCAGCAGC	CAGGGTAGACAGTTGGTTTCATTGGG	X59051
FAS	CCGTTGCCACAATTAAACTGCACG	CACGCTGAACGTGAATCAGCACACT	NM 017332
ACC	TTTGTTCCCACAAAGGCCCCGTAT	TGAGCTCCACGGTTCGGGTTTCTA	J03308
GK	TGGTGCTTTTGAGACCCGTTTCGT	CTTTCGCGCATGCGATTTATGACC	NM 012565

Treatment of hepatocytes with insulin

Quiescent hepatocytes from TG or control rats were stimulated without or with insulin (10^{-7} M) for 1 min. After stimulation, cells were harvested in lysis buffer. The extracts were centrifuged at 15,000 × *g* at 4 C for 20 min to remove insoluble material, and the supernatants were used as samples for immunoprecipitation or immunoblotting. The supernatant was diluted with lysis buffer to 1 mg protein per milliliter as a final concentration.

Amounts of insulin receptor and IRSs

Immunoblotting of the liver protein extracts and cell lysates using anti-IR β-subunit antibody (1:500), anti-IRS-1 antibody (1:1000), or anti-IRS-2 antibody (1:1000) were performed as described previously (26). All results of immunoblotting were quantitated using National Institutes of Health (NIH) Image computer program (version 1.61),and each experimental point represents the mean of three replicate dishes.

Autophosphorylation and tyrosine kinase activity of IR

Autophosphorylation of insulin receptor in WGA-adsorbed fraction prepared from liver extracts and cell lysates, and tyrosine kinase activity of IR in WGA-adsorbed fraction prepared from cell lysates were assayed according to methods described previously (27). In all experiments of tyrosine kinase activity assay, each experimental point represents the mean of three replicate dishes.

Tyrosine phosphorylation of IRSs

Immunoprecipitation of IRS-1 or IRS-2 followed by immunoblotting using antiphosphotyrosine or IRS-1 or IRS-2 antibody was performed according to methods described previously (24). Each experimental point represents the mean of three replicate dishes.

Amounts of p85 PI 3-kinase and activity of PI 3-kinase bound to IRSs

Amounts of p85 PI 3-kinase and their activities in the immunoprecipitates by anti-IRS-1 or IRS-2 antibody were analyzed according to previously reported methods (26). Each experimental point represents the mean of three replicate dishes.

Glycogen synthase activity

To start the reaction, extracts of hepatocytes from TG or control rats, which were stimulated by insulin for 0 or 20 min, were mixed with uridine diphosphate glucose-[^{14}C]glucose in the presence or absence of glucose 6-phosphate (G6P). Glycogen synthase activity index (GSI) was measured and calculated using the ratio of glycogen activity in the absence of G6P to that glycogen activity in the presence of G6P, as described previously (28).

mRNAs levels of enzymes to induce lipid synthesis

Quiescent hepatocytes from TG or control rats were treated without or with insulin (10^{-7} M) in the presence of dexamethasone (DEX). After 24 h incubation, total cellular RNA was isolated as described above. First-strand cDNA was synthesized from 2 μg total RNA with oligo-dT primers using the SuperScript2 RT-PCR kit (Invitrogen). Specific primers for each gene (Table 1) were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The real-time PCR contained, in a final volume of 20 μl, 50 ng reverse transcribed total RNA. PCR was carried out in microcapillary tubes using the LightCycler system (Roche). To compare transcript expression levels between RNA isolated from hepatocytes treated under different conditions, a dilution series of cDNA from hepatocytes of nonadditive control was made and assayed in each LightCycler run as a standard curve. RPS29 mRNA was used as the invariant control.

Statistical analysis

Results are expressed as means ± SEM. 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

Characteristics of hGH TG rats

mRNA of the hGH transgene was detected by RT-PCR in most of the organs of TG rats; however, we could not detect it in liver and primary cultured hepatocytes (Fig. 1). As shown in Table 2, BW and length of TG rats were significantly increased at 10 wk of age. In TG rats, IGF-I levels were 2.4-fold higher than that of control rats, in which growth was enhanced. Although 5-fold higher concentration of insulin was observed, plasma glucose level was noted to be the same as control (Fig. 2, A and B). Intraperitoneal glucose tolerance test results were also normal as were the controls (Fig. 2C). However, insulin tolerance testing demonstrated that TG rats were significantly resistant to the blood lowering effects of exogenously administered insulin (Fig. 2D). These results suggest that TG rats developed hyperinsulinemia but not hyperglycemia.

Glucose uptake into adipose tissue, muscle, and liver

Insulin-induced glucose uptake in adipocytes, skeletal muscles, and isolated hepatocytes prepared from TG and control rats were measured. In adipocytes, insulin-induced glucose uptake in TG rats was completely inhibited when compared with controls (Fig. 3A). We then measured glucose uptake in soleus muscle, which normally has higher insulin sensitivity, compared with the extensor digitorum longus

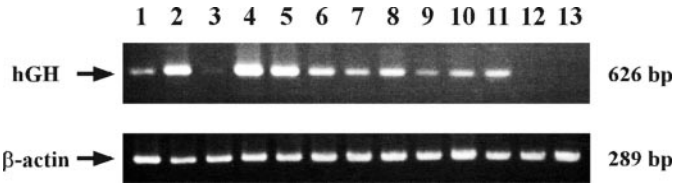

FIG. 1. Expression of hGH gene in TG rats. Total RNA was extracted from tissues of transgenic rats at 10 wk of age. A panel of organs was analyzed using RT-PCR as described in *Materials and Methods*. Lane 1, prostate; lane 2, lung; lane 3, testis; lane 4, epididymal fat; lane 5, mammary gland; lane 6, skeletal muscle; lane 7, brain; lane 8, tongue; lane 9, spleen; lane 10, heart; lane 11, kidney; lane 12, liver; lane 13, primary cultured hepatocytes.

