Diabetes In Press, published online March 15, 2007

Growth Hormone Regulation of p85α Expression and Phosphoinositide 3-Kinase Activity in Adipose Tissue: Mechanism for Growth Hormone-Mediated Insulin Resistance

Received for publication on 3 March 2006 and accepted in revised form 20 February 2007.

Juan-Pablo del Rincon[1], Keiji Iida[1], Bruce D. Gaylinn[1], Carrie E. McCurdy[2], J. Wayne Leitner[3], Linda A. Barbour[4,5], John J. Kopchick[6], Jacob E. Friedman[2], Boris Draznin[3,4], and Michael O. Thorner[1]

From the [1]Department of Internal Medicine, University of Virginia, Charlottesville, VA; [2]Department of Pediatrics, University of Colorado Health Sciences Center; [3]Research Service, Denver VA Medical Center, Denver, CO; [4]Department of Medicine, University of Colorado Health Sciences Center; [5]Department of Obstetrics and Gynecology, University of Colorado Health Sciences Center; and [6]Edison Biotechnology Institute and the Department of Biomedical Sciences, Ohio University, Athens, OH.

Running title: Regulation of PI 3-kinase in adipose tissue

Address correspondence and reprint requests to
Michael O. Thorner, Department of Medicine, University of Virginia, Box 800466, Charlottesville, VA 22908, E-mail: mot@virginia.edu, or to Juan-Pablo del Rincon, Department of Medicine, University of Virginia, Box 801411, Charlottesville, VA 22908,

Copyright American Diabetes Association, Inc., 2007

Phosphoinositide 3-kinase (PI3K) is involved in insulin-mediated effects on glucose uptake, lipid deposition and adiponectin secretion from adipocytes. Genetic disruption of the p85α regulatory subunit of PI3K increases insulin sensitivity, while elevated p85α levels are associated with insulin resistance through PI3K dependent and independent mechanisms. Adipose tissue plays a critical role in the antagonistic effects of growth hormone (GH) on insulin actions on carbohydrate and lipid metabolism through changes in gene transcription. Objective: Assess the role of the p85α subunit of PI3K and PI3K signaling in GH-mediated insulin resistance in adipose tissue. Research Design and Methods: p85α mRNA and protein expression and IRS-1-associated PI3K activity were measured in white adipose tissue (WAT) of mice with GH excess, deficiency and sufficiency. Additional studies using 3T3-F442A cells were conducted to confirm direct effects of GH on free p85α protein abundance. Results: p85α expression: 1) is decreased in WAT from mice with isolated GH deficiency; 2) is increased in WAT from mice with chronic GH excess; 3) is acutely up-regulated in WAT from GH-deficient and GH-sufficient mice following GH administration; 4) is directly up-regulated by GH in 3T3-F442A adipocytes. The insulin-induced increase in PI3K activity was robust in mice with GH-deficiency, but not in mice with GH excess. Conclusions: GH regulates p85α expression and PI3K activity in WAT and provides a potential explanation for: a) the insulin hypersensitivity and associated obesity and hyperadiponectinemia of GH-deficient mice; and b) the insulin resistance and associated reduced fat mass and hypoadiponectinemia of mice with GH excess.

GH has well known diabetogenic effects. As early as 1931, Houssay and Biasotti showed that injection of crude pituitary extracts induced diabetogenic effects in dogs (1). By 1949 it was confirmed that these effects were caused by GH (2). There is a 30 to 56% prevalence of overt diabetes mellitus in humans with acromegaly, a disease characterized by excessive GH production (3; 4). The molecular mechanism(s) of GH-induced insulin resistance is (are) not clear.

A large number of studies demonstrate that adipose tissue plays a critical role in the antagonistic effects of GH on insulin action on carbohydrate and lipid metabolism (reviewed in (5)). Studies showing direct actions of GH in adipose cells or tissue include: kinetic studies in pigs which demonstrated that GH-induced insulin resistance results predominantly from reduced incorporation of glucose into adipose tissue (6; 7), studies in 3T3-L1 and 3T3-F442A adipocytes showing that GH inhibits insulin action on glucose transport and lipid accumulation (5; 8), and studies in human adipose tissue and adipocytes showing that GH reduces the incorporation of glucose into triglycerides (9). It has been proposed that the GH-induced insulin resistance in adipocytes is caused by a mechanism involving the phosphoinositide 3-kinase (PI3K) signaling pathway (10).

The PI3K pathway has an essential role in insulin induced glucose uptake and lipid metabolism in adipocytes (11), and it is also involved in the insulin-mediated increase in secretion of the adipocyte-derived hormone adiponectin (12-14). PI3K is a heterodimer consisting of one p85 regulatory and one p110 catalytic subunit, each of which occurs in multiple isoforms. p85α and the p55α and p50α splice variants, represent 70-80% of the total PI3K regulatory subunits in insulin-sensitive tissues, and are encoded by the Pik3r1 gene (15). In addition to its role as part of the PI3K dimer, the p85α regulatory subunit is also recognized as an important negative regulator of PI3K and insulin action. This has been shown in mouse models with partial or total knockout of p85α that demonstrate the paradoxical phenotype of increased insulin sensitivity (16-18). The inverse correlation between p85 levels and insulin sensitivity is also applicable when this regulatory subunit is expressed at greater-than-normal levels. For instance, p85 overexpression in cells inhibited PI3K signaling and glucose uptake (19). This mechanism has been recently supported by studies of the insulin resistant states induced by human placental growth hormone (20), and in obesity and type 2 (21) and gestational diabetes (22).

