

# Compensatory alterations of insulin signal transduction in liver of growth hormone receptor knockout mice

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## Abstract

Growth hormone (GH) deficiency is associated with increased sensitivity to insulin, but the molecular mechanisms involved in this association are poorly understood. In the current work, we have examined the consequences of the absence of the biological effects of GH on the first steps of the insulin signaling system *in vivo* in liver of mice with targeted disruption of the GH receptor/GH binding protein gene (GHR-KO mice). In these animals, circulating insulin concentrations are less than 4  $\mu$ IU/ml, and glucose concentrations are low, concordant with a state of insulin hypersensitivity. The abundance and tyrosine phosphorylation state of the insulin receptor (IR), the IR substrate-1 (IRS-1), and Shc, the association between

IRS-1 and the p85 subunit of phosphatidylinositol (PI) 3-kinase, the IRS-1- and the phosphotyrosine-associated PI 3-kinase in liver were examined. We found that, in liver of GHR-KO mice, the lack of GHR and GH effects is associated with: (1) increased IR abundance, (2) increased insulin-stimulated IR tyrosine phosphorylation, (3) normal efficiency of IRS-1 and Shc tyrosine phosphorylation and (4) normal activation of PI 3-kinase by insulin. These alterations could represent an adaptation to the low insulin concentrations displayed by these animals, and may account for their increased insulin sensitivity.

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## Introduction

Insulin induces a wide diversity of growth and metabolic responses in many cell types. It acts as a major regulator of glucose, lipid and protein metabolism in liver and other peripheral tissues (Cheatham & Kahn 1995). After insulin binds to the  $\alpha$ -subunit of the insulin receptor (IR), the tyrosine kinase activity that resides in the  $\beta$ -subunit becomes activated, leading to autophosphorylation of tyrosine residues in several regions of the intracellular  $\beta$ -subunit and further activation of its tyrosine kinase towards intracellular substrates (Kasuga *et al.* 1982, White 1997). One of the principal substrates of the IR kinase is the insulin receptor substrate-1 (IRS-1), a 185-kDa cytosolic protein with many tyrosine phosphorylation sites which functions as the interfaces between the receptor and several Src homology 2 (SH2) domain-containing proteins (Sun *et al.* 1991), and has been shown to mediate multiple signaling pathways (White 1997). Phosphatidylinositol (PI) 3-kinase is one of the SH2 domain-containing proteins. When tyrosine phosphorylated, IRS-1 binds the p85 subunit of PI 3-kinase (p85), thereby activating this enzyme (Backer *et al.* 1992, Lavan *et al.* 1992). PI 3-kinase

activation seems to be a central event in the insulin action pathway (Shepherd *et al.* 1998). Activation of the mitogen-activated protein (MAP) kinase cascade is another major insulin signaling pathway. Although it has been shown that IRS-1 participates in this pathway, several reports indicate that the transforming protein Shc (Src homology 2/ $\alpha$ -collagen related) is the main signaling molecule involved (Sasaoka *et al.* 1994, Yamauchi & Pessin 1994, White 1997).

It is accepted that GH modulates tissue responses to insulin. Thus, whereas GH excess leads to an impairment of insulin sensitivity and hyperinsulinemia (Bratusch-Marrain *et al.* 1982, Davidson 1987, Rizza *et al.* 1982), GH-deficiency is associated with increased insulin sensitivity, decreased insulin secretion and decreased fasting glucose concentrations (Hopwood *et al.* 1975, Bougneres *et al.* 1985, Daugaard *et al.* 1999). GH biological effects are initiated by binding and dimerization of its membrane receptor (Thomas 1998). The GH receptor (GHR) lacks intrinsic tyrosine kinase activity (Argetsinger *et al.* 1993, Thomas 1998), but instead stimulates the activity of the JAK2 tyrosine kinase (Argetsinger *et al.* 1993). After the activation of JAK2, several intracellular

proteins undergo tyrosine phosphorylation (Thomas 1998). One of them is IRS-1, which has been shown to be tyrosine phosphorylated and associated with the p85 subunit of PI 3-kinase in response to GH, both in cultured cells (Souza *et al.* 1994, Argetsinger *et al.* 1995, Ridderstråle *et al.* 1995) and in target tissues of intact animals (Yamauchi *et al.* 1998, Thirone *et al.* 1999).

GHR/GH binding protein gene knockout (GHR-KO) mice, which are an animal model of the Laron syndrome, have recently been produced (Zhou *et al.* 1997). In these mice, although GH is secreted in large quantities, its biological effects are absent because of the lack of GHR. We have previously reported that insulin-like growth factor-1 was not detectable in the circulation of these animals (Zhou *et al.* 1997). Thus they appear to be an excellent tool with which to study how the lack of GH effects influences the insulin signaling system in intact animals. Consequently, in the present study, we have examined the first steps of insulin action in liver of GHR-KO mice. We evaluated the tyrosine phosphorylation status of the IR and its substrates IRS-1 and Shc, the association between IRS-1 and p85, and the IRS-1- and total phosphotyrosine-associated PI 3-kinase activity after insulin stimulation *in vivo*. Furthermore, the abundance of the IR, IRS-1, Shc, and p85 in this tissue was determined.

## Materials and Methods

### Materials

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA, USA). HEPES, Tris, phenylmethylsulfonyl fluoride (PMSF), aprotinin, ATP, phosphatidylinositol, phosphatidylinositol 4-monophosphate, Triton X-100, Tween 20, porcine insulin, and bovine serum albumin (fraction V) (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Silica gel thin-layer chromatography plates were from Merck (Darmstadt, Germany). Protein A-Sepharose 6 MB was from Pharmacia (Uppsala, Sweden). Iodine-125-labelled protein A was purchased from ICN Biomedicals (Costa Mesa, CA, USA). [ $\gamma$ - $^{32}$ P]ATP was from Dupont-NEN (Boston, MA, USA). Immobilized P membranes were from Millipore (Bedford, MA, USA). The monoclonal anti-phosphotyrosine antibody ( $\alpha$ PY, PY99) and the polyclonal anti-IR  $\beta$ -subunit antibody ( $\alpha$ IR, C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-rat carboxy-terminal IRS-1 antibody ( $\alpha$ IRS-1), the antibody to the p85 subunit of PI-3-kinase ( $\alpha$ p85), and the anti-Shc antibody ( $\alpha$ Shc) were from Upstate Biotechnology (Lake Placid, NY, USA).

### Animals

GHR-KO mice (-/-) were obtained as described previously (Zhou *et al.* 1997). Adult normal female mice bred

in our animal facility were mated with either GHR-KO male mice or male mice with the +/- genotype, and the resulting female GHR-KO mice and non-GHR-KO littermates (control mice) were used in the present experiments. Animals were housed in a room with a controlled photoperiod of 12 h light : darkness (lights on from 0600 to 1800 h) and a temperature of 22–23 °C. Mice were given free access to a nutritionally balanced diet (LabDiet, PMI Feeds, Inc., St Louis, MO, USA) and tap water.