TABLE 2. Body size and plasma concentrations of GH and IGF-I

	Control	TG
BW (g)	360 ± 15	540 ± 5[a]
Nose-to-tail length (cm)	42.6 ± 0.2	47.2 ± 0.2[a]
Plasma hGH (ng/ml)		218 ± 32
Plasma IGF-I (ng/ml)	947 ± 141	2298 ± 279[a]

Values are the mean ± SEM.
[a] Significantly different ($P < 0.05$) from control rats.

muscle (25). In soleus muscle of control, glucose uptake was increased about 1.8-fold by insulin treatment; however, in muscle of TG rats, glucose uptake was unchanged after insulin administration (Fig. 3B). These data demonstrate that adipose tissue and soleus muscle in TG rats developed insulin resistance. On the contrary, in hepatocytes, basal glucose uptake was increased more than 1.5-fold, compared with control (Fig. 3C). Therefore, glucose uptake into hepatocytes was less sensitive to insulin, a result that is consistent with previous reports (29, 30).

Glycogen and lipid accumulation in liver of TG rats

To investigate metabolism of increased glucose incorporated into liver from TG rats, we analyzed the amount of glycogen and lipid in liver sections from TG and control rats in a normal feeding state. In liver from control rats, glycogen was not accumulated abundantly; however, in liver from TG rats, glycogen granules were increased surrounding the portal and central veins, compared with controls (Fig. 3D, *upper panel*). Our results suggested that more glycogen is produced and stored in the liver of TG rats than controls. Additionally, in the liver, lipids were especially increased in parenchymal cells around the portal vein (Fig. 3D, *lower panel*). In the liver of TG rats, overall lipid levels were visibly increased and size of lipid droplets was much larger, compared with controls. These results suggest that more glycogen and lipid are synthesized and stored in the liver of TG rats than controls and indicate that insulin signaling is potentiated in TG hepatocytes.

Insulin signaling in liver of TG rats

To analyze insulin signaling at an early stage in liver, control and TG rats were injected with insulin. WGA-adsorbed fraction prepared from liver extracts was subjected to immunoblot analysis with antibodies against phosphotyrosine or IR β-subunit (Fig. 4A). In TG liver, insulin-induced IR autophosphorylation was not significantly different, compared with control. Then immunoprecipitates by anti-IRS-1 or IRS-2 antibody were subjected to immunoblot analysis with antibodies against phosphotyrosine, IRS-1, or IRS-2. We found that insulin-induced IRS-1 phosphorylation was decreased, but IRS-2 phosphorylation was significantly increased in TG liver (Fig. 4, B and C). Because IRS-2 was reported to play important roles in insulin actions including glucose homeostasis in liver (31–34) and it became clear that IRS-2 tyrosine phosphorylation induced by insulin is enhanced in TG liver, we further studied insulin signaling and insulin action using primary cultured hepatocytes from control and TG rats.

Autophosphorylation and kinase activity of IR in hepatocytes prepared from TG rats

To examine intracellular signaling, control or TG hepatocytes were treated without or with insulin. First, we analyzed the autophosphorylation, tyrosine kinase activity, and levels of IR. WGA-adsorbed fraction, prepared from cell extracts of TG or control hepatocytes, was subjected to immunoblot analysis with antibodies against phosphotyrosine or IR β-subunit and tyrosine kinase activity assay. In hepatocytes, autophosphorylation after insulin treatment was not significantly different from control (Fig. 5A). Tyrosine kinase activity was slightly lower but not significantly

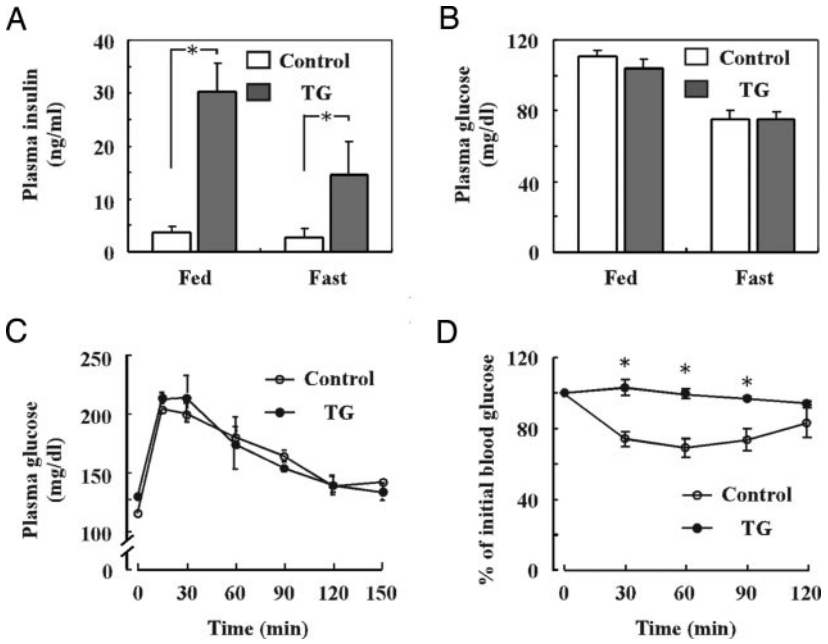

FIG. 2. Plasma insulin and glucose concentrations, and intraperitoneal glucose and insulin tolerance test in control and TG rats. A and B, Plasma insulin and glucose concentrations of TG or control rats were measured (fed or fasted overnight). C, Plasma glucose concentrations were measured immediately before and 15, 30, 60, 90, 120, and 150 min after ip injection of glucose (1 g/kg BW) at 10 wk of age in control or TG rats. Results are expressed as mean blood glucose concentration ± SEM from four rats. D, Plasma glucose concentrations were measured before and 30, 60, 90, and 120 min after ip injection of insulin (0.5 U/kg BW) at 10 wk of age in control or TG rats. Results are expressed as mean percent of basal blood glucose concentration ± SEM from four male rats. *, Statistical difference by Student's t test (n = 4). These experiments were repeated three times with similar results.

