Liver-Specific *igf-1* Gene Deletion Leads to Muscle Insulin Insensitivity

Shoshana Yakar,[1] Jun-Li Liu,[1] Ana M. Fernandez,[1] Yiping Wu,[1] Andrew V. Schally,[2] Jan Frystyk,[3] Steve D. Chernausek,[4] Wilson Mejia,[1] and Derek Le Roith[1]

Insulin and insulin-like growth factors (IGFs) mediate a variety of signals involved in mammalian development and metabolism. To study the metabolic consequences of IGF-I deficiency, we used the liver IGF-I–deficient (LID) mouse model. The LID mice show a marked reduction (~75%) in circulating IGF-I and elevated growth hormone (GH) levels. Interestingly, LID mice show a fourfold increase in serum insulin levels (2.2 vs. 0.6 ng/ml in control mice) and abnormal glucose clearance after insulin injection. Fasting blood glucose levels and those after a glucose tolerance test were similar between the LID mice and their control littermates. Thus, the high levels of circulating insulin enable the LID mice to maintain normoglycemia in the presence of apparent insulin insensitivity. Insulin-induced autophosphorylation of the insulin receptor and tyrosine phosphorylation of insulin receptor substrate (IRS)-1 were absent in muscle, but were normal in liver and white adipose tissue of the LID mice. In contrast, IGF-I–induced autophosphorylation of its cognate receptor and phosphorylation of IRS-1 were normal in muscle of LID mice. Thus, the insulin insensitivity seen in the LID mice is muscle specific. Recombinant human IGF-I treatment of the LID mice caused a reduction in insulin levels and an increase in insulin sensitivity. Treatment of the LID mice with GH-releasing hormone antagonist, which reduces GH levels, also increased insulin sensitivity. These data provide evidence of the role of circulating IGF-I as an important component of overall insulin action in peripheral tissues. *Diabetes* 50:1110–1118, 2001

Insulin resistance is defined as the failure of target cells to respond to normal levels of circulating insulin. This defect is central to the pathology of a number of diseases and syndromes, including obesity, diabetes, acromegaly, and polycystic ovary syndrome. At the molecular level, insulin resistance is correlated with impaired insulin signaling. This can involve dysfunction of cell surface components, such as the insulin receptor (IR), as well as intracellular components like the insulin receptor substrate (IRS) family of docking proteins, phosphatidylinositol 3′-kinase (PI3′-K), and other elements of the insulin signaling and glucose transport pathways (1).

There is a reciprocal relationship between circulating insulin levels and IR levels on target cells (2–4). Over the last few years, transgenic and knockout mouse models with various defects in the IR and other key elements in the insulin signaling pathways have been developed (1). These animal models have greatly facilitated our understanding of the molecular mechanisms of insulin resistance. Compared with wild-type mouse lines, transgenic mouse lines overexpressing insulin exhibited chronic hyperinsulinemia and decreased levels of the IR, which resulted in insulin resistance and further compensatory hyperinsulinemia. Mice that had the IR disrupted in all tissues (5) or even specifically in liver, muscle (6), or pancreatic β-cells (7) displayed impaired responses to insulin. Disruption of various genes encoding downstream components of the insulin signaling pathway, such as IRS-1 (8), IRS-2, or both (9,10), resulted in marked impairments in insulin-induced activation of PI3′-K, glucose transport, p70 S6 kinase, and mitogen-activated protein kinase in muscle.

In contrast to these studies on insulin and the IR, total deletion of the *igf-1* or *igf-2* gene resulted primarily in growth retardation (11,12). Deletion of the *igf-1* receptor gene in mice was lethal because of severe defects in cell and tissue differentiation (13). Therefore, insulin-like growth factor (IGF)-I is classified primarily as a mitogenic hormone and insulin as a metabolic hormone. Nevertheless, increasing evidence suggests that IGF-I plays a role in glucose homeostasis, lipolysis, proteolysis, and protein oxidation (14). It is important to note that two of the insulin-sensitive tissues, namely liver and adipose tissue, express little if any IGF-I receptors. Thus, any direct effect of IGF-I on glucose metabolism would primarily be mediated by its effects on either pancreatic insulin secretion or on glucose uptake by muscle.

To assess the role of circulating IGF-I in metabolism, we studied mice that lack the *igf-1* gene specifically in liver (liver IGF-I–deficient [LID] mice). LID mice were generated using the Cre/*loxP* system, as described previously (15). These mice exhibit extremely low levels of circulating IGF-I and high levels of growth hormone (GH). Interestingly, the LID mice showed hyperinsulinemia, which

From the [1]Section on Cellular and Molecular Physiology, Cellular Endocrinology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland; [2]Veterans Affairs Medical Center and the Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana; [3]Institute of Experimental Clinical Research, Aarhus University Hospital, Aarhus, Denmark; and [4]Department of Endocrinology, Children's Hospital Medical Center, Cincinnati, Ohio.

Address correspondence and reprint requests to Derek Le Roith, MD, PhD, Clinical Endocrinology Branch, NIDDK, NIH, Room 8D12, Bldg. 10, Bethesda, MD 20892. E-mail: derek@helix.nih.gov.

Received for publication 27 July 2000 and accepted in revised form 29 January 2001.

GH, growth hormone; GHRH, growth hormone–releasing hormone; HPLC, high-performance liquid chromatography; IGF, insulin-like growth factor; IGFBP, IGF-I–binding protein; IR, insulin receptor; IRS, insulin receptor substrate; LID, liver IGF-I–deficient; NIH, National Institutes of Health; PI3′-K, phosphatidylinositol 3′-kinase; rh, recombinant human; RIA, radioimmunoassay; TBS, Tris-buffered saline.

was associated with muscle insulin insensitivity. It is important to note that whereas GH hypersecretion has been linked to worsening of insulin resistance, most studies have not been able to show a direct association between serum IGF-I levels and insulin insensitivity (16,17). Here, we demonstrate that circulating low levels of IGF-I that cause GH hypersecretion can affect insulin action in vivo. We suggest that IGF-I plays an important role in the hormonal balance between GH and insulin. This model provides solid evidence of the importance of the interrelations between IGF-I, GH, and insulin in maintaining normal glucose metabolism.

RESEARCH DESIGN AND METHODS

Animal husbandry and polymerase chain reaction genotyping. The generation of LID mice has been described previously (15). The mice designated as control and LID in these studies express exon four of the *igf-1* gene flanked by two *loxP* sites (designated as LL in the work of Yakar et al. [15]). The LID mice also express the Cre transgene under control of the albumin/enhancer promoter sequence, exclusively in the liver (LL+ in the work of Yakar et al. [15]). Animal genotyping was performed using tail DNA and polymerase chain reaction as described elsewhere (18). All procedures were approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health (NIH) in Bethesda, Maryland.

Insulin, IGF-I, and glucose tolerance tests. Male mice, 6 to 8 weeks old, were anesthetized with pentobarbital (45 μg/g body wt) and maintained on a heating pad for the duration of the procedure. Insulin tolerance tests were performed on fed animals at noon. Human insulin (0.5 IU/kg; Sigma, St. Louis, MO) was injected intraperitoneally after anesthesia set in. At the indicated time points, blood was drawn from the tail vein and glucose levels were measured using a glucometer (Surestep; Lifescan, Milpitas, CA). For IGF-I tolerance tests, the animals were fasted for 6 h, and recombinant human (rh) IGF-I (1 mg/kg; Genentech, San Francisco, CA) was injected intraperitoneally. For the glucose tolerance tests, the animals were fasted overnight, and glucose (1 g/kg) was given orally by gavage.