### Measurement of plasma insulin and glucose concentrations

Fasting plasma insulin concentration was determined using a solid-phase radioimmunoassay kit from DPC (Diagnostic Products Inc., Los Angeles, CA, USA).

Plasma glucose was measured with the glucose oxidase procedure (Trender; Sigma Chemical).

### Methods

Female GHR-KO mice and their normal siblings (3–5 months of age) were starved overnight, and 15 min before the experiment they were anesthetized by the intraperitoneal administration of 100 mg of sodium pentobarbital per kg of body weight. After anesthesia was induced, the portal vein was exposed and 10 IU insulin per kg body weight in normal saline (0.9% NaCl) in a final volume of 0.1 ml was injected via this vein. GHR-KO and control mice that had been injected with diluent only were used to evaluate changes under basal conditions. Approximately 50 s after injection, the liver was removed, coarsely minced, and homogenized in 10 volumes of solubilization buffer A (1% Triton, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.1 mg/ml aprotinin) at 4 °C. Liver extracts were centrifuged at 100 000  $\times g$  at 4 °C in a Beckman 90 Ti rotor (Palo Alto, CA, USA) for 1 h to eliminate insoluble material. Protein concentration was determined by the method described by Bradford (1976), and the supernatants were subjected to immunoprecipitation with  $\alpha$ IR,  $\alpha$ IRS-1 and protein A-Sepharose 6 MB.

### Immunoprecipitation

Liver homogenates containing 8 mg total protein prepared as described in Methods were incubated at 4 °C overnight with  $\alpha$ IR,  $\alpha$ IRS-1 or  $\alpha$ Shc (4  $\mu$ g/ml final concentration for all antibodies). After incubation, 50  $\mu$ l protein A-Sepharose (50%, vol/vol) was added to the mixture. The preparation was further incubated with constant rocking for 2 h and centrifuged at 12 000 r.p.m. for 1 min at 4 °C. The precipitate was washed three times with lysis buffer. The final precipitate was boiled for 5 min in 60  $\mu$ l of reducing sample buffer (final concentrations: 62.5 mM Tris, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS,

0.02% bromophenol blue), and stored at  $-70^{\circ}\text{C}$  until required for electrophoresis.

#### Immunoblotting

Samples were subjected to SDS-PAGE on a 6% polyacrylamide gel using a Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Electrotransfer of proteins from the gel to Immobilon P membranes was performed for 2 h at 100 V (constant) using the Bio-Rad miniature transfer apparatus in 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, and 0.02% SDS. To reduce non-specific antibody binding, the membranes were incubated at  $4^{\circ}\text{C}$  overnight in a blocking buffer composed of Tris-buffered saline-Tween 20 (TBS-T) buffer (10 mM Tris HCl (pH 7.6), 150 mM NaCl, and 0.02% Tween 20) containing either 3% BSA (for phosphotyrosine detection) or 5% non-fat dry milk (for protein detection). The membranes were then incubated for 4 h at room temperature with  $\alpha\text{PY}$  (1  $\mu\text{g}/\text{ml}$ ),  $\alpha\text{IR}$  (1  $\mu\text{g}/\text{ml}$ ),  $\alpha\text{IRS-1}$  (1  $\mu\text{g}/\text{ml}$ ) or  $\alpha\text{Shc}$  (1  $\mu\text{g}/\text{ml}$ ) diluted in the corresponding blocking buffer. The membranes were subjected to four 5-min washes in TBS-T buffer and were then incubated with 3  $\mu\text{Ci}$   $^{125}\text{I}$ -protein A (30  $\mu\text{Ci}/\mu\text{g}$ ) in 15 ml blocking buffer for 1 h at room temperature and then washed again for 60 min as described above.  $^{125}\text{I}$ -Protein A bound to antibodies was detected by autoradiography using pre-flashed Kodak XAR film (Eastman Kodak, Rochester, NY, USA) at  $-70^{\circ}\text{C}$  for 6–72 h. Band intensities were quantitated by optical densitometry (model CS-930, Shimadzu, Japan) of the developed autoradiographs.

The amount of the p85 subunit of the PI 3-kinase in  $\alpha\text{IRS-1}$  immunoprecipitates was evaluated by stripping the membranes and reblotting as follows: The blots that had been blotted with  $\alpha\text{PY}$  were rinsed, incubated in 2% SDS, 60 mM Tris (pH 6.7), 100 mM 2-mercaptoethanol at  $50^{\circ}\text{C}$  for 45 min, and reprobed again without adding additional primary antibody to check that all antibody had been removed. The membranes were then blocked for 2 h at room temperature with 5% non-fat dry milk in TBS-T buffer and incubated with an antibody to the p85 subunit of PI-3-kinase ( $\alpha\text{p85}$ ; 1:2000 final concentration) in TBS-T plus 1% non-fat dry milk. Bound antibodies were detected by incubation with  $^{125}\text{I}$ -protein A as described previously.

To determine the abundance of p85 in liver, equal amounts of solubilized proteins (120  $\mu\text{g}$ ) were denatured by being boiled in reducing sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with  $\alpha\text{p85}$ . Bound antibodies were detected by incubation with  $^{125}\text{I}$ -protein A. Quantitation of specific protein bands was performed by densitometry.

#### Determination of PI 3-kinase activity in immunoprecipitates

This determination was performed essentially as described previously (Dominici *et al.* 1999a, b). Livers of control and

GHR-KO mice that had been injected with or without insulin as described in Methods were extracted and homogenized in solubilization buffer B (50 mM HEPES (pH 7.4), 137 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 100 mM NaF, 2 mM EDTA, 2 mM PMSF, 2 mM aprotinin, 2 mM leupeptin, 10% (vol/vol) glycerol, and 1% Triton X-100) at  $4^{\circ}\text{C}$ . The homogenates were centrifuged at 100 000  $g$  for 1 h at  $4^{\circ}\text{C}$ . Liver homogenates containing 8 mg total protein were incubated overnight at  $4^{\circ}\text{C}$  with  $\alpha\text{PY}$  (5  $\mu\text{g}$ ) or with  $\alpha\text{IRS-1}$  (4  $\mu\text{g}$ ). Immunocomplexes were collected by addition of 100  $\mu\text{l}$  of a 50% (vol/vol) slurry of protein A-Sepharose in phosphate-buffered saline (PBS) and further incubation for 2 h at  $4^{\circ}\text{C}$ . After centrifugation, bound complexes were washed three times with ice-cold PBS (pH 7.4) containing 1% Triton X-100 and 100  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$ , three times in ice-cold 100 mM Tris (pH 7.5) containing 100 mM  $\text{LiCl}_2$  and 100  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$ , and twice with ice-cold 10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 100  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$ . The precipitates were then resuspended in a mixture containing 50  $\mu\text{l}$  10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, 10  $\mu\text{l}$  100 mM  $\text{MgCl}_2$ , and 10  $\mu\text{l}$  phosphatidylinositol (2  $\mu\text{g}/\mu\text{l}$ ) sonicated in 10 mM Tris (pH 7.5), EDTA 1 mM. Reaction was initiated by the addition of 10  $\mu\text{l}$  440  $\mu\text{M}$  ATP containing 30  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (6000 Ci/mmol). After 10 min at room temperature with constant shaking, the reaction was terminated by the addition of 20  $\mu\text{l}$  8 N HCl and 160  $\mu\text{l}$  methanol-chloroform (1:1). Mixtures were centrifuged and the lower organic phase applied to silica TLC plates (Merck) coated with 1% (w/v) potassium oxalate. TLC plates were developed in  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{NH}_4\text{OH}$  (60:47:11:3:2) and visualized by autoradiography. Phosphatidylinositol 4-phosphate, which co-migrates with 3-phosphorylated phosphatidylinositol, was used as standard. The band corresponding to phosphatidylinositol 3-phosphate was excised and its radioactivity quantitated by liquid scintillation counting.