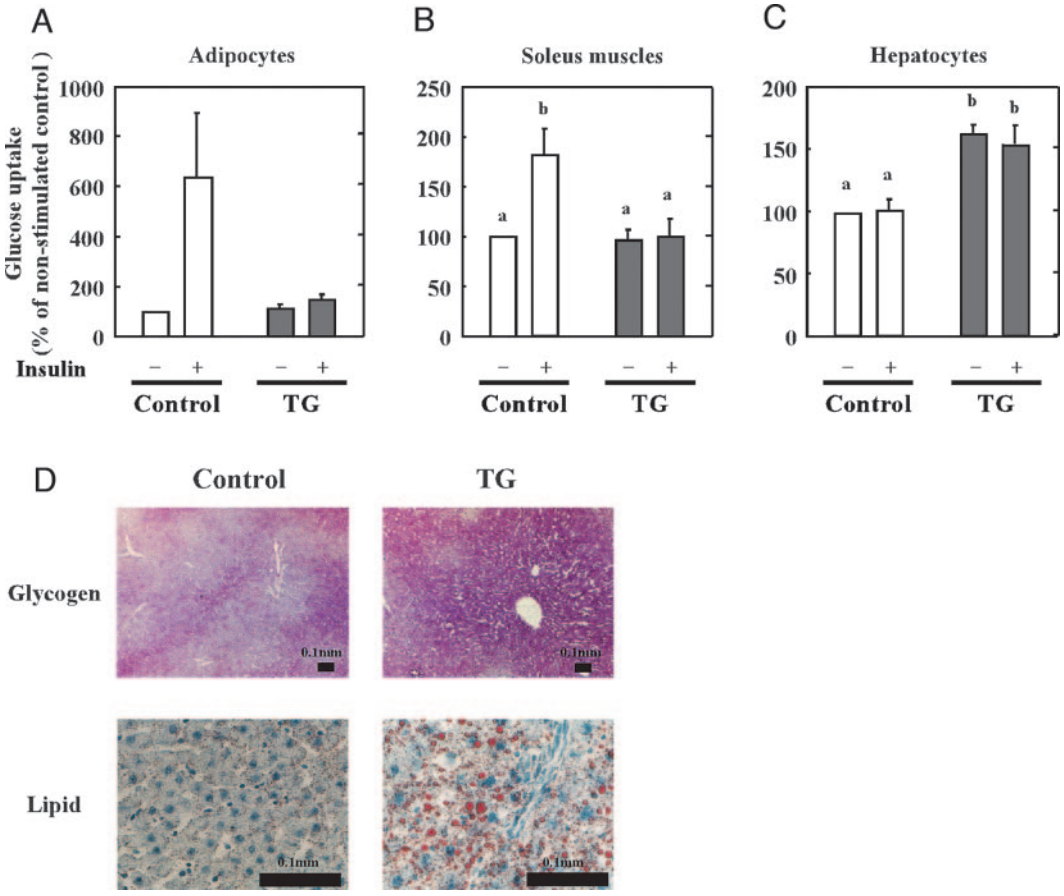

FIG. 3. Insulin-stimulated glucose uptake in adipocytes, soleus muscles, and isolated hepatocytes and accumulation of glycogen and lipids in liver from control and TG rats. A–C, Glucose uptake was determined in isolated adipocytes (A), soleus muscles (B), and isolated hepatocytes (C) by measuring incorporation of 2-deoxy-[^{3}H]glucose into each cell. These cells were treated with 10^{-7} M insulin. Values are the mean ± SEM of four different experiments and expressed as relative to nonstimulated control. *Bars with different superscript letters* are significantly different at $P < 0.05$. D, *Upper panel*, The liver sections from feeding, 12-month-old male control (*left panel*) and TG (*right panel*) rats were stained with periodic acid-Schiff reagent. Magnification, ×4. *Lower panel*, Oil red-O staining of liver sections from feeding, 12-month-old male control (*left panel*) and TG (*right panel*) rats. Magnification, ×10.

lower, compared with control hepatocytes (Fig. 5B). As shown in Fig. 5C, we also found no significant differences in the amounts of IR in TG hepatocytes, compared with control hepatocytes.

Tyrosine phosphorylation of IRSs in hepatocytes prepared from TG rats

We next examined the tyrosine phosphorylation of IRS-1 and IRS-2 induced by insulin in control and TG hepatocytes. Immunoprecipitates by anti-IRS-1 or IRS-2 antibody were subjected to immunoblot analysis with antiphosphotyrosine antibody. Tyrosine phosphorylation of IRS-1 increased after insulin stimulation, although there was not a significant difference between control and TG rats (Fig. 6A). In the case of IRS-2, tyrosine phosphorylation in response to insulin was clearly increased by 2-fold in TG hepatocytes (Fig. 7A). However, under these conditions the levels of these proteins did not differ when analyzed by immunoblotting with antibodies against IRS-1 and IRS-2 (Figs. 6B and 7B).

PI 3-kinase activity bound to IRSs in hepatocytes prepared from TG rats

We also tested whether GH excess affected binding of tyrosine-phosphorylated IRS-1 or IRS-2 to a p85 regulatory subunit of PI 3-kinase, using coimmunoprecipitation with an antibody against each IRS. Results showed that the PI 3-kinase p85 subunit was associated with tyrosine-phosphorylated IRSs in response to insulin and that in hepatocytes prepared from TG rats, PI 3-kinase p85 subunit binding to IRS-2 but not IRS-1 was increased (Figs. 6C and 7C). Under these conditions, insulin-induced PI 3-kinase activity bound to IRS-1 was not changed; however, PI 3-kinase activity bound to IRS-2 was enhanced by 2-fold in hepatocytes from TG rats (Figs. 6D and 7D).

Glycogen synthase activity in hepatocytes prepared from TG rats

Measurements of glycogen synthase activity were carried out using an *in vitro* assay. The basal activity of glycogen synthase in TG hepatocytes was 1.7-fold greater than that of