Serum insulin, GH, and IGF-I levels. Blood was collected from the retroorbital sinus of 6- to 8-week-old male mice. Serum insulin levels were measured using a highly sensitive rat insulin radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO). Serum concentrations of GH in individual mouse samples were determined using an RIA kit (mouse growth hormone assay system; National Hormone and Pituitary Program, Harbor–UCLA Medical Center, Torrance, CA). Serum IGF-I levels were measured by high-performance liquid chromatography (HPLC) as described elsewhere (19). Briefly, samples were incubated overnight at 4°C in an equal volume of 2 × mobile phase (0.2 mol/l acetic acid/0.1 mol/l trimethylamine, pH 2–3) to ensure dissociation from IGF-binding proteins before HPLC. Subsequently, samples were loaded on a Waters Protein-Pak 125 (10 μm) 7.8 × 300–mm column with 1 × mobile phase. Localization of the IGF-I peak was determined by collecting 1-min fractions (flow rate = 0.5 ml/min), and assaying for IGF-I immunoreactivity was determined by RIA using a rabbit anti–IGF-I primary antibody (provided by the National Pituitary Agency of the NIH) and a goat anti–rabbit IgG for precipitation. Low detection value is ~7.5 pg per tube, and the coefficient of variation is <10%.

Free IGF-I levels in serum. To determine the concentration of free IGF-I, sera from three to four mice from the same litter and genotype were pooled. All samples were analyzed in duplicate (20). In brief, serum samples were adjusted to pH 7.4 by being gassed with CO_2. Aliquots of 400 μl were then applied to Amicon YMT30 membranes and MPS-1 supporting devices (Amicon Division, Beverly, MA), incubated 30 min at 37°C, and centrifuged (1,500 rpm at 37°C; model Rotixa/RP; Hettich Zentrifugen, Tuttlingen, Germany). Free IGF-I levels in serum were determined directly from the ultrafiltrates by RIA using a polyclonal rabbit antibody (Nichols Institute Diagnostics, San Capistrano, CA) and rhIGF-I as a standard (Amgen Biologicals, Thousand Oaks, CA).

Levels of serum IGF-I–binding proteins. The levels of IGF-I–binding proteins (IGFBPs) in serum were analyzed by Western ligand blotting assay using I^{125}-IGF-I (Amersham Life Science, Buckinghamshire, U.K.). We mixed 2 μl of serum with 2 μl of protein loading buffer in nonreducing conditions and boiled for 3 min. Serum samples were separated on 4–20% gradient SDS-PAGE followed by Western blotting. Membranes were blocked with Tris-buffered saline (TBS) 1% bovine serum albumin and incubated with I^{125}-IGF-I (1.5×10^6 cpm in TBS 0.1% Tween-20) overnight at 4°C followed by three washes of TBS 0.1% Tween-20. Signals were quantified by phosphoimaging.

In vivo insulin/IGF-I stimulation. Experiments were carried out in 6- to 8-week-old male mice fasted overnight. Animals were anesthetized with pentobarbital (45 μg/g body wt) followed by injection with human insulin (5 IU insulin) or IGF-I (2 mg/kg) through the inferior vena cava. Liver, abdominal white adipose tissue, and hindlimb muscle were removed 1 min after injection and homogenized in homogenization buffer (10 mmol/l Tris pH 7.6, 1% triton X-100, 0.5% NP-40, 150 mmol/l NaCl, 10 mmol/l sodium orthovanadate, 10 mmol/l Na-pyrophosphate, 100 mmol/l Na-fluoride, 1 mmol/l EDTA, 1 mmol/l EGTA, and a cocktail of protease inhibitors [Boehringer Mannheim]). Samples were allowed to solubilize for 30 min on ice, and particulate matter was removed by centrifugation at 42,000 rpm for 1 h at 4°C in a Beckman Ti-70 rotor. Then, 5 mg of tissue extracts was immunoprecipitated with anti–IR-β subunit, anti–IGF-IR-β subunit (Santa Cruz Biotechnology, Santa Cruz, CA), and anti–IRS-1 (Upstate Biotechnology, Lake Placid, NY) antibodies. Immunoprecipitated samples were subjected to SDS-PAGE and Western blotting. Blots were probed with an antiphosphotyrosine antibody (RC20; Transduction Laboratories, Lexington, KY) and signals were detected by enhanced chemiluminescence. Blots were then stripped and reprobed with the above-referenced antibodies for detection of total protein levels. Signals were scanned and band intensities were quantified by optical densitometry of the developed autoradiograms.

Islet morphology. Pancreatic tissues were removed and fixed in 4% paraformaldhyde. Tissues were embedded in paraffin and consecutive 5-μm sections were mounted on slides. Sections were stained with hematoxylin and eosin. For quantification of islet area, sections were viewed using a Zeiss Axiovert S100 TV microscope and video camera magnification of 20×. Two sections of three control and three LID mice were checked systematically by accumulating images from six different fields of 1.5×10^6 μ^2. Analyses of islet size were performed using the MacBAS 2.51 software.

Therapy with IGF-I. Male LID mice were injected intraperitoneally with rhIGF-I (1 mg/kg) twice a day for 20 days. Insulin tolerance tests were performed on day 20. Insulin stimulation via the inferior vena cava, followed by tissue dissection and protein extraction, was performed on day 21 as described above.

Therapy with GH-releasing hormone antagonist. GH-releasing hormone (GHRH) antagonist MZ4-71, [isobutyryl^0, D-arginyl^2, *para*-chlorophenylalanyl^6, a-aminobutyryl^{15}, norleucyl^{25}]hGHRH 1–28 Agm, was synthesized by solid-phase methods and purified as described elsewhere (21). For daily injections, MZ4-71 was dissolved in 0.1% demethyl sulfoxide in 10% propylene glycol/saline solution (Sigma). MZ4-71–GHRH antagonist was injected subcutaneously at 40 μg twice a day for 28 days into 4-week-old male LID mice. Insulin tolerance tests were performed on day 28 as described above.

RESULTS

Metabolic consequences of LID. Liver-specific deletion of the IGF-I gene resulted in a complete abrogation of liver IGF-I mRNA and a 75% reduction in circulating total IGF-I levels (15). However, the LID mice showed normal growth and development. Measurements of body weight, body length, femur length, and organ weight were not different between LID and control mice (15). Extrahepatic IGF-I gene expression was similar in LID and control mice (15). Therefore, we considered the possibility that the local autocrine/paracrine expression of IGF-I maintains normal growth and development, despite the marked reductions in circulating IGF-I levels. On the other hand, despite the dramatic fall in total IGF-I levels, the serum concentrations of free IGF-I did not differ significantly between LID mice (7.35 ± 4.32 ng/ml) and control littermates (5.05 ± 1.24 ng/ml) as shown in Table 1. Thus, the normal growth observed in those mice might be promoted by the free IGF-I.

The low levels of circulating IGF-I in LID mice provide inadequate negative feedback at the level of the hypothalamus and/or pituitary (Table 1). This, in turn, results in GH hypersecretion during puberty and a decrease in insulin sensitivity, particularly in the muscle. To assess the effect of GH hypersecretion on insulin sensitivity in the LID mice, we determined insulin levels in the circulation. As shown in Fig. 1*A*, the LID mice displayed hyperinsulinemia

TABLE 1
Serum levels of IGF-I and GH

Genotype	Total IGF-I (ng/ml)	Free IGF-I (ng/ml)	GH (ng/ml)
Control	260 ± 34	5.05 ± 1.24	2.8 ± 0.5
LID	62 ± 26*	7.35 ± 4.2	17.8 ± 11*

Sera were collected from the retro-orbital sinus of 6- to 8-week-old control and LID mice. Serum levels of total IGF-I, free IGF-I, and GH were determined as indicated in RESEARCH DESIGN AND METHODS. *$P <$ 0.01.

with circulating insulin levels of 2.2 ng/ml, compared with 0.6 ng/ml in control littermates in the fed state ($P < 0.001$). However, blood glucose levels were normal in LID mice (188 ± 9.1 mg/dl) compared with those in their control littermates (192 ± 9.6 mg/dl) (Fig. 1*B*) in the fed state. Insulin tolerance tests showed that the LID mice responded less efficiently to exogenous administration of insulin than did control mice (Fig. 1*C*). In control mice, 30 and 60 min after insulin injections, blood glucose levels fell to 85 and 71% of basal levels, respectively ($P = 0.001$). In contrast, in LID mice, blood glucose levels were reduced to only 90% of their basal levels even 60 min after insulin injection ($P = 0.2$), suggesting that the LID mice have peripheral insulin insensitivity. However, intraperitoneal injection of IGF-I into the LID mice caused a reduction in blood glucose similar to that in their control littermates (Fig. 1*D*). Furthermore, the LID mice were able to clear a bolus of glucose with the same efficiency as control littermates (Fig. 1*E*). Thus, the high levels of insulin seen in the LID mice maintain normoglycemia. Taken together, these data demonstrate that the LID mice show peripheral insulin insensitivity.