Since studies with inhibitors of RNA and protein synthesis suggest that many (perhaps all) of the anti-insulin effects of GH involve mRNA transcription and protein synthesis (5; 23; 24), we investigated the effects of GH on the p85α subunit of PI3K and its potential involvement in GH mediated insulin resistance. Our results strongly support that GH modulates insulin action through regulation of the expression of the p85α subunit of PI3K in adipose tissue.

RESEARCH DESIGN AND METHODS

Generation of the giant transgenic mouse expressing bovine GH (bGH) under the regulation of the metallothionein I promoter was previously described (25). Mice with this transgene show high serum bGH levels (25). C57BL/6J lit/lit and lit/+ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The little (lit) autosomal recessive mutation of the GH-releasing hormone (GHRH) receptor causes isolated GH deficiency in lit/lit mice, while heterozygous lit/+ mice are GH sufficient (26). lit/lit and lit/+ mice received a subcutaneous bolus (120 ng/g body weight) of rat recombinant GH (rrGH) (gift from Genentech Inc., South San Francisco, CA) or vehicle (0.9% saline, 100 μl) 4 h before sacrifice. bGH and lit mice

were sacrificed at 3 months of age using halothane. WAT depots were snap-frozen and stored at -80°C. Male mice were used in all the studies. Animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Virginia, Ohio University and the University of Colorado Health Sciences Center.

Measurement of blood parameters. Blood was obtained by cardiac puncture. Plasma glucose and insulin were measured after a six h fast in lit/lit and lit/+ mice using the glucose oxidase method (YSI Incorporated, Yellow Springs, OH) and the Sensitive Rat Insulin RIA Kit (Linco Research, St. Charles, MO), respectively. Adiponectin concentrations were measured in serum from non-fasted lit/lit and bGH mice and their respective controls using the Mouse Adiponectin RIA Kit (Linco Research, Inc., St. Charles, MO). The insulin resistance index was calculated by multiplying the fasting blood glucose level (mg/dl) by the fasting insulin level (ng/ml) (27).

Determination of adipocyte size in lit/lit and lit/+ mice. Epididymal adipose tissue removed from lit/lit and lit/+ mice was fixed in a 4% paraformaldehyde/PBS solution, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. Cross-sectional areas of at least 100 adipocytes per mouse, from at least 7 microscopic fields, were measured using the Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). The mean area value from each mouse was subsequently used for comparison between groups (n=5 mice per group).

Cells. 3T3-F442A cells were provided by Dr. P.J. Bertics (University of Wisconsin) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% calf serum and a mixture of penicillin and streptomycin. Confluent cells were differentiated by adding DMEM containing 10% fetal bovine serum (Invitrogen) and 172 nM insulin (Eli Lilly, Indianapolis, IN). Medium was renewed every second day. After 10 days, differentiated cells were incubated overnight in a serum-free media (DMEM with 0.1% bovine serum albumin) and then treated with media with or without recombinant human GH (rhGH) (Genentech, South San Francisco, CA) (200 ng/ml) at 37° C for 3, 6 and 24 h. In experiments performed to determine the effects of GH on the abundance of free p85α protein, 3T3-F442 adipocytes were incubated with and without 500 ng/ml rrGH for 24 h.

Real-time Reverse Transcriptase-PCR. Total RNA was extracted using Tri Reagent (Molecular Research Center, Inc. Cincinnati, OH) followed by further purification using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNA digestion with the RNase-Free DNase Set (Qiagen). RNA concentrations were measured using the RiboGreen Quantitation Kit (Molecular Probes, Eugene, OR). Primers for the mouse Pik3r1 gene targeted exons 14 (5'-CTTGTCCGGGAGAGCAGTAAGCA-3') and 15 (5'-CAGGTTGTAGGGCTCGGCAAAG-3'), as the C-terminal region, encoded by nine exons (E7 to E15), is common to all variants (p85α, p55α and p55α) of the Pik3r1 gene (28). Primers targeted exon 5 of the Pik3r1 gene (5'-TCATCAGTGTTGGCTTACGCTTCAGTA-3') for p85α, exon E1-55 (5'-CACGGTTTGGACTATGGAAGACCTG-3') for p55α and exon E1-50 (5'-TTCTCTGTGGGATACTGGCAGTTCAA-3') for p50α. A reverse primer common to the three splice variants targeted the C-terminal region on exon 7 (5'-TTGTTGGCTACAGTAGTGGGCTTGG-3'). Primers for the insulin-like growth factor (IGF)-1 gene and 18S rRNA were as described (29).

Plasmids were constructed with the PCR products generated with the above-mentioned specific primer pairs and were cloned into the pGEM-T vector (Promega, Madison, WI) and introduced in Escherichia coli JM109 (Promega). From a selected

transformant containing the desired construct, plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen). A serial dilution of each plasmid was used to make a standard curve for quantification. RNA reverse transcription and real-time PCR were done as described (29). The obtained values (copy number) were normalized to 18S rRNA.