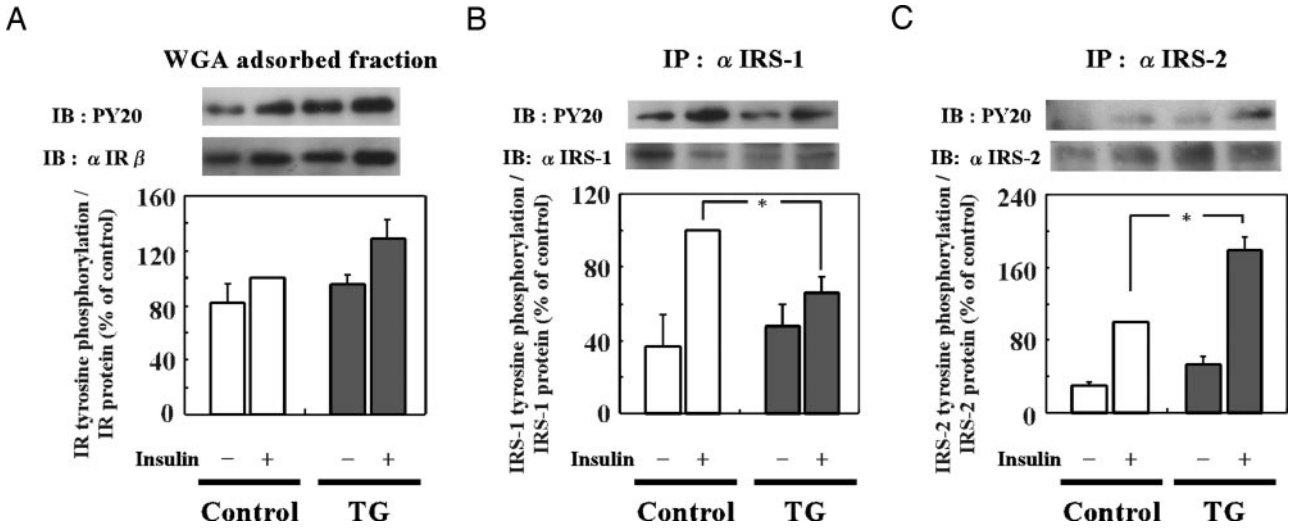

FIG. 4. Insulin signaling at an early stage in liver of control and TG rats. Overnight fasted rats were injected with insulin (2 U/kg BW) or PBS via the portal vein. Liver was excised 2 min after injection and liver extracts were prepared. A, WGA-adsorbed fraction that was prepared from liver extracts was subjected to 8% SDS-PAGE and immunoblotting (IB) with anti-IR β-subunit and antiphosphotyrosine antibody. B and C, Liver extracts were subjected to immunoprecipitation (IP) with anti-IRS-1 or anti-IRS-2 antibody, followed by 8% SDS-PAGE and IB with antiphosphotyrosine antibody, anti-IRS-1, or anti-IRS-2 antibody. The values were expressed as the mean ± SEM of three rats. *, Significant difference ($P < 0.05$) between values obtained in control and TG rats injected with insulin (unpaired t test).

control hepatocytes. Moreover, insulin-induced activation of glycogen synthase in TG hepatocytes was significantly enhanced, compared with control hepatocytes (Fig. 8A).

mRNA levels of lipogenic enzymes in hepatocytes prepared from TG rats

To compare the lipogenic response with insulin between control and TG hepatocytes, we measured the ability of insulin to induce expression of mRNA of glucokinase (GK), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) in the presence of synthetic glucocorticoid, DEX, because DEX is known to enhance insulin-induced FAS mRNA expression (35–37). As shown in Fig. 8, B–D, insulin-induced GK, ACC, and FAS gene expression in TG hepatocytes was significantly higher than in control hepatocytes.

Discussion

To date, various strains of GH transgenic animals have been developed and their growth and metabolism been carefully scrutinized (11–15). Consequently we know that these animal models produce varying phenotypes in varied species and varied tissues and hormone and metabolic indicator levels. For example, Dominici *et al.* (11) reported that bovine GH-transgenic mice had normal levels of blood glucose, despite hyperinsulinemia, whereas Costa *et al.* (12) showed that TG rabbits expressing the bovine GH gene (in liver and

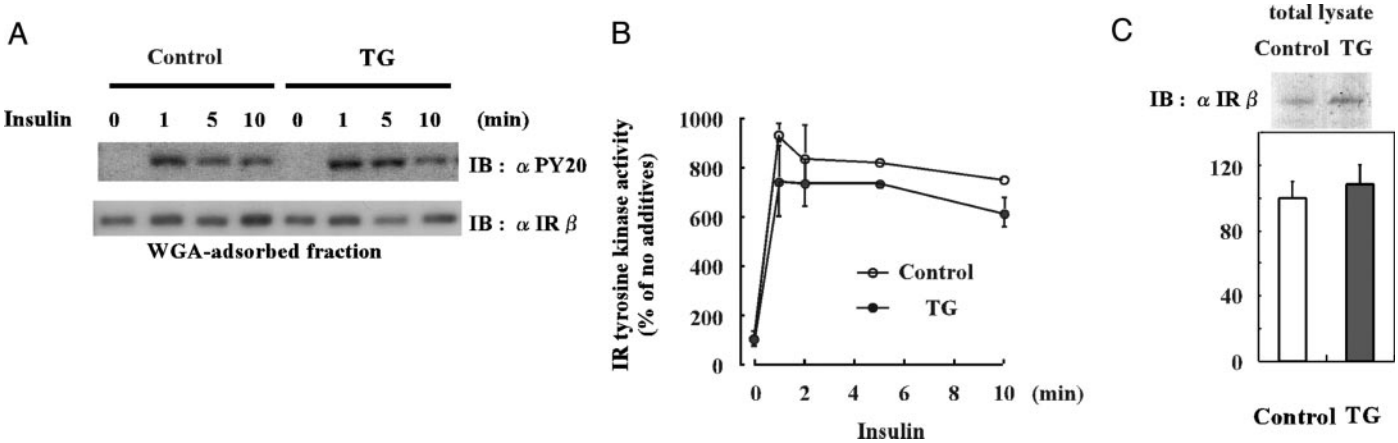

FIG. 5. Insulin-induced IR autophosphorylation and tyrosine kinase activity in primary cultured hepatocytes of control and TG rats. A, WGA-adsorbed fraction of the lysates from primary cultured hepatocytes of control or TG rats treated with insulin (10^{-7} M) for various times were subjected to 8% SDS-PAGE, and immunoblotting (IB) with antiphosphotyrosine antibody. B, Quiescent hepatocytes were treated without or with insulin (10^{-7} M) for 0, 1, 2, 5, and 10 min. After treatment, cells extracts were subjected to tyrosine kinase assay. The values were the mean ± SEM of three independent experiments. C, The cell extracts from quiescent hepatocytes of control and TG rats were subjected to SDS-PAGE followed by IB with anti-IR β-subunit antibody. A representative blot is shown in the *upper panel*. In *lower panels*, the results of densitometric analysis of three independent experiments were indicated.