IGFBPs. In the circulation, IGF-I is bound to IGFBPs, six of which have been well characterized. IGFBPs increase the half-life of IGF-I in circulation and regulate its action.

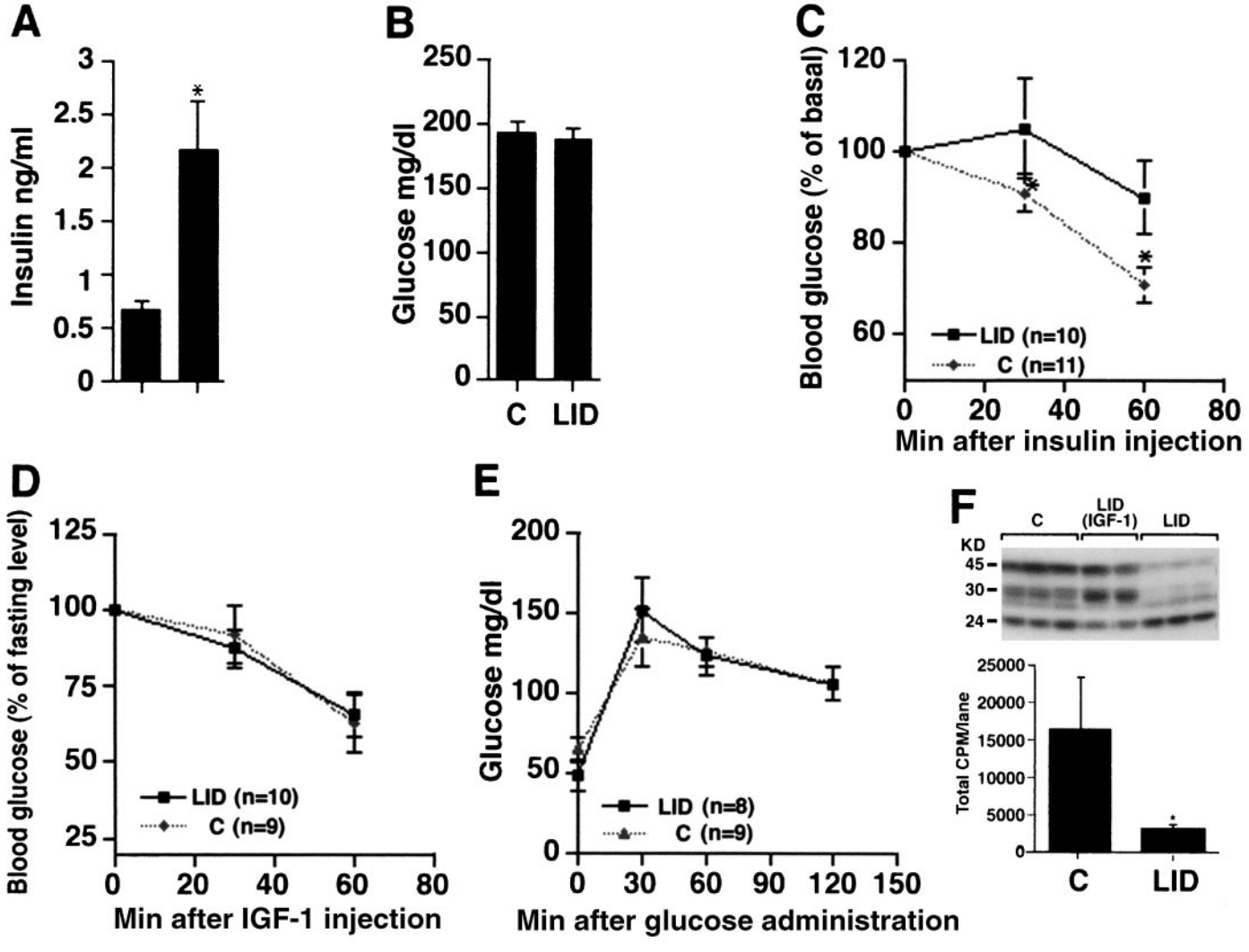

FIG. 1. LID mice are insulin resistant. *A*: Serum insulin levels, measured in the fed state, are fourfold higher in LID mice ($n = 26$) compared with controls (C) ($n = 19$). *B*: Blood glucose levels in the fed state are normal in LID mice ($n = 16$) as compared with their control littermates ($n = 11$). *C*: Insulin tolerance tests were performed on control and LID mice. Results are expressed as the mean percentage of basal blood glucose concentration ± SE. *D*: IGF-I tolerance tests are similar between control and LID mice. Results are expressed as mean percentage of fasted blood glucose concentration ± SE. *E*: The LID mice maintain normoglycemia. Results of glucose tolerance tests are expressed as mean blood glucose concentration ± SE (*$P < 0.01$). *F*: Serum IGFBP-3 and IGFBP-1 are decreased in LID mice. Representative ligand blot assay of serum from control, LID, and LID mice after IGF-I treatment. Proteins were separated on 4–20% SDS-PAGE and IGFBPs were detected by incubation with I^{125}–IGF-I; the graph below represents quantification of total I^{125}–IGF-I binding per lane.

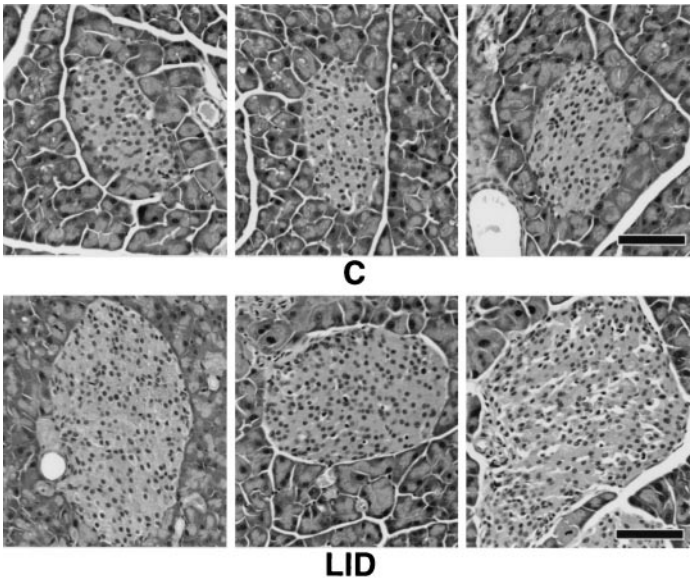

FIG. 2. Islet morphology in control (C) and LID mice. Hematoxylin and eosin staining of pancreatic sections. Representative islets from three control and three LID mice. Images are shown as 20× magnification; the bar represents 50 μm.

Determination of serum IGFBP levels using a ligand blot assay with I^{125}–IGF-I revealed the presence of three distinct IGFBP populations in serum of control mice: IGFBP-1, -3, and -4 (Fig. 1*F*). The doublet at 45 kDa represents glycosylation variants of IGFBP-3. The band of 30 kDa corresponds to IGFBP-1, whereas the band at 24 kDa corresponds to IGFBP-4. The abundance of IGFBP-3 and IGFBP-1 was decreased to ~15% of control levels. In contrast, the abundance of IGFBP-4 was not significantly affected by the dramatic reduction of serum IGF-I levels in the LID mice. Measurements of IGFBPs after IGF-I treatment for 20 days (LID [IGF-I]) revealed that IGFBP-3 and -1 levels in the LID mice were restored to control levels (Fig. 1*F*).

Islet morphology in LID mice. In view of the differences in circulating insulin and GH levels between control and LID mice, we analyzed islet morphology. As shown in Fig. 2, LID mice showed an approximately twofold increase in islet size, $(163 \pm 23) \times 10^2$ μm^2 in LID mice versus $(86 \pm 11) \times 10^2$ μm^2 in control animals ($P < 0.003$). Quantitative analysis of pancreatic sections indicates that the increase in islet size was due to an increase in β-cell number (i.e., hyperplasia).