Western blots. Polyclonal anti-p110α (07-658) and anti-p110β (06-568) antibodies used for co-immunoprecipitation of p85α, and a specific anti-p85α monoclonal antibody (Anti-PI 3 Kinase p85 N-SH3 clone AB6) used for p85α detection were from Upstate USA, Inc. Tissues or 3T3-F442A adipocytes were homogenized in an ice-cold modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) with a protease inhibitor cocktail (Calbiochem, La Jolla, CA), followed by two cycles of centrifugation at 10,000g for 10 min at 4 C. The supernatant was removed from the centrifugation tube. Proteins were measured using the BCA assay reagent kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg) from each sample were mixed 2:1 with 4x Laemmli sample buffer, boiled for 5 min, separated on a 7.5% SDS-PAGE gel, and transferred to nitrocellulose membranes (Protran BA85, Schleicher & Schuell, Keene, NH). Nitrocellulose blots were stained with Ponceau S (0.5% in 1% acetic acid) to confirm equal protein loading, destained with TBS-T (0.15 M NaCl, 0.1% Tween 20, 50 mM Tris, pH 7.6), and incubated for 1 h at room temperature with blocking buffer (5% bovine serum albumin in TBS-T) and then incubated for an additional 1 h at room temperature with anti-p85α antibody at 1:500 dilution. After washing 3 times in TBS-T, membranes were incubated for 2 h with a goat anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz ,CA). Bands were visualized using SuperSignal® West Pico Chemiluminescence Substrate (Pierce) and exposed to CL-XPosure Film (Pierce). Band densitometry was analyzed with Image Master TotalLab (Amersham Pharmacia Biotech, Piscataway, NJ). To assess the effects of GH on free p85α abundance, p85α-p110 dimers were immunodepleted by subjecting protein lysates to three rounds of sequential immunoprecipitation with a combination of anti-p110α (1:100) and anti-p110β (1:100) antibodies. Immunoprecipitates were captured with ImmunoPure Immobilized Protein A/G (Pierce). p85α protein remaining in the supernatant, representing the p85 monomer, was detected with an anti-p85α antibody by Western blot.

IRS-1 associated PI3K activity. PI3K activity was measured in epididymal fat from mice with: 1) chronic GH deficiency (lit/lit) and their littermates controls (lit/+); 2) chronic GH excess (bGH) and their wild-type littermates; 3) short-term GH excess (C57BL/6 wild-type mice). The latter C57BL/6, 2-3 months of age, were injected with rrGH (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Los Angeles, CA) (1 μg/g) subcutaneously twice daily for 3 days. At the time of sacrifice, mice were fasted for 6 hours and anesthetized with ketamine (150 mg/kg) and acepromazine (5 mg/kg). Their right-sided epididymal fat pad was rapidly removed and frozen immediately in liquid nitrogen. An insulin bolus of 10 U/kg body weight was then injected into the portal vein. At 5 minutes after injection, the epididymal fat pad from the opposite side was excised and frozen immediately. The level of IRS-1 associated PI3K activity was determined as described (30).

Statistical analysis. Results are presented as means ± SEM. Unpaired t-test and one-way ANOVA were used for comparisons between groups as appropriate, and a non-parametrical (Spearman) method was used for correlation analysis. Statistical significance was considered at P values < 0.05.

RESULTS

Mice with chronic GH excess show increased p85α expression in WAT and reduced serum

adiponectin. To test the hypothesis that GH modulates insulin action in WAT through regulation of Pik3r1 expression, we studied bGH transgenic mice. These mice showed increased Pik3r1 mRNA (Fig. 1A) and concomitantly increased p85α protein levels in WAT (Fig. 1B). Circulating adiponectin concentrations are in general negatively correlated with insulin resistance and with fat mass (31). In the bGH mice that are lean but insulin resistant (32; 33), adiponectin concentrations were 55% of those in their wild-type littermates (Fig. 1C).

Mice with chronic GH deficiency show reduced p85α expression in WAT, hypoinsulinemia, hyperadiponectinemia and adipocyte hypertrophy. p85α protein in WAT of lit/lit mice was reduced by ~60% as compared to lit/+ mice (Fig. 2A). While plasma glucose concentrations were not significantly different between lit/lit and lit/+ mice (Fig. 2B), fasting insulin concentrations in lit/lit were 38% of those in lit/+ mice (Fig. 2C). Estimation of insulin action using the insulin resistance index (fasting blood glucose level (mg/dl) multiplied by the fasting insulin level (ng/ml)) (27) supports that lit/lit mice have enhanced insulin sensitivity as compared to lit/+ mice controls (P=0.006) (Fig. 2D). Similarly significant results were obtained with other commonly used formulas for insulin action estimation including QUICKI (34) or HOMA-IR (35) (not shown). The cross-sectional area of adipocytes was significantly higher in lit/lit than in lit/+ mice (947.7 ± 99.28 vs. 456.6 ± 91.68 μm^2) (Fig. 2E). Circulating adiponectin has previously been inversely correlated with adipocyte size (31), however, lit/lit mice in spite of their enlarged adipocyte size showed higher serum adiponectin concentrations compared to those in lit/+ mice (30.33 ± 1.36 vs. 11.28 ± 0.97 μg/ml) (Fig. 2F).

Effects of chronic GH deficiency and acute GH replacement on total Pik3r1 and its p85α, p55α and p50α splice variants in WAT. As mice with heterozygous disruption of the Pik3r1 gene have shown increased insulin sensitivity, we explored whether the enhanced insulin sensitivity of GH-deficient mice might be related to a decreased expression of the Pik3r1 gene. lit/lit mice showed Pik3r1 mRNA levels (that included the three products, p85α, p55α and p50α, of the gene) ~ half of those in their GH-sufficient lit/+ littermates (Fig. 3A). As we found that mice with chronic GH excess had increased p85α expression, we analyzed the response at 4 h after an acute dose of rrGH on Pik3r1 expression. Pik3r1 mRNA levels in GH-treated lit/lit mice were 169% higher than in vehicle-treated lit/lit mice (Fig. 3A). Pik3r1 mRNA levels also increased in GH-treated lit/+ mice (Fig. 3A). To elucidate the response of each of the splice variants of the Pik3r1 gene under different levels of GH action, we measured p85α, p55α and p50α mRNA levels. p85α, the most abundant product, showed very similar changes to those found for Pik3r1 (Fig. 3B). In contrast, p55α (Fig. 3C) and p50α (Fig. 3D) mRNA levels did not show significant differences between any of the studied groups. Correlation analysis between Pik3r1 and p85α, p55α and p50α mRNA levels showed respectively values of 0.89 ($P<0.001$), 0.31 (P=N.S.) and 0.03 (P=N.S.) (not shown), confirming that the observed effects on total Pik3r1 expression can be accounted for by changes in p85α expression. Since the IGF-1 gene is an established target of GH, we validated our experimental model by analyzing IGF-1 mRNA levels in the same groups of mice (Fig. 3E), and obtained results similar to those of the Pik3r1 gene. A strong correlation between IGF-1 and Pik3r1 mRNA levels (Spearman r=0.79) was found, suggesting that GH regulates both Pik3r1 and IGF-1 expression in WAT.