#### Statistical analysis

Results are presented as means  $\pm$  S.E.M. Experiments were performed by analyzing all groups of animals in parallel. Statistical analyses were performed by ANOVA followed by the Tukey-Kramer test using the InStat statistical program by GraphPad Software, Inc. (San Diego, CA). Student's *t* test was used when the values of two groups were analyzed. The level of significance used was  $P < 0.05$ .

## Results

#### Characteristics of the animals studied

Data on body weight and plasma glucose and insulin concentrations of the studied animals are presented in

**Table 1** Characteristics of the experimental animals. Values are means  $\pm$  S.E.M.;  $n=12$  per group

	Control	GHR-KO
Body weight (g)	25 $\pm$ 2	13 $\pm$ 1*
Glucose (mg/dl)	165 $\pm$ 8	97 $\pm$ 2*
Insulin ( $\mu$ IU/ml)	23 $\pm$ 6	<4*

\*Significantly different from the control value ( $P<0.001$ ).

Table 1. The lack of GHR led to a dramatic reduction in fasting insulin plasma concentrations and to a marked decrease in glucose plasma concentrations in GHR-KO mice. Insulin concentrations in GHR-KO mice were below limit of detection of the assay (less than 4  $\mu$ IU/ml); glucose concentrations averaged 58% of values measured in normal animals.

#### *Insulin receptor abundance and tyrosine phosphorylation in liver*

To determine the abundance of IR in liver, homogenates of liver of the GHR-KO mice and their control littermates were subjected to immunoprecipitation using an antibody to the IR  $\beta$ -subunit ( $\alpha$ IR), followed by immunoblotting with the same antibody. We found a major up-regulation of insulin receptor immunoreactivity in the liver of GHR-KO mice (Fig. 1A, B). Insulin receptor levels in GHR-KO animals were increased to 180  $\pm$  14% ( $n=10$ ;  $P<0.01$ ) of values measured in the normal controls.

To evaluate the tyrosine phosphorylation status of the IR, liver homogenates were obtained from control and GHR-KO mice that had been injected with saline vehicle or insulin. Extracts were subjected to immunoprecipitation with  $\alpha$ IR and immunoblotted with an anti-phosphotyrosine antibody ( $\alpha$ PY). The amount of tyrosine phosphorylation of the IR  $\beta$ -subunit was quantitated by densitometry. Comparisons were made setting the value of insulin-stimulated controls as 100%. Basal phosphorylation of the IR was similar for control and GHR-KO mice (6  $\pm$  4 vs 6  $\pm$  3% respectively; Fig. 1C, D). Insulin stimulated the tyrosine phosphorylation of the 95 kDa  $\beta$ -subunit of the IR 16.6-fold in liver of control animals and 21.3-fold in GHR-KO mice. Thus, in the liver of GHR-KO mice, insulin-stimulated IR phosphorylation was increased by 28  $\pm$  7% ( $n=5$ ;  $P<0.01$ ) compared with that in control animals (Fig. 1C, D).

Although IR abundance was increased in liver of GHR-KO mice, when the data were normalized for the amount of receptor protein in liver, the hepatic receptor phosphorylation in liver of GHR-KO mice was reduced to 71% of control values.

#### *IRS-1 abundance and tyrosine phosphorylation in liver*

To evaluate IRS-1 protein abundance, liver homogenates were subjected to immunoprecipitation with a specific

anti-IRS-1 antibody ( $\alpha$ IRS-1) followed by immunoblotting with the same antibody. In liver of GHR-KO mice, there was no statistically significant change in the abundance of IRS-1, as evaluated by scanning densitometry ( $n=10$ ; Fig. 2A, B).

To assess the extent of IRS-1 tyrosine phosphorylation under basal conditions and after insulin administration, liver homogenates were immunoprecipitated with  $\alpha$ IRS-1 and subjected to immunoblotting with  $\alpha$ PY. Basal IRS-1 tyrosine phosphorylation was low in both groups of animals (11  $\pm$  4% and 15  $\pm$  6% for control and GHR-KO mice respectively). There was a slight but not statistically significant increase in the insulin-stimulated phosphorylation of IRS-1 in liver of GHR-KO mice (121  $\pm$  10% of control values;  $n=5$ ).

#### *p85 association and p85 abundance in liver*

To assess p85 association with IRS-1, samples that had been immunoprecipitated with  $\alpha$ IRS-1 were immunoblotted with  $\alpha$ p85 as described in Methods. As shown in Fig. 3A, in the liver of GHR-KO mice, there was a slight increase in the amount of p85 present in  $\alpha$ IRS-1 immunoprecipitates under basal conditions, but this apparent change was not statistically significant (Fig. 3B). The association of insulin-stimulated p85 with IRS-1 in liver of GHR-KO mice reached levels similar to those found in control mice ( $n=5$ ; Fig. 3A, B).