Insulin signaling in LID mice. To define the molecular mechanism(s) of insulin resistance in the LID mice, we compared the early signaling events after insulin stimulation in liver, white adipose tissue, and skeletal muscle from LID mice with that of control littermates. As shown in Fig. 3*A*, insulin stimulated a twofold increase in tyrosine phosphorylation of the IR in liver of both control and LID mice. Similarly, insulin stimulated tyrosine phosphorylation of IRS-1 by approximately twofold in liver samples from control and LID mice. Analysis of early insulin signaling events in white adipose tissue revealed that insulin stimulated a twofold increase of tyrosine phosphorylation of both the IR β subunit and the IRS-1 associated with the IR in control and LID mice (Fig. 3*B*). In contrast, while insulin also stimulated a twofold increase in tyrosine phosphorylation of IR and IRS-1 in muscle of control mice, there was little or no effect of insulin on IR and IRS-1 phosphorylation in skeletal muscle from LID mice (Fig. 4*A*). Moreover, in most of the uninjected LID mice, the basal tyrosine phosphorylation of IRS-1 was elevated (compared with control animals, $P < 0.01$). This basal increase in IRS-1 phosphorylation is most likely due to constitutively elevated levels of insulin and/or GH in the circulation. Determination of IR protein levels in muscle of LID and control mice by Western blotting revealed no change in the IR protein expression (data not shown). It remains somewhat surprising that hyperinsulinemia did not cause downregulation in the IR.

Analysis of early IGF-I signaling events in skeletal muscle revealed that IGF-I stimulated a twofold increase of tyrosine phosphorylation of both the β-subunit of the IGF-I receptor and IRS-1 in control and LID mice (Fig. 4*B*). Taken together, these studies show that tyrosine phosphorylation of the IR in response to insulin stimulation is virtually abolished in muscle but remains normal in the liver and white adipose tissues of LID mice. Furthermore, the insulin insensitivity seen in skeletal muscle of LID mice is specific to the IR, since signaling through the IGF-IR (i.e., tyrosine phosphorylation of the IGF-IR and IRS-1) was normal.

Increased insulin sensitivity in muscle of LID mice after IGF-I administration. In order to examine whether administration of IGF-I could increase sensitivity to insulin in muscle, LID mice were injected with rhIGF-I for 20 days. After IGF-I treatment, insulin tolerance tests showed that blood glucose fell to 40% of basal levels compared with LID mice treated with saline, in which blood glucose fell to only 90% of basal levels (Fig. 5*A*). After IGF-I injections, serum insulin levels fell by sevenfold, as compared with saline-treated LID mice (Fig. 5*B*). These results are consistent with an IGF-I–induced increase in sensitivity to insulin and possible inhibition of insulin secretion. Serum GH levels, determined after IGF-I treatment, were reduced to normal levels (Fig. 6*A*). Furthermore, after IGF-I treatment, insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 in muscle of the LID mice (Fig. 5*C* and *D*) was comparable to that seen in the control mice. This is in striking contrast to the lack of insulin-induced tyrosine phosphorylation of the IR and IRS-1 in naive LID mice (Fig. 4*A*).

Increased peripheral insulin sensitivity in the LID mice after treatment with GHRH antagonist. To examine whether suppression of endogenous GH secretion will restore insulin sensitivity, we injected LID mice with GHRH antagonist MZ4-71. This antagonist was studied in mouse and rat models of GH hypersecretion and was proved to suppress endogenous GH secretion both in basal and pulsatile levels (22–24). After treatment with MZ4-71 for 28 days, insulin tolerance tests showed that blood glucose fell to 80% of basal levels compared with LID mice treated with vehicle, in which blood glucose did not change even 60 min after insulin injection (Fig. 6*B*). Serum GH levels, determined after GHRH antagonist treatment, were reduced but were not inhibited completely as seen with IGF-I treatment (Fig. 6*A*). These data suggest that GH plays a major role in reducing insulin sensitivity. However, the insulin tolerance test demonstrates that inhibition of

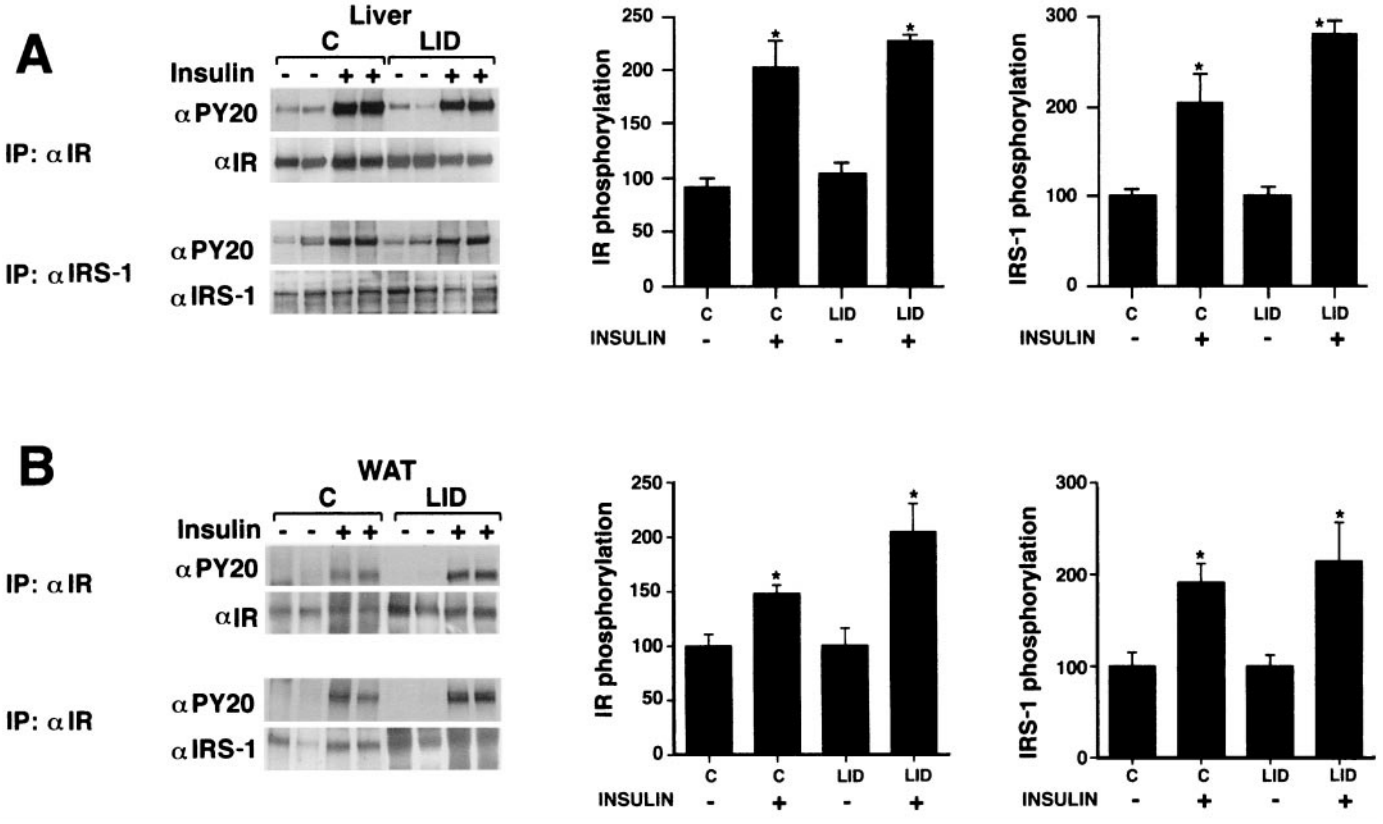

FIG. 3. Normal insulin signaling in liver and white adipose tissue of LID mice. The tyrosine phosphorylation state of the IR β-subunit and IRS-1 in liver and white adipose tissue were analyzed in LID mice and control (C) littermates. *A*: Insulin-stimulated tyrosine phosphorylation of both IR and IRS-1 in liver of control (n = 3 nonstimulated, n = 4 stimulated) and LID (n = 3 nonstimulated, n = 3 stimulated) mice. *B*: Insulin stimulated tyrosine phosphorylation of both IR and IRS-1 in white adipose tissue (WAT) of control (n = 4 nonstimulated, n = 4 stimulated) and LID (n = 7 nonstimulated, n = 10 stimulated) mice. The levels of phosphotyrosine signals are normalized to total immunoreactivity of IR or IRS-1. (*P < 0.01).