GH increases Pik3r1 mRNA levels in differentiated 3T3-F442A cells. To gain further insight into the possibility of a direct effect of GH on Pik3r1, we analyzed 3T3-F442A adipocytes incubated with or without GH. Pik3r1 mRNA levels increased to 191%, 244% and 247% within 3, 6 and 24 h of

incubation with GH, respectively (Fig. 4), supporting a direct effect of GH on Pik3r1 expression in adipose cells.

Insulin-stimulated IRS-1 PI3K activity is increased in WAT of mice with chronic GH deficiency and decreased in WAT of mice with chronic or with short-term GH excess. In order to analyze if the GH-induced changes in p85α expression had concomitant effects on PI3K activity in WAT, epididymal fat from mice with diverse levels of GH action were analyzed. While lit/+ and lit/lit mice showed respectively a 57% and 207% increase in IRS-1-associated-PI3K activity in response to insulin (Fig. 5A), bGH mice did not have a significant response (Fig. 5B). In order to asses a shorter term effect of GH, wild-type mice were exposed to rrGH or vehicle for a period of 3 days. While insulin significantly increased PI3K activity in wild-type mice (Fig. 5C), this effect was abrogated in mice treated for 3 days with rrGH (Fig. 5C), demonstrating a GH-inhibitory effect on insulin action on IRS-1-associated PI3K activation.

Effects of GH on the abundance of the p85α monomer. To gain further insight into the effects of GH on abundance of the p85α monomer, we measured p85α protein levels in supernatants of 3T3-F442A adipocyte lysates following immunodepletion of p110α and p110β protein, resulting in co-immunoprecipitation of their bound p85α protein. Free p85α was increased by ~290% in GH-treated cells (Fig. 6A). Using the same technique, we found increased free p85α in WAT from bGH mice (Fig. 6B), and decreased free p85α in WAT from lit/lit mice (Fig. 6C).

DISCUSSION

GH can exert divergent actions on carbohydrate and lipid metabolism, showing acute insulin-like effects and delayed anti-insulin effects. The insulin-like effects are observed under conditions of previous GH deprivation (36); however, these effects are short-lived and their physiological significance, remains unclear (5). In contrast, the insulin-opposing effects of GH are present after a lag period of a few hours and persist chronically if exposure to GH continues (5; 36). The main hypothesis in the present study was that GH antagonizes insulin actions through regulation of p85α expression in adipose tissue. Our results show that GH up-regulates the expression of the p85α-encoding gene, Pik3r1, both directly in adipose cells, and in adipose tissue in mice. Several lines of evidence support that p85α can reciprocally regulate insulin action; PI3K dependent and independent mechanisms have been proposed as explanations for these p85α-mediated effects (reviewed in (37)). We found in the present study that PI3K is involved in the insulin opposing effects of GH in WAT, as shown by the fact that the normal increase in insulin-stimulated IRS-1-associated PI3K activity was abrogated in mice exposed to exogenous GH. We observed similar changes in bGH mice, a model of life-long GH excess, as IRS-1-associated-PI3K activity did not increase in response to insulin in these mice. The latter result confirms insulin resistance and is consistent with a previous analysis of the same strain of bGH mice, showing an 80% decrease in insulin-simulated glucose uptake in WAT (33). Mice with GH excess demonstrate dissociation between body mass and insulin sensitivity, as despite being lean (32) and resistant to high-fat diet induced obesity (38), they are severely insulin resistant (33). Since PI3K is involved in up-regulation of glucose uptake and lipid deposition in adipocytes (11; 39), an increase of p85α, a negative regulator of insulin action, specifically in adipose tissue potentially explains the dissociation between body mass and insulin resistance in mice with GH excess.

Insulin hypersensitivity in hypophysectomized animals was described in the 1920s (1). Mice with chronic GH deficiency (40; 41) and mice with genetically reduced p85α show insulin hypersensitivity