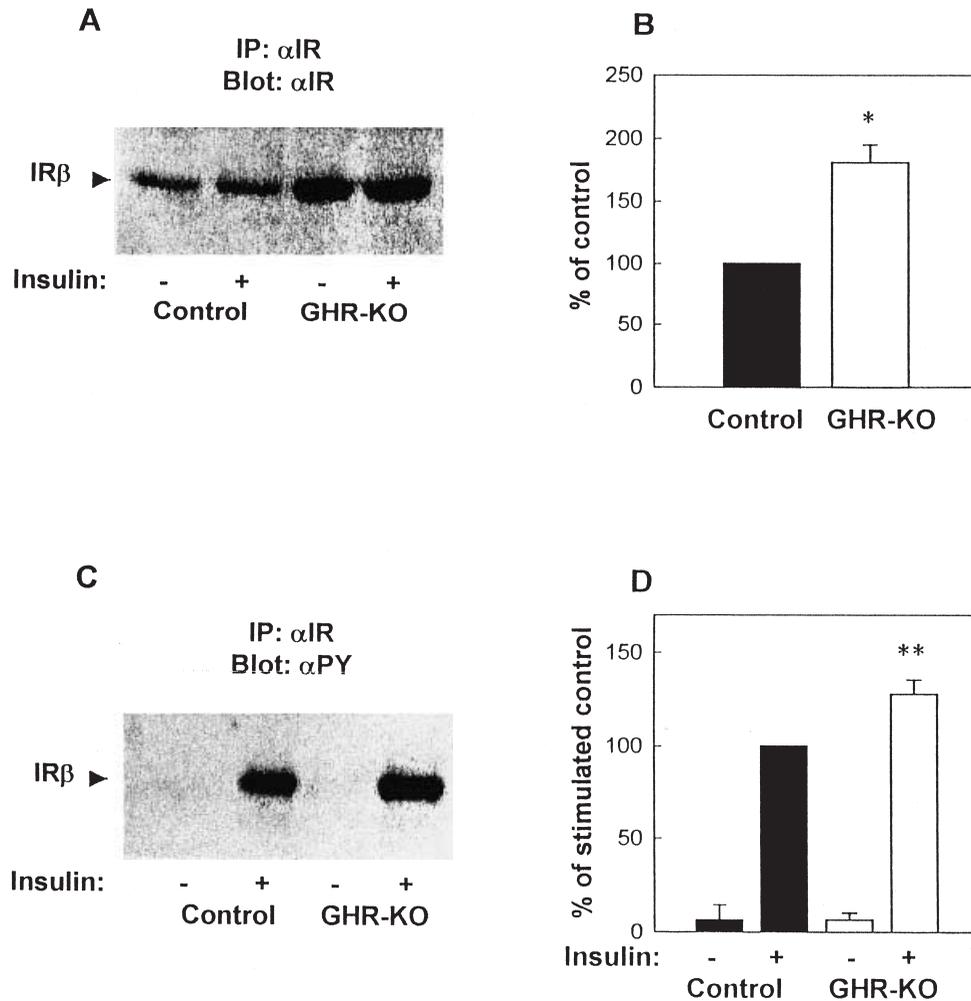
To determine if the lack of GHR was associated with changes in the abundance of p85 in liver, liver homogenates were subjected to immunoblotting with  $\alpha$ p85. A representative autoradiogram showing p85 protein from two control and two GHR-KO mice is shown in Fig. 3C. Densitometric analysis showed that there was no change in the abundance of p85 in the liver of GHR-KO mice ( $n=8$ ; Fig. 3D).

#### *PI 3-kinase activity in liver*

A low basal PI 3-kinase activity was present in  $\alpha$ IRS-1 immunoprecipitates from liver of GHR-KO and control mice (Fig. 4A, B). As the figure shows, insulin-stimulated IRS-1-associated PI 3-kinase activity in liver of GHR-KO mice did not differ significantly from that found in their control littermates (109  $\pm$  7% of control values;  $n=4$ ). Concordant with the IRS-1-associated PI 3-kinase activity, the total phosphotyrosine-associated PI 3-kinase activity in liver of GHR-KO mice reached values that were comparable to those detected in the control group. The insulin-stimulated PI 3-kinase activity measured in  $\alpha$ PY immunoprecipitates from liver of GHR-KO mice was 106  $\pm$  9% of control values ( $n=4$ ; Fig. 4C, D).

#### *Shc abundance and tyrosine phosphorylation in liver*

To evaluate Shc protein abundance, liver homogenates were subjected to immunoprecipitation with a specific