GH secretion using the MZ4-71 antagonist did not increase peripheral insulin sensitivity to the same level as in control mice (Fig. 1*C*). Moreover, determination of serum insulin levels after the MZ-4-71 antagonist treatment showed that insulin levels did not reduce to normal levels (1.84 ± 0.25 ng/ml). These data suggest that circulating IGF-I may play a direct role in increasing insulin sensitivity.

DISCUSSION

In this study, we demonstrate that LID mice, which have a liver-specific deletion of the IGF-I gene, exhibit peripheral insulin insensitivity at the level of the IR. These mice display very low levels of circulating IGF-I together with GH hypersecretion, which is secondary to the IGF-I deficiency. It has been well documented that GH has antagonistic effects on insulin actions. For example, although insulin increases lipogenesis and glucose uptake, GH promotes lipolysis, increases serum levels of nonesterified fatty acids, and decreases glucose metabolism (25). Moreover, in humans, whereas insulin is released in response to high circulating levels of glucose, GH is secreted in response to hypoglycemia (26). Chronic elevations of circulating GH, such as that seen in acromegaly (27) or GH-producing tumors (28), lead to the development of insulin resistance, impaired glucose tolerance, hyperinsulinemia, and, in some cases, diabetes (29). However, although the anti-insulin effects of GH are well known, the exact molecular mechanism by which GH induces insulin insensitivity has not yet been elucidated. As demonstrated in Figs. 1 and 3, LID mice show hyperinsulinemia and peripheral insulin insensitivity as determined by the insulin tolerance test. However, these mice maintain normoglycemia. Similarly, GH (human GH and bovine GH) transgenic mice, despite their hyperinsulinemia, always show normal blood glucose levels and rarely develop diabetes (25).

The metabolic characteristics of the LID mice suggested that they were relatively insensitive to insulin in peripheral tissues. We examined the effect of insulin on liver, white adipose tissue, and muscle. In liver and white adipose tissue, we found that insulin similarly stimulated the phosphorylation of the IR and IRS-1 in LID and control mice. In muscle, however, there was no detectable stimulation of tyrosine phosphorylation of either IR or IRS-1 in the LID mice in response to insulin injections. Thus, these mice clearly have impaired insulin signaling in muscle. Comparable with our results, bovine GH transgenic mice, which also exhibit peripheral insulin insensitivity, show normal response to insulin in liver as determined by IR β-subunit phosphorylation during insulin stimulation (30). However, in the muscle of these mice there is a reduction in IR protein levels as well as in IR tyrosine phosphorylation during insulin stimulation. Additionally, resembling our results, the bovine GH transgenic mice have increased basal phosphorylation of IRS-1 in muscle, which may also contribute to the insulin insensitivity seen in those mice.

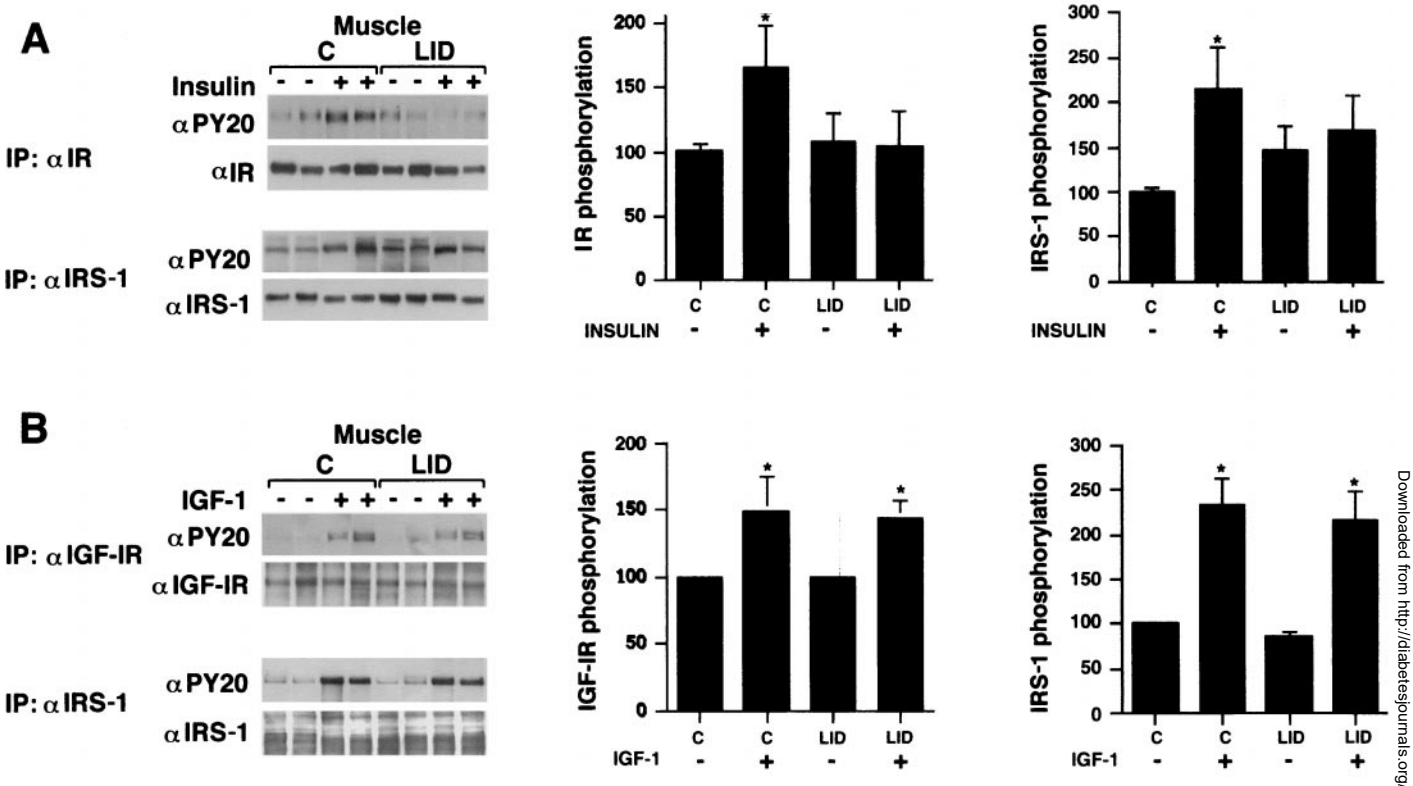

FIG. 4. LID mice show impaired insulin signaling in skeletal muscle. *A*: Insulin failed to stimulate IR and IRS-1 phosphorylation in muscle of LID mice ($n = 10$ nonstimulated, $n = 9$ stimulated) as compared with control littermates ($n = 3$ nonstimulated, $n = 6$ stimulated). *B*: IGF-I stimulated tyrosine phosphorylation of both IGF-IR and IRS-1 in muscle of control ($n = 3$ nonstimulated, $n = 3$ stimulated) and LID ($n = 3$ nonstimulated, $n = 3$ stimulated) mice. The levels of phosphotyrosine signals are normalized to total immunoreactivity of IR, IGF-IR, or IRS-1. (*$P < 0.01$).

Other studies performed in rats have shown that chronic administration of GH caused impaired insulin signaling, specifically in muscle (31,32).