(18). We questioned whether the enhanced insulin sensitivity of GH-deficient mice was related to a decrease in p85α expression in WAT. mRNA levels of the Pik3r1 gene (in a region amplifying not only p85α but also the splice forms p55α and p50α) were decreased in WAT of lit/lit mice. Further real-time RT-PCR analysis in lit/lit and lit/+ mice treated with rrGH or vehicle demonstrated that the observed effects of GH action on Pik3r1 were accounted for by changes in its most abundant isoform, p85α. Consistent with changes in p85α mRNA, lit/lit mice showed reduced p85α protein levels and a substantial (above 200%) increase in insulin-induced IRS-1-associated-PI3K activity. The increased insulin sensitivity found in mice with GH deficiency occurs in spite of an increase in adiposity (32; 40; 41). A previous study in lit/lit mice showed that body fat accounted for 52% of body weight at age 12 months vs. 25% in lit/+ mice (26) and we found that lit/lit mice adipocytes are hypertrophic. As mentioned above, PI3K is involved in up-regulation of glucose uptake and lipid deposition in adipose tissue, therefore a decrease in p85α in this tissue provides a potential explanation for the dissociation between increased insulin sensitivity and adiposity in GH deficiency. This notion is consistent with results from a recent study where liver IGF-1–deficient (LID) mice – having increased plasma GH due to lack of IGF-1 negative feedback on GH secretion- were crossed with transgenic mice expressing a GH antagonist (GHa) to block GH action. The resulting LID + GHa mice showed improved total body insulin sensitivity and increased insulin-stimulated glucose uptake in WAT and muscle, in spite of a two fold increase in adipose tissue mass (42). We propose that the dissociation between insulin sensitivity and adiposity in mouse models with altered GH action is best explained by direct effects of GH action in adipose tissue. This concept is consistent with the physiological role of GH in switching energy away from adipose tissue (through decreased lipogenesis and increased lipolysis) resulting in increased lipid oxidation and preservation of body protein (43). Do the adipocytes of GH deficient animals reach a limit in their capacity to accumulate energy that would result in reduction of insulin sensitivity? We did not investigate this question; however increased insulin sensitivity, in spite of obesity, is also observed in children with GH deficiency, an effect that diminishes with increasing age; paradoxically, GH deficient adults demonstrate insulin resistance even prior to GH replacement therapy (43; 44). Of note too, is a recent study in lit/lit mice in which insulin sensitivity was analyzed up to 18 months of age; at age 12 months lit/lit still had greater insulin sensitivity than control mice, but by age 18 months they showed a blunted response to insulin (45).

A larger adipocyte size has been related with reduced serum adiponectin concentrations (31); this was not the case for lit/lit mice as in spite of their enlarged adipocyte size they showed hyperadiponectinemia. As PI3K has been implicated in up-regulation of adiponectin secretion in 3T3-L1 adipocytes (12-14), it is possible that decreased p85α in WAT (accompanied by greater responsiveness to insulin in PI3K activity) contributes to the increased serum adiponectin concentrations found in lit/lit mice. In the case of bGH mice, an increase in p85α in WAT (with a resultant decrease in PI3K responsiveness to insulin) might contribute to the reduced adiponectinemia. Consistent with our results in the lit/lit model, mice with specific disruption of the p85α subunit of Pik3r1 ($p85\alpha^{-/-}$) (16) placed on a high-fat diet developed greater adipocyte size than their littermates but yet showed higher adiponectin in WAT (and greater insulin sensitivity) compared to their wild-type littermates on the same diet (27). We acknowledge that other factors beside adiponectin secretion, including adiponectin turnover, determine serum adiponectin concentrations and may be playing a role in these mouse models (46).

Besides changes in adipocytokines (e.g. adiponectin) (47), other mechanisms have been proposed to explain the modulatory effects of GH on insulin (5; 48), including up-regulation of suppressor of cytokine signaling (SOCS) proteins (e.g. SOCS-3). However, a role for SOCS is still undefined. It was suggested that SOCS-3 may be involved in termination of insulin-like effects in 3T3-F442A cells incubated with GH, but due to its transient expression and rapid turnover it is unlikely to play a relevant role in GH-mediated insulin resistance (24).

Since it has been reported that insulin per se can induce expression of p85α (49), we analyzed the effect of GH in 3T3-F442A adipocytes in the absence of insulin; the resulting rapid and sustained increase in expression of the p85α gene supports a direct effect of GH in adipocytes. Additional experiments to measure the abundance of the p85α monomer, further confirmed that GH per se induces p85α protein expression. Monomeric or "free" p85α protein, this being p85α not bound to the p110 catalytic subunit of PI3K, is the p85 fraction more specifically linked with insulin resistance (37). In experiments performed to gain further insight into the direct effects of GH on p85α in adipose cells, we exposed 3T3-F442A adipocytes to rrGH for 24 h and analyzed the amount of p85α in lysate supernatants after 3 rounds of immunoprecipitation with anti-p110α and anti-p110β antibodies. The amount of p85α monomer was markedly increased in GH-treated as compared to control cells, thus demonstrating direct up-regulation by GH of this critical negative regulator of insulin action.

Taken together, our results demonstrate that GH regulates the expression of p85α, a critical modulator of insulin action. GH-deficient mice showed decreased p85α expression and a concomitant increase in insulin-induced IRS-1-associated PI3K activity, while mice with GH excess showed increased p85α levels and a concomitant suppression of the IRS-1-associated PI3K activity response to insulin. The GH-induced up-regulation of p85α expression may explain the GH-induced insulin resistance and reduced fat mass found in mice with GH excess. A schematic representation of a proposed model explaining GH effects on insulin action in adipose tissue is shown in Fig. 7. In the context of previous studies showing that genetic manipulations of Pik3r1 result in profound effects on insulin action (16-18; 27; 50), the results of the present study strongly support a role for regulation of p85α expression in WAT as a mechanism involved in the insulin hypersensitivity of GH deficiency and in GH-mediated insulin resistance.

ACKNOWLEDGMENTS

We thank Pattie Hellman and Edward O. List for expert technical assistance, Dr. Corinne M. Silva for expert advice, and the University of Virginia Diabetes and Endocrinology Research Center Core Facility for measurement of plasma insulin concentrations. This work was supported by grants from the Foundation for Growth Science in Japan and from the Uehara Memorial Foundation (K.I), DKF32-075252 (C.E.M.), support from the state of Ohio's Eminent Scholar Program that includes a gift by Milton and Lawrence Goll and by DiAthegen, LLC and NIH RO1 AG19899-03 (J.J.K.), NIH grant DK-062155 (J.F.), the Veterans Administration Research Service (B.D.), Pfizer, Inc. and a gift to the laboratory by Mr. Sal Ranieri (to M.O.T.).