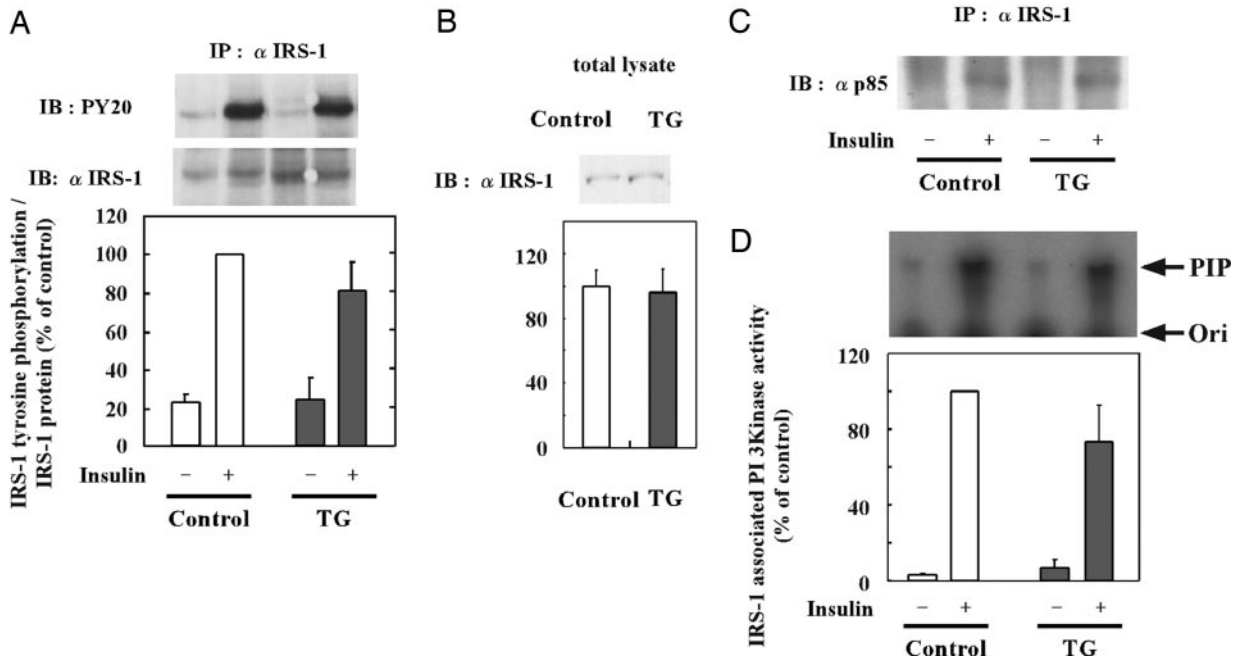

FIG. 6. Insulin-induced IRS-1 tyrosine phosphorylation and PI 3-kinase activation bound to IRS-1 in primary cultured hepatocytes from control and TG rats. A, *Upper panel*, Quiescent hepatocytes of control and TG rats were treated without or with insulin (10^{-7} M) for 1 min. After treatment, cell extracts were subjected to immunoprecipitation (IP) with anti-IRS-1 antibody, followed by 8% SDS-PAGE and immunoblotting (IB) with antiphosphotyrosine antibody or anti-IRS-1 antibody. *Lower panel*, The amounts of tyrosine-phosphorylated IRS-1 or total IRS-1 were quantified using NIH software and are expressed as a percentage of the amounts in the insulin-treated control hepatocytes. The values were expressed as the mean ± SEM of three independent experiments. B, The cell extracts from quiescent hepatocytes of control and TG rats were subjected to SDS-PAGE and IB with anti-IRS-1 antibody. A representative blot is shown in the *upper panel*. In the *lower panel*, the results of densitometric analysis of three independent experiments were indicated. C, Quiescent hepatocytes in primary cultures from control and TG rats were incubated without or with insulin (10^{-7} M) for 1 min. After treatment, cells were harvested and the cell lysate was subjected to the IP with anti-IRS-1 antibody before 8% SDS-PAGE and IB with anti-p85 subunit of PI 3-kinase antibody as described in *Materials and Methods*. These experiments were performed three times independently and a representative blot is shown. D, Quiescent hepatocytes in primary cultures from control and TG rats were incubated without or with insulin (10^{-7} M) for 1 min. PI 3-kinase activity in immunocomplex using anti-IRS-1 antibody was measured as described in *Materials and Methods*. Each treatment was performed in triplicate and in three independent experiments. PI 3-kinase activity is expressed as a percentage of activity in control hepatocytes followed by insulin treatment. PIP, Phosphatidylinositol phosphate; Ori, origin. Results represent the means ± SEM of three independent experiments.

kidney) were markedly hyperinsulinemic as well as hyperglycemic. In this study, we developed hGH TG rats using whey acidic protein promoter so that GH was overexpressed in various tissues except liver (Fig. 1). These GH TG rats were normoglycemic and had normal glucose tolerance (Fig. 2) but were also hyperinsulinemic, similar to results published about other GH excess models (2, 12, 13).

GH is known to negatively affect insulin signaling, which can lead to insulin resistance in various peripheral tissues including adipocytes (27, 38) and muscle (39). As shown in Fig. 3, we found that in the TG rats in this study, insulin-dependent glucose uptake was completely abolished in adipocytes and skeletal muscle, thus confirming results in previously published reports (16). Taken together with the data of hyperinsulinemia in our model, it seems that insulin resistance is induced in adipose tissues and muscles at an early stage of development, perhaps between 0 and 14 wk of age; however, hyperglycemia was not observed concomitantly with this insulin resistance (Fig. 2). These results suggest that other tissues compensate for the insulin resistance found in adipose tissue or muscle so as to prevent hyperglycemia. Liver has recently been shown to be important in maintaining glucose and lipid homeostasis in muscle-specific GLUT4 knockout mice (40).

The next logical question is regarding how other tissues prevent hyperglycemia. We found up-regulation of glucose uptake into the hepatocytes from TG rats (Fig. 3C), suggesting that liver compensates for the loss of ability of glucose uptake in adipocytes and muscle. Because glucose is incorporated into liver mainly through GLUT2, glucose uptake in liver is not dependent on insulin concentrations but instead on the difference between plasma and intracellular glucose concentrations. As expected, in hepatocytes, we found no insulin-dependent increase in glucose uptake. We therefore conclude that an increase in the use of glucose in liver causes up-regulation of glucose uptake into hepatocytes from TG rats.