The mechanism by which insulin signaling is impaired in LID mice is not known. However, inhibition of insulin action can be mediated by agents that increase serine/threonine (Ser/Thr) phosphorylation of the IR or its downstream effectors such as the IRS proteins (33). Ser/Thr–phosphorylated sites on IR are thought to interfere with the enzymatic tyrosine kinase activity of the IR and thereby its ability to phosphorylate downstream substrates. For example, it has been shown that chronic stimulation by insulin may cause activation of Akt (a Ser/Thr kinase), which was found to negatively regulate the insulin-induced association of IRS-1 and PI3′-K. This feedback inhibition of Akt appears to drive the development of insulin resistance that occurs during periods of hyperinsulinemia (34). Another mechanism by which insulin signaling can be downregulated is through the dephosphorylation of the IR and its substrates by protein tyrosine phosphatases. It has been shown that protein tyrosine phospatase 1B regulates both the mitogenic and metabolic actions of insulin (35). An elevation in the basal level of tyrosine phosphorylation of IRS-1 was seen in most of the LID mice, which most likely resulted from high circulating levels of GH and/or insulin. It has been shown that GH can activate members of the insulin signaling pathway, IRS-1 and IRS-2 (36,37). Although the nature of the interaction between the IRS molecules and the GHR/JAK2 complex is not clear, it appears that JAK2 activation is involved in the insulin-like effect of GH on tyrosine phosphorylation of IRS-1 and IRS-2. Moreover, activation of the GHR by high levels of circulating GH may also cause activation of Ser/Thr kinases that could negatively regulate the insulin receptor kinase activity (38). As demonstrated in Fig. 4, IGF-I induced tyrosine phosphorylation of the IGF-IR and its downstream effector IRS-1 to the same extent in muscle of LID and control mice. This finding suggests that the downstream components of the insulin signaling pathway, such as IRS-1, are functioning normally in response to stimulation of other receptor tyrosine kinases and strengthens the observation that the insulin insensitivity is at the level of IR.

The exact function of a large reservoir of bound IGF-I in serum is unknown. No other growth factor has been found to circulate at such high concentrations. IGFBP-3 is the primary carrier of IGF-I in serum and is an important regulator of IGF-I action. There appears to be a positive correlation between the amount of IGF-I produced by the liver and the serum levels of IGFBP-3. On the other hand, despite the marked reduction in bound IGF-I in serum, serum concentrations of free IGF-I were not changed. Because liver production of IGF-I in the LID mice is abrogated, it can be inferred that the free form of IGF-I is secreted by extrahepatic tissues such as fat, muscle, or bone. IGF-I bioactivity is usually related to the free form of IGF-I. However, it remains unclear how the normal levels of free IGF-I, which could partially explain the normal growth and development of the LID mice, were not capable of suppressing GH secretion at the level of pituitary. LID mice demonstrated a sixfold reduction in serum levels of IGFBP-1 and IGFBP-3. Insulin levels are inversely related

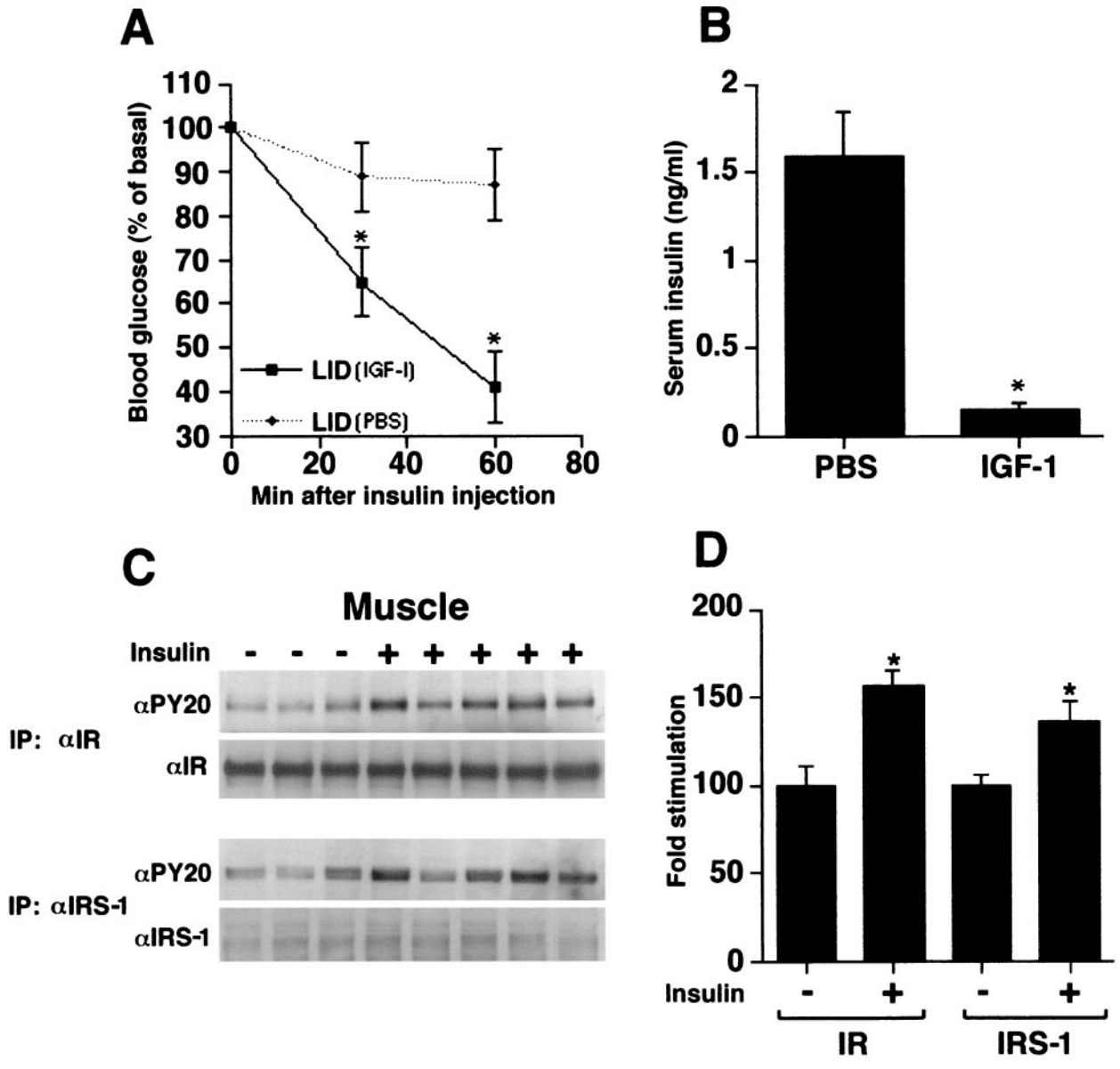

FIG. 5. Increased insulin sensitivity in muscle of LID mice after IGF-I treatment. *A*: Insulin tolerance tests were performed on LID mice treated with IGF-I (n = 16) or phosphate-buffered saline (n = 19) for 20 days. Results are expressed as mean percent of basal blood glucose concentration ± SE. *B*: Serum insulin levels were measured in LID mice treated with either IGF-I (n = 7) or saline (n = 4). *C*: The effect of insulin on tyrosine phosphorylation of the IR and IRS-1 was measured in muscle of LID mice after either saline (-) or IGF-I (+) treatment. *D*: Quantification of insulin-stimulated IR β-subunit and IRS-1 tyrosine phosphorylation in muscle of LID mice either untreated (n = 3) or treated with IGF-I (n = 5). The levels of phosphotyrosine signals are normalized to total immunoreactivity for either IR or IRS-1. (*P < 0.01).

to those of IGFBP-1, as it has been shown that insulin directly inhibits the production of IGFBP-1 in human hepatoma cell lines (39,40). It is therefore likely that the high levels of circulating insulin in LID mice may have caused the reduction in IGFBP-1 levels. Changes in IGFBP-3 in serum are often used as an index of GH action in humans. However, despite the elevated GH levels in the circulation, there was a marked reduction in IGFBP-3 levels in the LID mice, suggesting that the main regulator of IGFBP-3 in the circulation, at least in mice, is IGF-I.

In view of the elevated circulating levels of insulin in LID mice, we examined the morphology of the pancreas and found that these mice manifest pancreatic hyperplasia. This result could be secondary to the muscle insulin resistance seen in the LID mice. On the other hand, it was also shown that GH could increase β-cell DNA synthesis in cultures of isolated islets, and that the mitogenic effects of GH on islets are mediated in part by an increase in local release of IGF-I (41). The increase in GH secretion in the LID mice was detected even early in postnatal development (at 3–4 weeks). Increased GH may cause local release of IGF-I and thereby increase the mitogenic activity of the β-cells. Apart from inhibiting GH secretion, IGF-I also suppresses insulin secretion in isolated perfused rat pancreas and in humans (42–45). Restoration of circulating IGF-I levels in the LID mice, using a recombinant peptide, suppressed endogenous GH secretion, led to reductions in insulin levels, and improved peripheral insulin sensitivity. At the molecular level, insulin was able to stimulate tyrosine phosphorylation of the IR and IRS-1 in muscle of LID mice treated with IGF-I.