REFERENCES

1. Houssay BA, Biasotti A: The hypophysis, carbohydrate metabolism and diabetes. Endocrinology 15:511-523, 1931

2. Coates PM, Reid E, Young FG: Diabetogenic action of pure anterior pituitary growth hormone. Nature 164:209-211, 1949

3. Ezzat S, Forster MJ, Berchtold P, Redelmeier DA, Boerlin V, Harris AG: Acromegaly. Clinical and biochemical features in 500 patients. Medicine 73:233-240, 1994

4. Arya KR, Pathare AV, Chadda M, Menon PS: Diabetes in acromegaly -a study of 34 cases. J Indian Med Assoc 95:546-547, 1997

5. Davidson MB: Effect of growth hormone on carbohydrate and lipid metabolism. Endocr Rev 8:115-131, 1987

6. Dunshea FR, Harris DM, Bauman DE, Boyd RD, Bell AW: Effect of porcine somatotropin on in vivo glucose kinetics and lipogenesis in growing pigs. J Anim Sci 70:141-151, 1992

7. Wray-Cahen D, Bell AW, Boyd RD, Ross DA, Bauman DE, Krick BJ, Harrell RJ: Nutrient uptake by the hindlimb of growing pigs treated with porcine somatotropin and insulin. J Nutr 125:125-135, 1995

8. Schwartz J, Carter-Su C: Effects of growth hormone on glucose metabolism and glucose transport in 3T3-F442A cells: dependence on cell differentiation. Endocrinology 122:2247-2256, 1988

9. Nyberg G, Bostrom S, Johansson R, Smith U: Reduced glucose incorporation to triglycerides following chronic exposure of human fat cells to growth hormone. Acta Endocrinol (Copenh) 95:129-133, 1980

10. Takano A, Haruta T, Iwata M, Usui I, Uno T, Kawahara J, Ueno E, Sasaoka T, Kobayashi M: Growth hormone induces cellular insulin resistance by uncoupling phosphatidylinositol 3-kinase and its downstream signals in 3T3-L1 adipocytes. Diabetes 50:1891-1900, 2001

11. Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M: Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. J Biol Chem 269:3568-3573, 1994

12. Kamon J, Yamauchi T, Muto S, Takekawa S, Ito Y, Hada Y, Ogawa W, Itai A, Kasuga M, Tobe K, Kadowaki T: A novel IKKb inhibitor stimulates adiponectin levels and ameliorates obesity-linked insulin resistance. Biochem Biophys Res Commun 323:242-248, 2004

13. Bogan JS, Lodish HF: Two compartments for insulin-stimulated exocytosis in 3T3-L1 adipocytes defined by endogenous ACRP30 and GLUT4. J Cell Biol 146:609-620, 1999

14. Pereira RI, Draznin B: Inhibition of the phosphatidylinositol 3'-kinase signaling pathway leads to decreased insulin-stimulated adiponectin secretion from 3T3-L1 adipocytes. Metabolism 54:1636-1643, 2005

15. Ueki K, Fruman DA, Brachmann SM, Tseng YH, Cantley LC, Kahn CR: Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival. Mol Cell Biol 22:965-977, 2002

16. Terauchi Y, Tsuji Y, Satoh S, Minoura H, Murakami K, Okuno A, Inukai K, Asano T, Kaburagi Y, Ueki K, Nakajima H, Hanafusa T, Matsuzawa Y, Sekihara H, Yin Y, Barrett JC, Oda H, Ishikawa T, Akanuma Y, Komuro I, Suzuki M, Yamamura K, Kodama T, Suzuki H, Koyasu S, Aizawa S, Tobe K, Fukui Y, Yazaki Y, Kadowaki T: Increased insulin sensitivity and hypoglycaemia in mice lacking the p85a subunit of phosphoinositide 3-kinase. Nat Genet 21:230-235, 1999

17. Fruman DA, Mauvais-Jarvis F, Pollard DA, Yballe CM, Brazil D, Bronson RT, Kahn CR, Cantley LC: Hypoglycaemia, liver necrosis and perinatal death in mice lacking all isoforms of phosphoinositide 3-kinase p85 alpha. Nat Genet 26:379-382, 2000