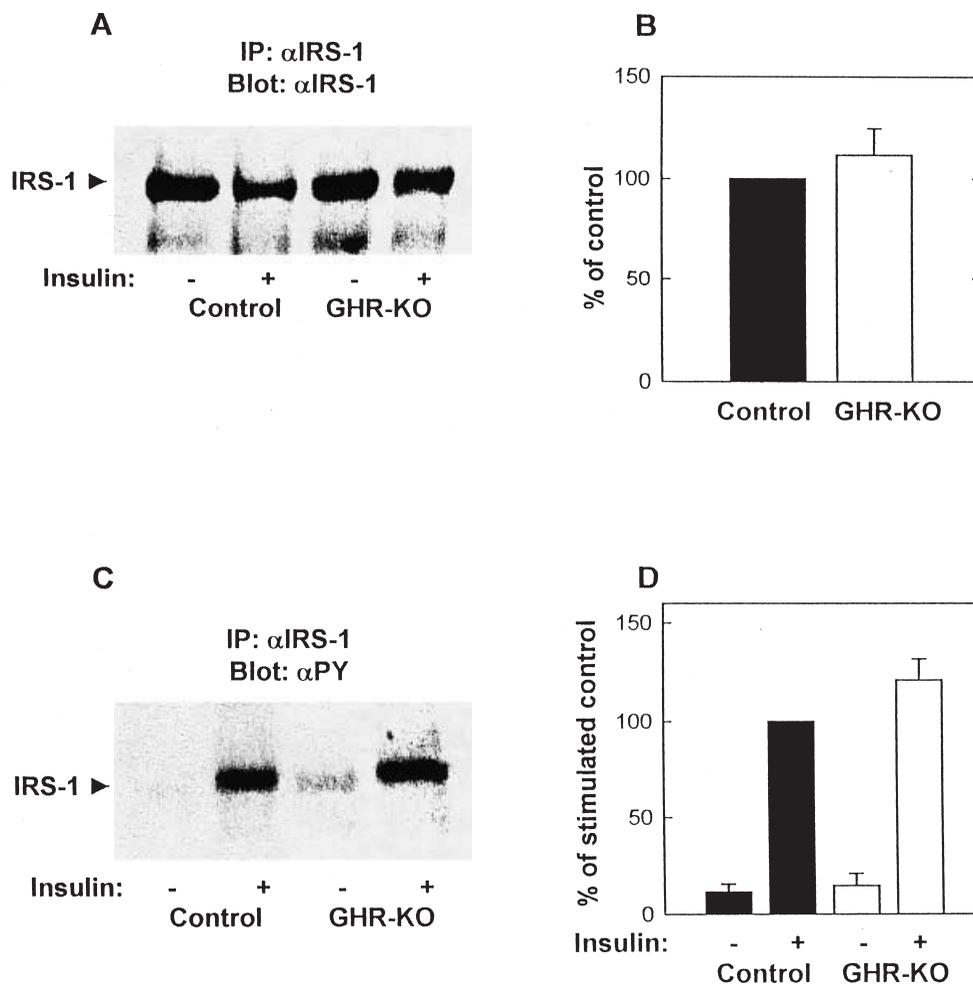


**Figure 1** Insulin receptor (IR) abundance and tyrosine phosphorylation in liver of GHR-KO and control mice. Animals were anesthetized, and normal saline (–) or insulin (10 IU/kg) (+) was injected into the portal vein. After 50 s, liver was excised and tissue extraction was performed as described in Materials and Methods. Equal amounts of solubilized liver protein (8 mg) were immunoprecipitated (IP) with an anti-IR  $\beta$ -subunit antibody ( $\alpha$ IR), separated by SDS-PAGE, and subjected to immunoblot analysis with the same antibody (A). (B) Data quantification by scanning densitometry: means  $\pm$  S.E.M. of five separate experiments ( $n=10$ /group), expressed as relative to control values, which were set at 100%. \* $P<0.01$  compared with control mice. (C) The same samples were immunoprecipitated with  $\alpha$ IR and immunoblotted with anti-phosphotyrosine antibody PY99 ( $\alpha$ PY) as described in Materials and Methods. (D) Quantification of IR tyrosine phosphorylation by scanning densitometry: means  $\pm$  S.E.M. of five different experiments ( $n=5$ /group), expressed as relative to control, assigning a value of 100% to the insulin-stimulated control mean. \*\* $P<0.01$  compared with insulin-stimulated control mice.

antibody that recognizes the 46-, 52- and 66-kDa isoforms of Shc ( $\alpha$ Shc), followed by immunoblotting with the same antibody. The predominant bands detected corresponded to the 46- and 52-kDa isoform of Shc, whereas the 66-kDa isoform was barely detected (Fig. 5A). In liver of GHR-KO mice, there was no statistically significant change in the abundance of p46 and p52 isoforms of Shc, as evaluated by scanning densitometry ( $n=6$ ; Fig. 5A, B).

To assess the extent of Shc tyrosine phosphorylation under basal conditions and after insulin administration,

liver homogenates were immunoprecipitated with  $\alpha$ Shc and subjected to immunoblotting with  $\alpha$ PY. Consistent with previous reports (Paez-Espinosa *et al.* 1998), we found no detectable phosphorylation in the p46 and p66 isoforms of Shc (Fig. 5C). Basal tyrosine phosphorylation of the 52-kDa isoform of Shc was relatively high in both groups of animals ( $35 \pm 6\%$  and  $43 \pm 6\%$  for control and GHR-KO mice respectively). Consistent with the phosphorylation of IRS-1, there was a slight but not statistically significant increase in the insulin-stimulated



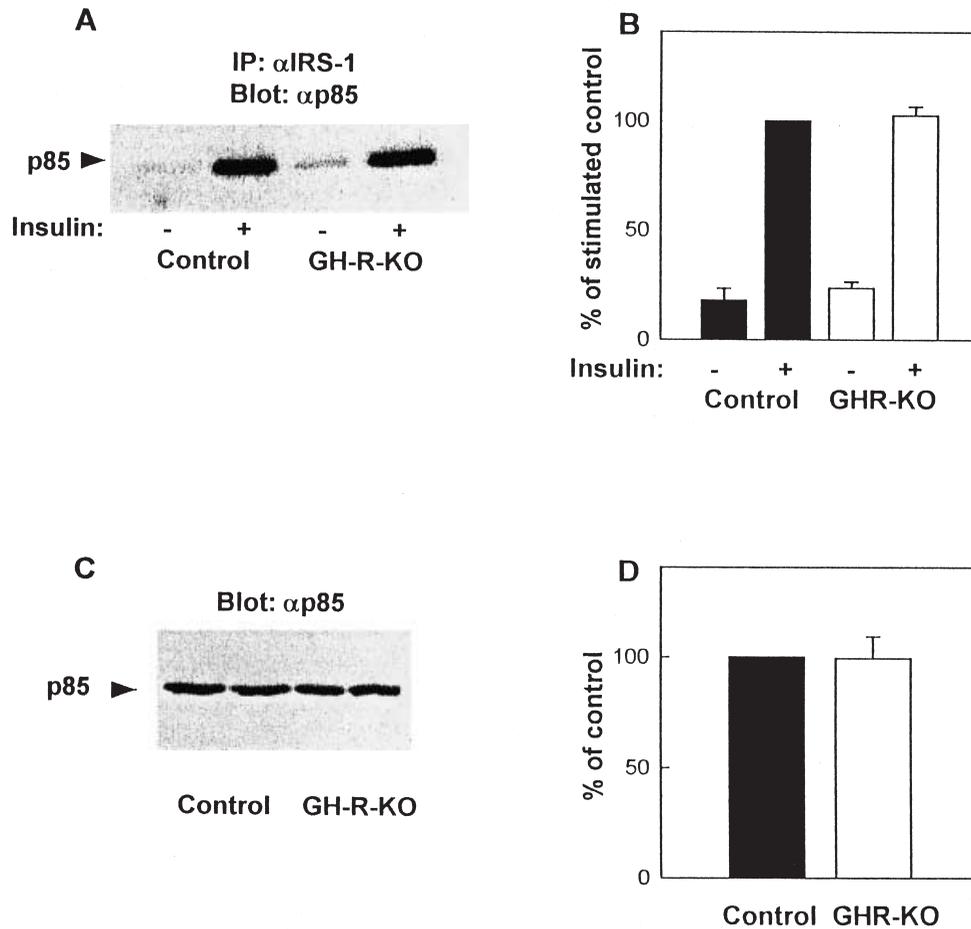
**Figure 2** IRS-1 abundance and tyrosine phosphorylation in liver of GHR-KO and control mice. Animals were anesthetized, and normal saline (–) or insulin (10 IU/kg) (+) was injected into the portal vein. After 50 s, liver was excised and tissue extraction was performed as described in Materials and Methods. Equal amounts of solubilized liver protein (8 mg) were immunoprecipitated (IP) with an anti-IRS-1 antibody ( $\alpha$ IRS-1), separated by SDS-PAGE, and subjected to immunoblot analysis with the same antibody (A). (B) Data quantification by scanning densitometry: means  $\pm$  S.E.M. of five separate experiments ( $n=10$ /group), expressed as relative to control values, which were set at 100%. (C) The same samples were immunoprecipitated with  $\alpha$ IRS-1 and immunoblotted with anti-phosphotyrosine antibody PY99 ( $\alpha$ PY) as described in Materials and Methods. (D) Quantification of IRS-1 tyrosine phosphorylation by scanning densitometry: means  $\pm$  S.E.M. of five different experiments ( $n=5$ /group), expressed as relative to control, assigning a value of 100% to the insulin-stimulated control mean.

phosphorylation of Shc in liver of GHR-KO mice ( $124 \pm 15\%$  of control values;  $n=3$ ) (Fig. 5C, D).

## Discussion

GHR-KO mice exhibit a pronounced decrease in fasting insulin concentrations and a moderate decrease in fasting glucose concentrations. This indicates that lack of GHR induces a state of insulin hypersensitivity, with glucose homeostasis being preserved with little circulating insulin.