Various experimental and clinical data demonstrate the

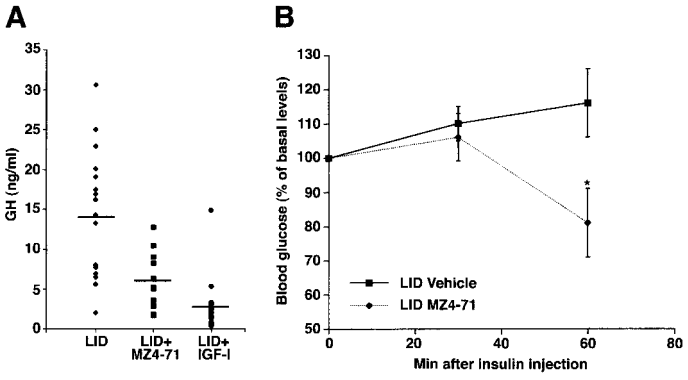

FIG. 6. *A*: GH levels determined using RIA in LID mice (n = 17), LID mice treated with IGF-I for 20 days (n = 17), and LID mice treated with GHRH antagonist, MZ4-71, for 28 days (n = 15). Horizontal lines represent the mean in each group. *B*: Increased insulin sensitivity in muscle of LID mice followed GHRH antagonist treatment. Insulin tolerance tests were performed on LID mice treated with MZ4-71 (n = 12) or phosphate-buffered saline (n = 5) for 28 days. Results are expressed as mean percent of basal blood glucose concentration ± SE.

essential role of GHRH in the control of GH secretion and regulation of linear growth. It has been shown that administration of GHRH antiserum to young rats can significantly retard their growth rate by inhibiting basal and/or pulsatile GH release. Recently, a potent and specific antagonist of GHRH was developed—MZ4-71. The MZ4-71 was proven to inhibit the growth rate of young rats. Chronic injections of the antagonist lowered the sensitivity of GH secretory response to GHRH and caused 85–91% reduction in circulating GH (22). Furthermore, in a mouse model of GHRH transgenic mice, in which overproduction of GHRH results in a dramatic increase in pituitary GH and GH mRNA contents coupled with increased GH secretion, the MZ4-71 was able to reduce serum GH concentrations by 70% (23,24). In an attempt to verify the role of GH hypersecretion in insulin insensitivity in LID mice, we treated those mice with the MZ4-71 antagonist. Treatment of the LID mice with GHRH antagonist, which reduces endogenous GH secretion, resulted in improved insulin sensitivity. Insulin tolerance tests showed that LID mice, treated with the antagonist, were able to respond to exogenous administration of insulin and significantly reduced blood glucose levels to 80% of basal state, compared with mice not receiving the antagonist in which blood glucose levels did not change. However, this treatment was not able to fully restore the insulin sensitivity to the extent seen in control mice in which blood glucose levels were reduced to 70% of basal levels after insulin administration. Unlike IGF-I treatment, in which insulin levels were decreased dramatically, determination of insulin levels after the MZ-4-71 treatment showed that insulin levels remained high and were not reduced to normal levels. This result might point to a direct role for circulating IGF-I in insulin sensitivity and action in addition to its effect on reducing circulating GH levels.

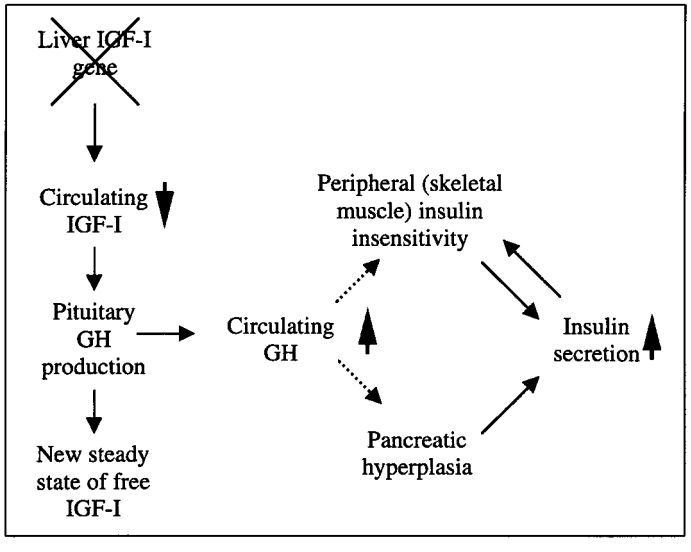

FIG. 7. A model for the role of liver-derived circulating IGF-I in muscle insulin sensitivity. Liver-specific *igf-1* gene deletion is associated with a marked reduction in circulating total IGF-I levels and elevated GH levels. Consequently, insulin insensitivity at the level of the muscle as well as islet cell hyperplasia are associated with hyperinsulinemia.

In the past, it has been argued that IGFs were unlikely to have any direct metabolic role, particularly with respect to glucose homeostasis. However, more recent data indicate that circulating IGF-I and its binding proteins, particularly IGFBP-1, are capable of modulating glucose levels and may have direct effects on glucose homeostasis (14,39,40). Clinical studies performed on normal subjects as well as patients with type 1 and type 2 diabetes (44,46–49) showed that IGF-I enhances insulin action. rhIGF-I administration significantly lowered blood glucose as reflected by short-term and long-term indexes of glycemic control and increased insulin sensitivity. It has been shown that rhIGF-I directly suppresses insulin and GH secretion in the treated subjects.

In summary, the results of the present study provide strong support for an important role of circulating IGF-I in maintaining the hormonal balance between GH and insulin and in controlling glucose homeostasis (Fig. 7). Further studies will be required to determine whether the effect is indirect via GH suppression or a direct effect of IGF-I at the level of the muscle.

ACKNOWLEDGMENTS

Determination of free IGF-I was funded by the Aarhus University–Novo Nordisk Center for Research in Growth and Regeneration (Danish Health Research Council Grant no. 9700592).

The authors thank Dr. Oksana Gavrilova, Dr. Nicola Perrotti, and Dr. Joelle DuPont (Bethesda, MD) for helpful discussion; Bernice Samuels (Bethesda, MD) and Walter Banach (Cincinnati, OH) for technical assistance; and Dr. A.F. Parlow (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA) for help in establishing the GH RIA.

REFERENCES

1. Lamothe B, Baudry A, Desbois P, Lamotte L, Bucchini D, De Meyts P, Joshi RL: Genetic engineering in mice: impact on insulin signalling and action. *Biochem J* 335:193–204, 1998
2. Bar RS, Gorden P, Roth J, Kahn CR, De Meyts P: Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients: effects of starvation, refeeding, and dieting. *J Clin Invest* 58:1123–1135, 1976
3. Gavin JRD, Roth J, Neville DM Jr, De Meyts P, Buell DN: Insulin-dependent regulation of insulin receptor concentrations: a direct demonstration in cell culture. *Proc Natl Acad Sci U S A* 71:84–88, 1974
4. Soll AH, Kahn CR, Neville DM Jr: Insulin binding to liver plasma mem-