As mentioned above, other groups have reported that insulin signaling is impaired in GH excess mice and chronic GH-treated rats. They proposed that the hepatic response to insulin might be decreased because of GH increased IRS-1 tyrosine phosphorylation at the basal level, which in turn leads to a decrease in changes in IRS-1 tyrosine phosphorylation induced by insulin (15, 38). Although we could not detect an increase in the basal levels of IRS-1 phosphorylation, we showed insulin-induced IRS-1 phosphorylation was decreased or not significantly changed in TG liver or hepatocytes (Figs. 4 and 6). Surprisingly, insulin-dependent IRS-2

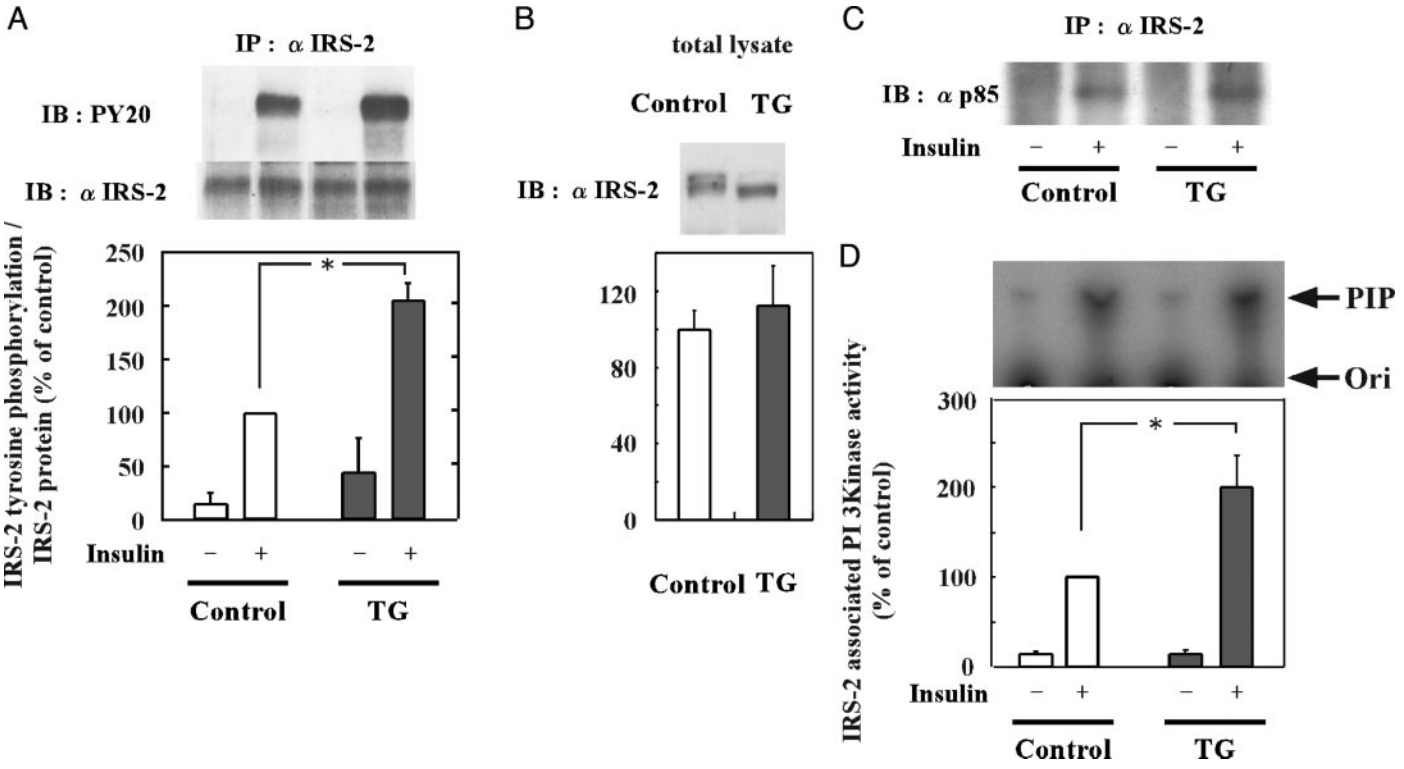

FIG. 7. Insulin-induced IRS-2 tyrosine phosphorylation and PI 3-kinase activation bound to IRS-2 in primary cultured hepatocytes from control and TG rats. A, *Upper panel*, Quiescent hepatocytes of control and TG rats were treated without or with insulin (10^{-7} M) for 1 min. After treatment, cell extracts were subjected to immunoprecipitation (IP) with anti-IRS-2 antibody, followed by 8% SDS-PAGE and immunoblotting (IB) with antiphosphotyrosine antibody or anti-IRS-2 antibody. *Lower panel*, The amounts of tyrosine-phosphorylated IRS-2 or total IRS-2 were quantified using NIH software and are expressed as a percentage of the amounts in the insulin-treated control hepatocytes. The values were expressed as the mean ± SEM of three independent experiments. *, Significant difference ($P < 0.05$) between values obtained in control hepatocytes treated with insulin and those of TG hepatocytes (unpaired *t* test). B, The cell extracts from quiescent hepatocytes from control or TG rats and subjected to SDS-PAGE and immunoblotting with anti-IRS-2 antibody. A representative blot is shown in the *upper panel*. In *lower panels*, the results of densitometric analysis of three independent experiments are indicated. C, Quiescent hepatocytes in primary cultures from control and TG rats were incubated without or with insulin (10^{-7} M) for 1 min. After treatment, cells were harvested and the cell lysate was subjected to IP with anti-IRS-2 antibody before 8% SDS-PAGE and IB with anti-p85 subunit of PI 3-kinase antibody as described in *Materials and Methods*. These experiments were performed three times independently and a representative blot is shown in the *upper panel*. In the *lower panel*, densitometric analysis of the amount of p85 PI 3-kinase was performed. *, Significant difference ($P < 0.05$) between values obtained in cells from control and TG rats (unpaired *t* test). D, Quiescent hepatocytes in primary cultures from control and TG rats were incubated without or with insulin (10^{-7} M) for 1 min. PI 3-kinase activity in immunocomplex using anti-IRS-2 antibody was measured as described in *Materials and Methods*. Each treatment was performed in triplicate and in three independent experiments. PI 3-kinase activity is expressed as a percentage of activity in control hepatocytes followed by insulin treatment. PIP, Phosphatidylinositol phosphate; Ori, origin. Results represent the means ± SEM of three independent experiments. *, Significant difference ($P < 0.05$) between insulin-dependent activities in cells from control and TG rats (unpaired *t* test).