Association of GH deficiency with hypersensitivity to insulin has long been recognized in humans and animals (Davidson 1987). In GH-deficient patients there is a tendency to hypoglycemia (Hopwood *et al.* 1975), apparently mainly caused by reduced hepatic glucose output (Bougneres *et al.* 1985). GH deficiency in rats also leads to increased insulin sensitivity (Daugaard *et al.* 1999). As demonstrated in insulin tolerance tests, the sensitivity to insulin is indeed enhanced in GHR-KO mice (Coschigano *et al.* 1999). When an intraperitoneal injection of insulin (0.75 IU/kg) is given, blood glucose

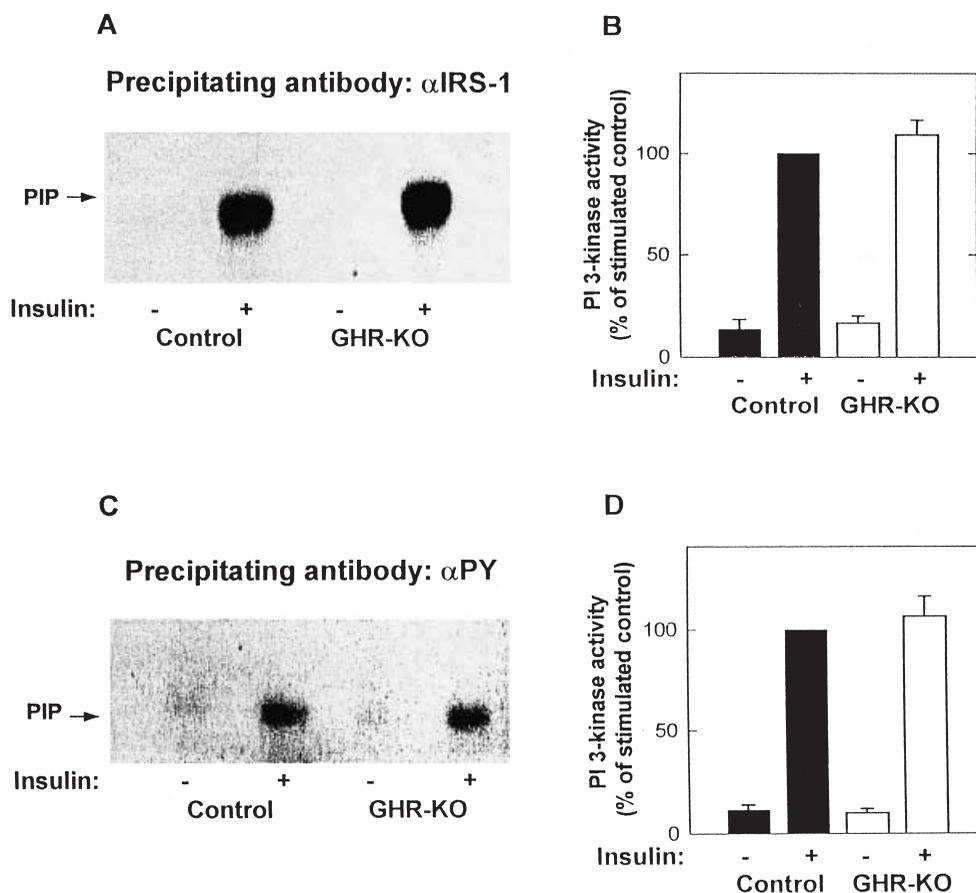


**Figure 3** Association of IRS-1 with p85 and p85 abundance in liver of GHR-KO and control mice. Animals were injected as described in Figs 1 and 2. Equal amounts of liver protein were subjected to immunoprecipitation with  $\alpha$ IRS-1, separated by SDS-PAGE, and subjected to immunoblot analysis with an antibody to the p85 subunit of PI 3-kinase ( $\alpha$ p85) (A). (B) Scanning densitometry on autoradiograms from five separate experiments ( $n=5$ /group): means  $\pm$  S.E.M. expressed as relative to control, assigning a value of 100% to the insulin-stimulated control mean. (C) Aliquots from total liver extracts containing equal amounts of protein (120  $\mu$ g) separated by SDS-PAGE and immunoblotted with  $\alpha$ p85 as described in Materials and Methods. (D) Quantification of p85 abundance by scanning densitometry: means  $\pm$  S.E.M. of four separate experiments ( $n=8$ /per group).

concentrations decrease significantly more steeply in fasted GHR-KO mice than in controls (Coschigano *et al.* 1999). Moreover, preliminary observations suggest that this finding may be of physiological relevance as, in the fed state, glucose plasma concentrations are significantly lower in GHR-KO mice than in control animals (F P Dominici *et al.*, unpublished data).

In addition, GHR-KO mice display impaired tolerance to glucose, as determined in glucose tolerance tests. Insulin sensitivity assays suggest that the glucose intolerance is not the result of a reduced tissue sensitivity to insulin. Insulin requirements in terms of glucose homeostasis are dramatically reduced in GHR-KO mice. Thus, in these animals,  $\beta$ -cell function might be reduced, reflecting an adaptive

response to the extremely low need for insulin. The impaired handling of a glucose load may thus, presumably, reflect reduced synthesis or release of insulin (Coschigano *et al.* 1999). Because of these characteristics, GHR-KO mice have a role as a very useful animal model in which to study how the absence of GHR-mediated biological effects of GH influences the insulin signaling pathway. Thus, in the present study we have examined the status of the first steps of the insulin signaling system in liver of GHR-KO *in vivo*. We have previously analyzed insulin signal transduction in liver of transgenic mice overexpressing bovine GH, and showed that this is a suitable tissue in which to study GH and insulin interactions *in vivo* (Dominici *et al.* 1999b).