branes in the obese hyperglycemic (*ob/ob*) mouse: demonstration of a decreased number of functionally normal receptors. *J Biol Chem* 250: 4702–4707, 1975
5. Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H: Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet* 12:106–109, 1996
6. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR: A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* 2:559–569, 1998
7. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR: Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329–339, 1999
8. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S: Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182–186, 1994
9. Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF, Accili D: Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *J Clin Invest* 105:199–205, 2000
10. Rother KI, Imai Y, Caruso M, Beguinot F, Formisano P, Accili D: Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. *J Biol Chem* 273:17491–17497, 1998
11. Powell-Braxton L, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, Gillett N, Stewart TA: IGF-I is required for normal embryonic growth in mice. *Genes Dev* 7:2609–2617, 1993
12. Baker J, Liu JP, Robertson EJ, Efstratiadis A: Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75:73–82, 1993
13. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A: Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75:59–72, 1993
14. Froesch ER, Hussain MA, Schmid C, Zapf J: Insulin-like growth factor I: physiology, metabolic effects and clinical uses. *Diabete Metab Rev* 12:195–215, 1996
15. Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B, LeRoith D: Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proc Natl Acad Sci U S A* 96:7324–7329, 1999
16. Dunger DB, Cheetham TD: Growth hormone insulin-like growth factor I axis in insulin-dependent diabetes mellitus. *Horm Res* 46:2–6, 1996
17. Dunger DB, Acerini CL: IGF-I and diabetes in adolescence. *Diabete Metab* 24:101–107, 1998
18. Liu JL, Grinberg A, Westphal H, Sauer B, Accili D, Karas M, LeRoith D: Insulin-like growth factor-I affects perinatal lethality and postnatal development in a gene dosage-dependent manner: manipulation using the Cre/loxP system in transgenic mice. *Mol Endocrinol* 12:1452–1462, 1998
19. Chernausek SD, Dickson BA, Smith EP, Hoath SB: Suppression of insulin-like growth factor I during epidermal growth factor-induced growth retardation. *Am J Physiol* 260:E416–E421, 1991
20. Frystyk J, Delhanty PJ, Skjaerbaek C, Baxter RC: Changes in the circulating IGF system during short-term fasting and refeeding in rats. *Am J Physiol* 277:E245–E252, 1999
21. Zarandi M, Horvath JE, Halmos G, Pinski J, Nagy A, Groot K, Rekasi Z, Schally AV: Synthesis and biological activities of highly potent antagonists of growth hormone–releasing hormone. *Proc Natl Acad Sci U S A* 91:12298–12302, 1994
22. Kovacs M, Zarandi M, Halmos G, Groot K, Schally AV: Effects of acute and chronic administration of a new potent antagonist of growth hormone–releasing hormone in rats: mechanisms of action. *Endocrinology* 137: 5364–5369, 1996
23. Kovacs M, Kineman RD, Schally AV, Zarandi M, Groot K, Frohman LA: Effects of antagonists of growth hormone–releasing hormone (GHRH) on GH and insulin-like growth factor I levels in transgenic mice overexpressing the human GHRH gene, an animal model of acromegaly [see comments]. *Endocrinology* 138:4536–4542, 1997
24. Kovacs M, Schally AV, Zarandi M, Groot K: Inhibition of GH release of rats by new potent antagonists of growth hormone–releasing hormone (GH-RH). *Peptides* 18:431–438, 1997
25. Kopchick JJ, Bellush LL, Coschigano KT: Transgenic models of growth hormone action. *Annu Rev Nutr* 19:437–461, 1999
26. Casanueva FF: Physiology of growth hormone secretion and action. *Endocrinol Metab Clin North Am* 21:483–517, 1992
27. Hansen I, Tsalikian E, Beaufrere B, Gerich J, Haymond M, Rizza R: Insulin resistance in acromegaly: defects in both hepatic and extrahepatic insulin action. *Am J Physiol* 250:E269–E273, 1986
28. Moller DE, Flier JS: Insulin resistance: mechanisms, syndromes, and implications [see comments]. *N Engl J Med* 325:938–948, 1991
29. Sonksen PH, Salomon F, Cuneo R: Metabolic effects of hypopituitarism and acromegaly. *Horm Res* 36:27–31, 1991
30. Dominici FP, Cifone D, Bartke A, Turyn D: Alterations in the early steps of the insulin-signaling system in skeletal muscle of GH-transgenic mice. *Am J Physiol* 277:E447–E454, 1999
31. Smith TR, Elmendorf JS, David TS, Turinsky J: Growth hormone–induced insulin resistance: role of the insulin receptor, IRS-1, GLUT-1, and GLUT-4. *Am J Physiol* 272:E1071–E1079, 1997
32. Thirone AC, Carvalho CR, Brenelli SL, Velloso LA, Saad MJ: Effect of chronic growth hormone treatment on insulin signal transduction in rat tissues. *Mol Cell Endocrinol* 130:33–42, 1997
33. Qiao LY, Goldberg JL, Russell JC, Sun XJ: Identification of enhanced serine kinase activity in insulin resistance. *J Biol Chem* 274:10625–10632, 1999
34. Li J, DeFea K, Roth RA: Modulation of insulin receptor substrate-1 tyrosine phosphorylation by an Akt/phosphatidylinositol 3-kinase pathway. *J Biol Chem* 274:9351–9356, 1999
35. Shalev A: The crucial role of a phosphatase in insulin resistance and obesity. *Eur J Endocrinol* 141:323–324, 1999
36. Argetsinger LS, Hsu GW, Myers MG Jr, Billestrup N, White MF, Carter-Su C: Growth hormone, interferon-gamma, and leukemia inhibitory factor promoted tyrosyl phosphorylation of insulin receptor substrate-1. *J Biol Chem* 270:14685–14692, 1995
37. Argetsinger LS, Norstedt G, Billestrup N, White MF, Carter-Su C: Growth hormone, interferon-gamma, and leukemia inhibitory factor utilize insulin receptor substrate-2 in intracellular signaling. *J Biol Chem* 271:29415–29421, 1996
38. Cengel KA, Freund GG: JAK1-dependent phosphorylation of insulin receptor substrate-1 (IRS-1) is inhibited by IRS-1 serine phosphorylation. *J Biol Chem* 274:27969–27974, 1999
39. Ooi GT, Tseng LY, Tran MQ, Rechler MM: Insulin rapidly decreases insulin-like growth factor-binding protein-1 gene transcription in streptozotocin-diabetic rats. *Mol Endocrinol* 6:2219–2228, 1992
40. Powell DR, Suwanichkul A, Cubbage ML, DePaolis LA, Snuggs MB, Lee PD: Insulin inhibits transcription of the human gene for insulin-like growth factor-binding protein-1. *J Biol Chem* 266:18868–18876, 1991
41. Hill DJ, Hogg J: Growth factor control of pancreatic B cell hyperplasia. *Baillieres Clin Endocrinol Metab* 5:689–698, 1991
42. Leahy JL, Vandekerkhove KM: Insulin-like growth factor-I at physiological concentrations is a potent inhibitor of insulin secretion. *Endocrinology* 126:1593–1598, 1990
43. Guler HP, Schmid C, Zapf J, Froesch ER: Effects of recombinant insulin-like growth factor I on insulin secretion and renal function in normal human subjects. *Proc Natl Acad Sci U S A* 86:2868–2872, 1989
44. Zenobi PD, Graf S, Ursprung H, Froesch ER: Effects of insulin-like growth factor-I on glucose tolerance, insulin levels, and insulin secretion. *J Clin Invest* 89:1908–1913, 1992
45. Hussain MA, Schmitz O, Mengel A, Keller A, Christiansen JS, Zapf J, Froesch ER: Insulin-like growth factor I stimulates lipid oxidation, reduces protein oxidation, and enhances insulin sensitivity in humans. *J Clin Invest* 92:2249–2256, 1993
46. Clemmons DR, Moses AC, McKay MJ, Sommer A, Rosen DM, Ruckle J: The combination of insulin-like growth factor I and insulin-like growth factor-binding protein-3 reduces insulin requirements in insulin-dependent type 1 diabetes: evidence for in vivo biological activity. *J Clin Endocrinol Metab* 85:1518–1524, 2000
47. Kuzuya H, Matsuura N, Sakamoto M, Makino H, Sakamoto Y, Kadowaki T, Suzuki Y, Kobayashi M, Akazawa Y, Nomura M, et al.: Trial of insulin-like growth factor I therapy for patients with extreme insulin resistance syndromes. *Diabetes* 42:696–705, 1993
48. Moses AC, Young SC, Morrow LA, O'Brien M, Clemmons DR: Recombinant human insulin-like growth factor I increases insulin sensitivity and improves glycemic control in type II diabetes. *Diabetes* 45:91–100, 1993
49. Rennert NJ, Caprio S, Sherwin RS: Insulin-like growth factor I inhibits glucose-stimulated insulin secretion but does not impair glucose metabolism in normal humans. *J Clin Endocrinol Metab* 76:804–806, 1993