18. Mauvais-Jarvis F, Ueki K, Fruman DA, Hirshman MF, Sakamoto K, Goodyear LJ, Iannacone M, Accili D, Cantley LC, Kahn CR: Reduced expression of the murine p85a subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. J Clin Invest 109:141-149, 2002
19. Ueki K, Algenstaedt P, Mauvais-Jarvis F, Kahn CR: Positive and negative regulation of phosphoinositide 3-kinase-dependent signaling pathways by three different gene products of the p85a regulatory subunit. Mol Cell Biol 20:8035-8046, 2000
20. Barbour LA, Shao J, Qiao L, Leitner W, Anderson M, Friedman JE, Draznin B: Human placental growth hormone increases expression of the p85 regulatory unit of phosphatidylinositol 3-kinase and triggers severe insulin resistance in skeletal muscle. Endocrinology 145:1144-1150, 2004
21. Bandyopadhyay GK, Yu JG, Ofrecio J, Olefsky JM: Increased p85/55/50 expression and decreased phosphotidylinositol 3-kinase activity in insulin-resistant human skeletal muscle. Diabetes 54:2351-2359, 2005
22. Catalano PM, Nizielski SE, Shao J, Preston L, Qiao L, Friedman JE: Downregulated IRS-1 and PPARg in obese women with gestational diabetes: relationship to FFA during pregnancy. Am J Physiol Endocrinol Metab 282:E522-533, 2002
23. Borland CA, Barber MC, Travers MT, Vernon RG: Growth hormone inhibition of lipogenesis in sheep adipose tissue: Requirement for gene transcription and polyamines. J Endocrinol 142:235-243, 1994
24. Ridderstrale M: Signaling mechanism for the insulin-like effects of growth hormone -another example of a classical hormonal negative feedback loop. Curr Drug Targets Immune Endocr Metabol Disord 5:79-92, 2005
25. Chen WY, Wight DC, Wagner TE, Kopchick JJ: Expression of a mutated bovine growth hormone gene suppresses growth of transgenic mice. Proc Natl Acad Sci U S A 87:5061-5065, 1990
26. Donahue LR, Beamer WG: Growth hormone deficiency in 'little' mice results in aberrant body composition, reduced insulin-like growth factor-I and insulin-like growth factor-binding protein-3 (IGFBP-3), but does not affect IGFBP-2, -1 or -4. J Endocrinol 136:91-104, 1993
27. Terauchi Y, Matsui J, Kamon J, Yamauchi T, Kubota N, Komeda K, Aizawa S, Akanuma Y, Tomita M, Kadowaki T: Increased serum leptin protects from adiposity despite the increased glucose uptake in white adipose tissue in mice lacking p85a phosphoinositide 3-kinase. Diabetes 53:2261-2270, 2004
28. Fruman DA, Cantley LC, Carpenter CL: Structural organization and alternative splicing of the murine phosphoinositide 3-kinase p85 alpha gene. Genomics 37:113-121, 1996
29. Iida K, del Rincon JP, Kim DS, Itoh E, Nass R, Coschigano KT, Kopchick JJ, Thorner MO: Tissue-specific regulation of growth hormone (GH) receptor and insulin-like growth factor-I gene expression in the pituitary and liver of GH-deficient (lit/lit) mice and transgenic mice that overexpress bovine GH (bGH) or a bGH antagonist. Endocrinology 145:1564-1570, 2004
30. Barbour LA, Mizanoor Rahman S, Gurevich I, Leitner JW, Fischer SJ, Roper MD, Knotts TA, Vo Y, McCurdy CE, Yakar S, LeRoith D, Kahn CR, Cantley LC, Friedman JE, Draznin B: Increased p85a is a potent negative regulator of skeletal muscle insulin signaling and induces in vivo insulin resistance associated with growth hormone excess. J Biol Chem 280:37489-37494, 2005
31. Yamauchi T, Kamon J, Waki H, Murakami K, Motojima K, Komeda K, Ide T, Kubota N, Terauchi Y, Tobe K, Miki H, Tsuchida A, Akanuma Y, Nagai R, Kimura S, Kadowaki T: The mechanisms by which both heterozygous peroxisome proliferator-activated receptor g (PPARg) deficiency and PPARg agonist improve insulin resistance. J Biol Chem 276:41245-41254, 2001

32. Berryman DE, List EO, Coschigano KT, Behar K, Kim JK, Kopchick JJ: Comparing adiposity profiles in three mouse models with altered GH signaling. Growth Hormone & IGF Research 14:309-318, 2004
33. Higashimori T, Kim HJ, Coschigano K, Park SY, Choi H, Kopchick J, Shulman G, Kim J: Transgenic mice overexpressing growth hormone are insulin resistant due to decreased insulin-stimulated glucose uptake in skeletal muscle and adipose tissue. Diabetes Suppl. 1, 52:A539, 2003
34. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, Quon MJ: Quantitative insulin sensitivity check index: A simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 85:2402-2410, 2000
35. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and b-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28:412-419, 1985
36. Goodman HM: Separation of early and late responses of adipose tissue to growth hormone. Endocrinology 109:120-129, 1981
37. Taniguchi CM, Emanuelli B, Kahn CR: Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 7:85-96, 2006
38. Olsson B, Bohlooly YM, Fitzgerald SM, Frick F, Ljungberg A, Ahren B, Tornell J, Bergstrom G, Oscarsson J: Bovine growth hormone transgenic mice are resistant to diet-induced obesity but develop hyperphagia, dyslipidemia, and diabetes on a high-fat diet. Endocrinology 146:920-930, 2005
39. Wang D, Sul HS: Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway. Involvement of protein kinase B/Akt. J Biol Chem 273:25420-25426, 1998
40. Dominici FP, Hauck S, Argentino DP, Bartke A, Turyn D: Increased insulin sensitivity and upregulation of insulin receptor, insulin receptor substrate (IRS)-1 and IRS-2 in liver of Ames dwarf mice. J Endocrinol 173:81-94, 2002
41. Coschigano KT, Holland AN, Riders ME, List EO, Flyvbjerg A, Kopchick JJ: Deletion, but not antagonism, of the mouse growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased life span. Endocrinology 144:3799-3810, 2003
42. Yakar S, Setser J, Zhao H, Stannard B, Haluzik M, Glatt V, Bouxsein ML, Kopchick JJ, LeRoith D: Inhibition of growth hormone action improves insulin sensitivity in liver IGF-1-deficient mice. J Clin Invest 113:96-105, 2004
43. Mauras N, Haymond MW: Are the metabolic effects of GH and IGF-I separable? Growth Hormone & IGF Research 15:19-27, 2005
44. Husbands S, Ong KKL, Gilbert J, Wass JAH, Dunger DB: Increased insulin sensitivity in young, growth hormone deficient children. Clin Endocrinol (Oxf) 55:87-92, 2001
45. Fleenor D, Oden J, Kelly PA, Mohan S, Alliouachene S, Pende M, Wentz S, Kerr J, Freemark M: Roles of the lactogens and somatogens in perinatal and postnatal metabolism and growth: Studies of a novel mouse model combining lactogen resistance and growth hormone deficiency. Endocrinology 146:103-112, 2005
46. Combs TP, Pajvani UB, Berg AH, Lin Y, Jelicks LA, Laplante M, Nawrocki AR, Rajala MW, Parlow AF, Cheeseboro L, Ding Y-Y, Russell RG, Lindemann D, Hartley A, Baker GRC, Obici S, Deshaies Y, Ludgate M, Rossetti L, Scherer PE: A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. Endocrinology 145:367-383, 2004