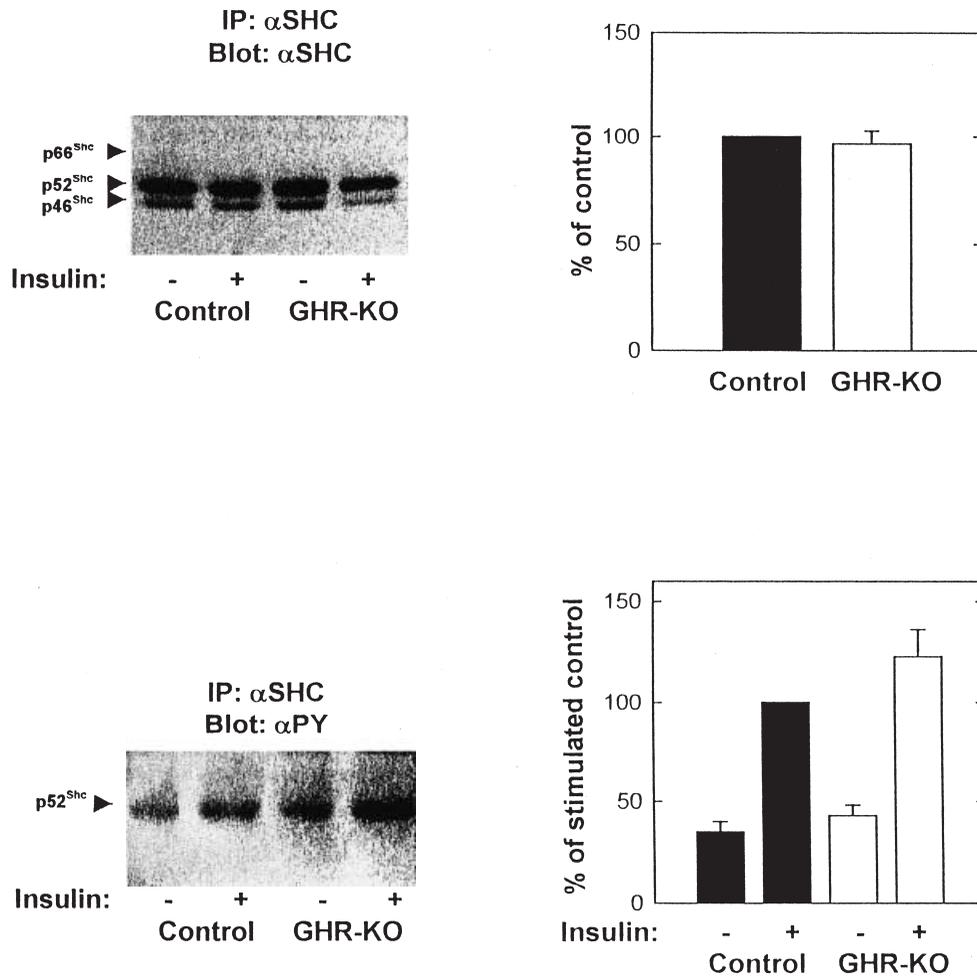


**Figure 4** IRS-1, and phosphotyrosine-associated PI 3-kinase activities in liver of GHR-KO and control mice. Animals were injected with insulin (10 IU/kg) (+) or its diluent (-) in the portal vein. Equal amounts of liver proteins (8 mg), isolated as described in Materials and Methods were incubated with  $\alpha$ IRS-1 (A) or with  $\alpha$ PY (C) and protein A-Sepharose. PI 3-kinase was determined in immunoprecipitates in presence of phosphatidylinositol and [ $\gamma$ - $^{32}$ P]ATP as described in Materials and Methods. Lipid products were separated by thin-layer chromatography (A and C). (B, D) The radioactivity present in spots that co-migrated with a PI 4-phosphate (PIP) standard was quantified by liquid scintillation. Values are expressed as relative to control, assigning a value of 100% to the insulin-stimulated control mean. Data shown in (B) and (D) are means  $\pm$  S.E.M. of four separate experiments in each case ( $n=4$ /group).

The abundance of IR in liver of GHR-KO, as measured by immunoblotting, was markedly increased. This up-regulation could be a long-term consequence of the very low plasma concentrations of insulin observed in GHR-KO mice. A negative relationship between insulin concentrations and the number of IRs has been demonstrated both *in vivo* (Kahn *et al.* 1978, Vigneri *et al.* 1978, Almira & Reddy 1979) and *in vitro* (Gavin *et al.* 1974). Chronic hypoinsulinemic states induced by streptozotocin (STZ) administration, prolonged fasting or hypophysectomy in rodents, are known to produce an increase in the number of IRs in liver (Kahn *et al.* 1978, Vigneri *et al.* 1978, Almira & Reddy 1979). Moreover, although GH concentrations are increased in GHR-KO mice, it is highly unlikely that the increase in this hormone could directly increase the levels of IR in liver. Studies *in vitro*

(Lesniak & Roth 1976, Maloff *et al.* 1980) and *in vivo* (Dominici *et al.* 1998) have demonstrated that GH does not directly affect IR concentration and absence of GHR probably precludes any actions of GH. Transgenic mice overexpressing GH exhibit reduced insulin binding to liver membranes as well as reduced IR protein abundance in liver (Balbis *et al.* 1996, Dominici *et al.* 1998). We have demonstrated that this reduction in GH-transgenic mice is the result of an increase in plasma concentrations of insulin, and that GH, despite being present in large amounts, does not influence the IR levels in liver (Dominici *et al.* 1998).

Our data showed a significant (28%) increase in the insulin-dependent phosphorylation of the IR in liver of GHR-KO mice. However, insulin-stimulated IRS-1 phosphorylation levels in liver of GHR-KO mice were



**Figure 5** Shc abundance and tyrosine phosphorylation in liver of GHR-KO and control mice. Animals were anesthetized, and normal saline (–) or insulin (10 IU/kg) (+) was injected into the portal vein. After 50 s, liver was excised and tissue extraction was performed as described in Materials and Methods. Equal amounts of solubilized liver protein (8 mg) were immunoprecipitated (IP) with an anti-Shc antibody ( $\alpha$ Shc), separated by SDS-PAGE, and subjected to immunoblot analysis with the same antibody (A). (B) Data quantification by scanning densitometry: means  $\pm$  S.E.M. of three separate experiments ( $n=6$ /group), expressed as relative to control values, which were set at 100%. (C) The same samples were immunoprecipitated with  $\alpha$ Shc and immunoblotted with anti-phosphotyrosine antibody PY99 ( $\alpha$ PY) as described in Materials and Methods. (D) Quantification of Shc tyrosine phosphorylation by scanning densitometry: means  $\pm$  S.E.M. of three different experiments ( $n=3$ /group), expressed as relative to control, assigning a value of 100% to the insulin-stimulated control mean.

comparable to those found in normal mice, which suggest that the ability of the IR to use IRS-1 as a substrate is intact in these animals. Similar findings were reported in rats that had been exposed to a 72-h fast, a condition in which, similar to the findings in GHR-KO mice, both insulin and glucose concentrations are less than normal (Saad *et al.* 1992).

Our finding that basal phosphorylation of IRS-1 was normal, with a tendency to be increased, in liver of GHR-KO mice was unexpected. GH has been shown to induce the tyrosine phosphorylation of IRS-1 in liver of

rats *in vivo* (Yamauchi *et al.* 1998, Thirone *et al.* 1999). Basal IRS-1 tyrosine phosphorylation is greatly increased in liver of transgenic mice overexpressing GH (Dominici *et al.* 1999b). Chronic administration of GH also leads to an increase in the basal tyrosine phosphorylation of IRS-1 in liver of intact rats (Thirone *et al.* 1996). On the basis of these findings, we expected that the absence of GH activity would lead to a reduced tyrosine phosphorylation of IRS-1 under basal conditions. However, it was recently shown that prolactin (PRL) is also able to promote the tyrosine phosphorylation of IRS-1, both in 293-PRL

receptor cells (Berlanga *et al.* 1997) and in liver of intact rats (Yamauchi *et al.* 1998). We have recently demonstrated that circulating PRL concentrations are increased almost threefold in GHR-KO mice (Bartke *et al.* 1999, Chandrashekar *et al.* 1999), and thus it is possible to speculate that our current results may be the consequence of the interaction of PRL with IRS-1 through the PRL receptor.