tyrosine phosphorylation was higher in TG than in control, whereas the basal level of IRS-2 tyrosine phosphorylation was same (Figs. 4 and 7). Withers *et al.* (41) produced IRS-2 knockout mice, in which IRS-2 was not expressed in liver and thus demonstrated that deficiency of IRS-2 leads to noninsulin-dependent diabetes mellitus. Valverde *et al.* (42) showed that IRS-2 plays an essential role in insulin-regulated glycogen synthesis and hepatic glucose production in hepatocytes. Therefore, it is thought that IRS-2 in liver might be an important insulin signaling molecule for maintaining glucose homeostasis.

It is well known that tyrosine-phosphorylated IRSs are recognized by the p85 regulatory subunit of PI 3-kinase, leading to activation of PI 3-kinase. In TG hepatocytes, we found that enhancement of IRS-2 tyrosine phosphorylation induced by insulin caused increases in insulin-dependent binding of the p85 regulatory subunit of PI 3-kinase to IRS-2, resulting in potentiation of PI 3-kinase activation bound to IRS-2 (Fig. 7). We therefore conclude that amplification of IRS-2 tyrosine phosphorylation induces enhancement of signaling in the PI 3-kinase pathway in TG hepatocytes. In addition, we found that there were insulin-dependent increases in glycogen synthase activity and also that mRNA levels of lipogenic enzymes, such as GK, ACC, and FAS, were increased in TG hepatocytes (Fig. 8). Insulin-dependent increases are reportedly caused by activation of the PI 3-kinase pathway (43, 44); we are in agreement with this because we found that addition of LY294002 abolished insulin-dependent changes in these cells (data not shown). A series of our results strongly suggested that potentiation of insulin signals at IRS-2 induces additional increases in glycogen and lipid synthesis.

In TG rats, high concentrations of insulin might further accelerate glycogen and lipid synthesis via activated glyco-

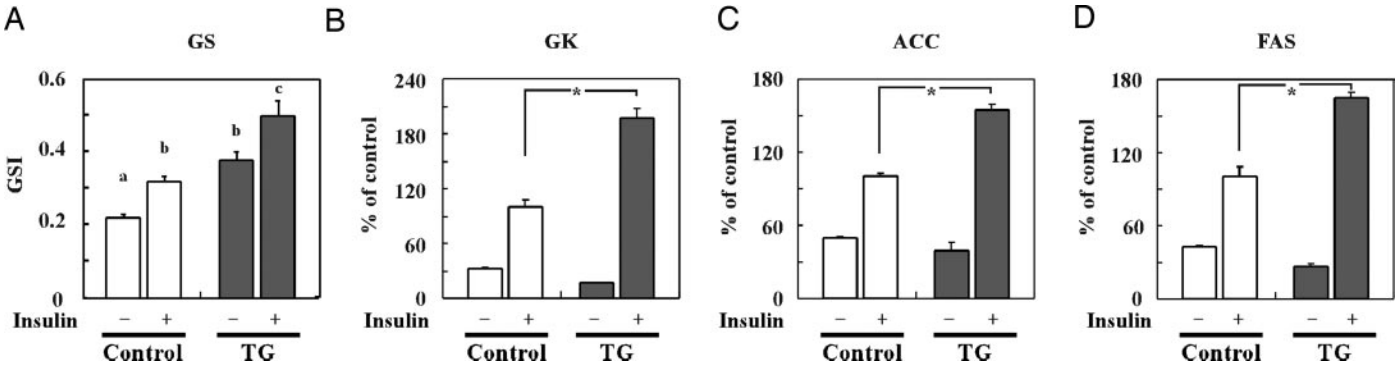

FIG. 8. Glycogen synthase activity and mRNA levels of lipogenic enzymes in insulin-treated primary cultured hepatocytes from control and TG rats. A, Quiescent hepatocytes were treated without or with insulin (10^{-7} M) for 20 min. After treatment, the cell extracts were obtained and glycogen synthase activity assay was performed as described in *Materials and Methods*. Results are expressed as means ± SEM of percentage of GSI. Glycogen synthase activity was determined as (activity without G6P/activity with G6P) × 100. Values are expressed as relative to nonstimulated hepatocytes from control rats. *, Significant difference ($P < 0.01$) from the GSI before insulin treatment (unpaired *t* test). B–D, Quiescent hepatocytes from control and TG rats were incubated without or with 10^{-7} M insulin (INS) in the presence of DEX for 24 h. Total RNA was isolated from the cells, real-time PCR analyses of GK, ACC (25), and FAS were performed as described in *Materials and Methods*. Values are the mean ± SEM of each three independent data. *, Significant difference ($P < 0.05$) between values obtained in control and TG hepatocytes, both of which were treated with insulin in the presence of DEX (unpaired *t* test).

gen synthase or increased levels of GK, ACC, and FAS. Reports showing that glycogen synthase activity is increased by long-term insulin treatment (45) and that hyperinsulinemia induces increased gene expression of lipogenic enzymes, such as FAS (46) support the present results. We did not find any significant differences in plasma free fatty acid levels between control and TG rats (data not shown), thus enabling us to draw the conclusion that the demonstrated accumulation of lipids was due to the increased presence of lipogenic enzymes. Other GH excess models are known to exhibit an enlarged liver, similarly to our TG rats. Excessive glycogen and lipid storage is one cause of liver enlargement (47, 48), and prolonged treatment with GH in dogs has also been reported to result in an increase in glycogen storage in liver (49). We therefore tested whether glycogen and lipid were accumulated in liver of TG rats by using histological analysis. As shown in Fig. 3D, glycogen granules and lipids were clearly increased in liver of TG rats, compared with control rats. Thus, we conclude that in TG rats, glucose is converted to glycogen and lipid induced by insulin in the liver and this increase in glucose use then causes up-regulation of glucose incorporation into the liver, resulting in prevention of hyperglycemia. Recently there are reports showing liver may play important roles to reduce insulin resistance in peripheral tissues partially by enhancing fatty acid oxidation when type II model animals were treated with fibrates or leptin (50, 51). However, compensation in our model possibly occurs through the different mechanisms from these models.