Some contradictory observations have been reported when the relationship between insulin and IRS-1 concentrations in liver *in vivo* was analyzed using various rodent models (Saad *et al.* 1992). IRS-1 protein concentrations were reported to be moderately increased in liver of STZ-treated rats, in which insulin is virtually absent (Saad *et al.* 1992). Conversely, hyperinsulinemic ob/ob mice showed reduced IRS-1 protein concentrations in liver (Saad *et al.* 1992), which led the authors to suggest that IRS-1 concentrations are inversely related to insulin concentrations *in vivo*. However, in the same work it was found that in rats made hypoinsulinemic by a 72-h fast, liver IRS-1 concentrations were not significantly different from those in control rats (Saad *et al.* 1992).

In the present study, we found that IRS-1 protein abundance was unchanged in liver of hypoinsulinemic GHR-KO mice compared with that in normal mice. This is in excellent agreement with our recent demonstration that GH-transgenic mice display normal concentrations of IRS-1 in liver despite being hyperinsulinemic (Dominici *et al.* 1999b). Together with the present results, those observations indicate that insulin may not be the only factor involved in the regulation of IRS-1 protein expression *in vivo*. Other factors, such as changes in the concentrations of glucose or counterregulatory hormones, might also be involved in this regulation.

Insulin-stimulated tyrosine phosphorylation of IRS-1 results in an intracellular signaling step (which involves the docking of the p85 subunit of PI 3-kinase) that parallels the increase in tyrosine phosphorylation of IRS-1. This association results in the activation of PI 3-kinase (Backer *et al.* 1992, Lavan *et al.* 1992). It is becoming apparent that PI 3-kinase plays a central role in signal-transduction pathways linking insulin with many of its specific endpoint cellular responses (Shepherd *et al.* 1998). One of the mechanisms by which insulin regulates glucose homeostasis is by controlling hepatic glucose output; attenuation of glucose-6-phosphatase activity is one of the processes involved, and PI 3-kinase has recently been shown to mediate this attenuation (Mithieux *et al.* 1998). Thus activation of PI 3-kinase represents a principal step in the pathway of insulin action in the liver. Therefore, to characterize the consequences of the lack of GH effects on the insulin signaling cascade further, we have measured the association of IRS-1 with p85 and the PI 3-kinase activity in liver of GHR-KO mice.

Consistent with the tyrosine phosphorylation of IRS-1, both the basal and insulin-stimulated association of p85

with IRS-1 in liver of GHR-KO mice showed normal values. Moreover, whereas basal IRS-1-associated PI 3-kinase activity was found to be slightly but not statistically significantly increased, insulin-stimulated IRS-1-associated activity was found to be unaltered in liver of GHR-KO mice. However, in IRS-1-deficient mice it was demonstrated that insulin may use other signaling molecules such as IRS-2 to activate PI 3-kinase (Araki *et al.* 1994, Tamemoto *et al.* 1994). Therefore, in the present study we also measured the total phosphotyrosine-associated PI 3-kinase in liver of GHR-KO mice. As was found for the IRS-1-associated PI 3-kinase activity, insulin stimulated the total phosphotyrosine-associated PI 3-kinase activity to a similar extent in liver of both control and GHR-KO mice. Taken together, our results suggest that the signaling pathway leading to activation of PI 3-kinase by insulin is not altered in the liver of GHR-KO mice.

The transforming protein Shc is also tyrosine phosphorylated by the IR in response to insulin stimulation (White *et al.* 1997). Although it was shown that insulin is capable of activating the MAP kinase cascade through IRS-1, various reports indicate that Shc has a dominant role in this activation (Sasaoka *et al.* 1994, Yamauchi & Pessin 1994, White 1997). Therefore, to have a more detailed indication of the status of insulin signaling in liver of GHR-KO mice, in the present work we also evaluated the tyrosine phosphorylation of Shc in liver of these mice. Consistent with data on IRS-1, both the abundance and phosphorylation of Shc were found to be unaltered in these animals. Therefore, as Shc and IRS-1 constitute major pathways by which insulin leads to activation of the MAP kinase cascade (Sasaoka *et al.* 1994, Yamauchi & Pessin 1994, White 1997), and in view of our present results showing a non-significant increase in the tyrosine phosphorylation of either of these proteins in liver of GHR-KO mice, it seems reasonable to think that there should be no significant alteration in the activity of MAP kinase in the liver of these animals.

We detected a large increase in the abundance of IR in liver, which most probably reflected receptor up-regulation due to hypoinsulinemia, together with an increase in the insulin-stimulated phosphorylation of the IR in this tissue. In contrast, the post-receptor steps analyzed – namely IRS-1 phosphorylation, Shc phosphorylation, p85-IRS-1 association and the activity of PI 3-kinase associated with IRS-1 and phosphotyrosine – showed values comparable to those found in normal mice. Interestingly, the changes detected on insulin signal transduction in liver of GHR-KO mice are close to being the opposite of those we recently found in liver of transgenic mice overexpressing GH (Dominici *et al.* 1999b). Exposure to chronic GH excess leads to hyperinsulinemia, which in turn produces a down-regulation of the IR in liver. Moreover, we detected that the basal tyrosine phosphorylation of the IR, the tyrosine phosphorylation of

the IRS-1, its association with the p85 subunit of PI 3-kinase, and the phosphotyrosine-associated activity of PI 3-kinase in GH transgenic mice are maximal in the basal state (Dominici *et al.* 1999b).

It appears that the increase in hepatic IR protein concentrations and phosphorylation in GHR-KO mice is sufficient to compensate for reduced insulin signaling in this tissue. Thus the liver of GHR-KO mice is adapted to function in the presence of low concentrations of insulin. We believe that results from the present study support the notion that the concentration and state of phosphorylation of the IR have critical roles in the intracellular signal transduction that leads to the biological actions of insulin on the metabolism (Wilden & Kahn 1994, Patti & Kahn 1998). Therefore, compensatory changes in the IR abundance and tyrosine kinase activity may be sufficient to maintain glucose homeostasis in the presence of a wide range of insulin concentrations.

In summary, we have analyzed the early steps of insulin signaling in liver of GHR-KO mice and found that the abundance of IR in this tissue is increased almost twofold. The *in vivo* insulin-stimulated tyrosine phosphorylation of the IR is increased also. However, in spite of these changes, the subsequent steps of the insulin signaling pathway, namely the insulin-induced tyrosine phosphorylation of Shc and IRS-1, the association of IRS-1 with p85, and the total phosphotyrosine- and IRS-1-associated PI 3-kinase activity are unaltered. These changes may reflect an adaptation to very low circulating concentrations of insulin and could account at least in part for the increased insulin sensitivity displayed by these animals.

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