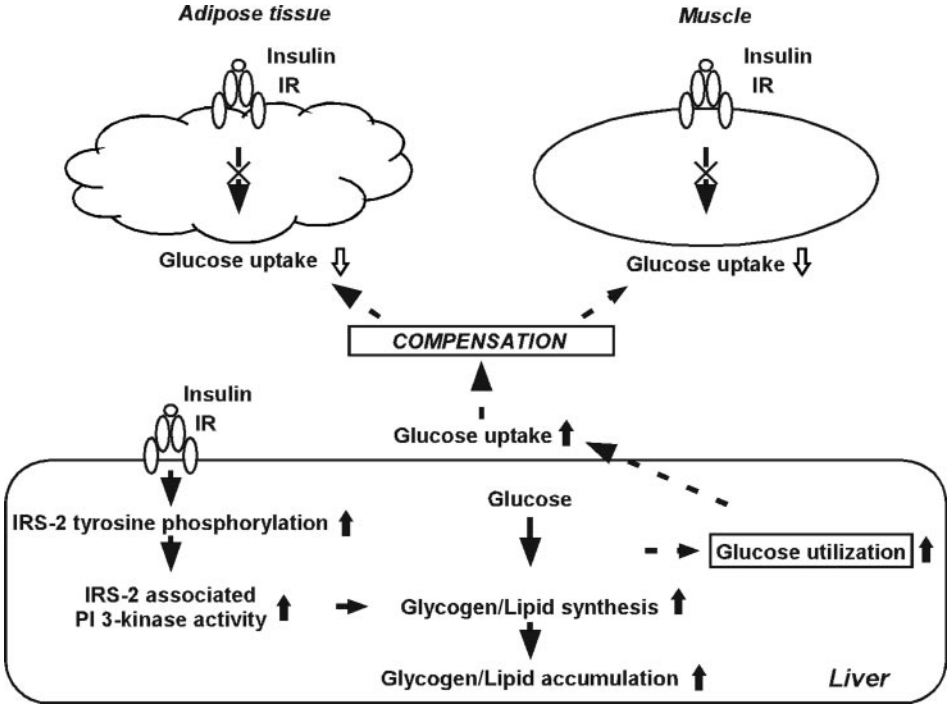

FIG. 9. Working hypothesis of the mechanisms in liver-dependent compensation of an increase in plasma glucose levels caused by insulin resistance in adipose tissue and muscle in hGH TG rats. A proposed model of liver compensation to prevent hyperglycemia caused by insulin resistance of adipose tissues and muscles. When high levels of GH induce insulin resistance in adipose tissues and muscles, in liver insulin signal through IRS-2 is up-regulated and glycogen synthase activity and gene expression of lipogenic enzyme were enhanced. Excessive glycogen and lipid are accumulated in liver from TG rats. On the other hand, use of glucose in liver causes up-regulation of glucose uptake into liver, leading to normalization of plasma glucose levels.

Next, we consider how IRS-2 tyrosine phosphorylation is enhanced in TG hepatocytes. GH treatment over a 24-h time period did not increase glucose uptake, enhance insulin signaling, or increase lipogenic enzyme expression in hepatocytes prepared from control rats (data not shown). These results suggest that up-regulation in liver is not directly induced by GH. This compensation might be mediated by circulating factors in the blood, such as glucose and cytokines. This system appears to be important in maintenance of glucose homeostasis. In addition, we observed up-regulation of insulin signals in intact liver as well as cultured hepatocytes from TG rats, suggesting that hepatocytes have been primed by circulating factors as mentioned above to increase insulin signaling in TG rats *in vivo*. Because IR kinase activity, IRS-1 tyrosine phosphorylation, and the amount of IRS-2 changes have not been noted in TG hepatocytes, we suspect that bioavailability of IRS-2 is increased to IR kinase. We incubated immunoprecipitated IRS-2 from control or TG hepatocytes with semipurified IR in the presence of ATP *in vitro*. Results of this preliminary study showed that tyrosine phosphorylation of IRS-2 from TG hepatocytes is increased, compared with control, suggesting that IRS-2 changes to a phosphorylated state are catalyzed by receptor kinase with posttranslational modifications (data not shown).

Regarding diabetic symptomology, acromegalic patients have been known to exhibit differing phenotypes; some of these patients are normoglycemic despite the presence of hyperinsulinemia, whereas others are both hyperglycemic and hyperinsulinemic (2, 52). The usual sequence for diagnosing diabetes in acromegalic patients is as follows: in the beginning stages of acromegaly, glucose tolerance remains normal, but as the disease progresses, massive insulin secretion due to insulin resistance occurs in peripheral tissues, thereby decreasing glucose tolerance, resulting in a diagnosis of diabetes. Hansen *et al.* suggested that liver played a critical role in the maintenance of normal blood glucose levels (2). Karlander *et al.* (52) demonstrated that glucose production is markedly increased in acromegalic patients despite hyperinsulinemia and normal glucose tolerance, compared with controls. In this study, 10-wk-old TG rats were normoglycemic, but the phenotype of older TG rats should be carefully analyzed. Further study using TG rats will be needed to learn how to maintain the relative health of acromegalic patients and those without this disorder and prevent or delay the onset of diabetes mellitus.

Thus, our results suggest that impairment of insulin-dependent glucose uptake by GH excess in adipose tissue and muscle is compensated by up-regulation of glucose uptake in liver and that potentiation of insulin signaling through IRS-2 in liver in conditions of GH excess causes increases in glycogen and lipid syntheses from incorporated glucose, resulting in accumulation of glycogen and lipids in liver (Fig. 9). This novel mechanism explains normalization of plasma glucose levels, at least in part, in the GH excess model.

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