47. del Rincon JP, Hellmann P, Coschigano KT, Kopchick JJ, Thorner MO: Low circulating adiponectin/Acrp30 levels in a mouse model of acromegaly (Abstract). In The Endocrine Society 85th Annual Meeting, Philadelphia, PA. Chevy Chase, MD, The Endocrine Society Press, 2003, p. 372
48. Dominici FP, Argentino DP, Munoz MC, Miquet JG, Sotelo AI, Turyn D: Influence of the crosstalk between growth hormone and insulin signalling on the modulation of insulin sensitivity. Growth Hormone & Igf Research 15:324-336, 2005
49. Bouzakri K, Roques M, Gual P, Espinosa S, Guebre-Egziabher F, Riou JP, Laville M, Le Marchand-Brustel Y, Tanti JF, Vidal H: Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes 52:1319-1325, 2003
50. Ueki K, Fruman DA, Yballe CM, Fasshauer M, Klein J, Asano T, Cantley LC, Kahn CR: Positive and negative roles of p85a and p85b regulatory subunits of phosphoinositide 3-kinase in insulin signaling. J Biol Chem 278:48453-48466, 2003

Figure legends

FIG. 1. Increased p85α expression in WAT and reduced adiponectin in serum of bGH mice. A: Pik3r1 mRNA levels in mesenteric fat from bGH and wild-type littermates (WT). Percent values are expressed as means ± SEM (n=5-6/group). B: Western blot of p85α protein in perirenal fat of bGH mice. Experiments were done as described in Research Design and Methods. The upper panel shows a representative picture, and the lower panel shows the mean ± SEM of arbitrary densitometry units of 3 independent experiments. C: Serum adiponectin concentrations in bGH mice. **P≤0.01; ***P<0.001.

Figure 1A

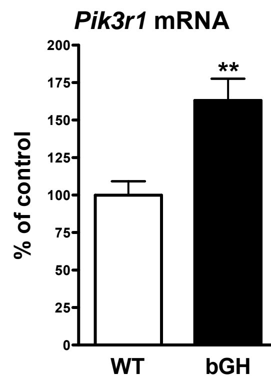

Figure 1B

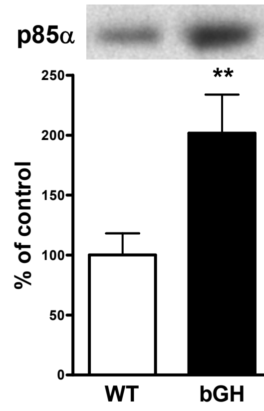

Figure 1C

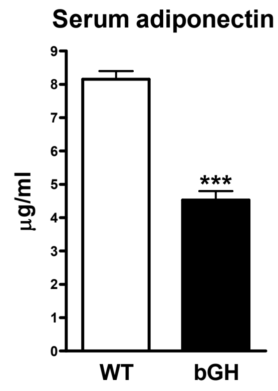

FIG. 2. Reduced p85α protein, normoglycemia, hypoinsulinemia, hyperadiponectinemia and increased adipocyte size in GH deficient mice. p85α protein in epididymal fat (A), fasting plasma glucose (B) and insulin concentrations (C), insulin resistance index (glucose mg/dl x insulin ng/ml) (D), cross sectional adipocyte area (with representative picture in upper panel) (E), and serum adiponectin concentrations (F) in lit/lit compared to lit/+ mice (n=5/group). p85α was measured in epididymal fat protein pooled from 5 mice/group in 3 independent experiments. Values are expressed as means ± SEM. **P<0.01; ***P<0.001.

Figure 2A

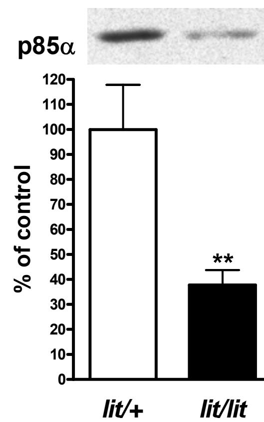

Figure 2B

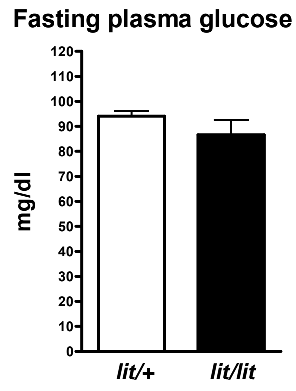

Figure 2C

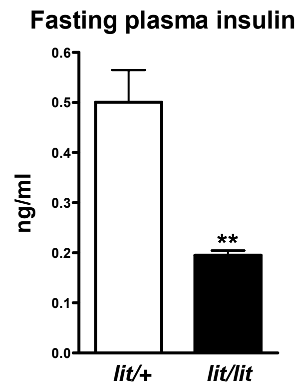

Figure 2D

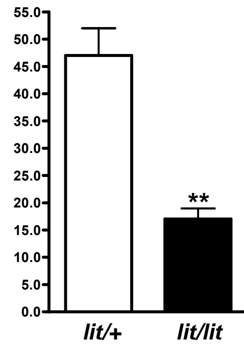

Figure 2E

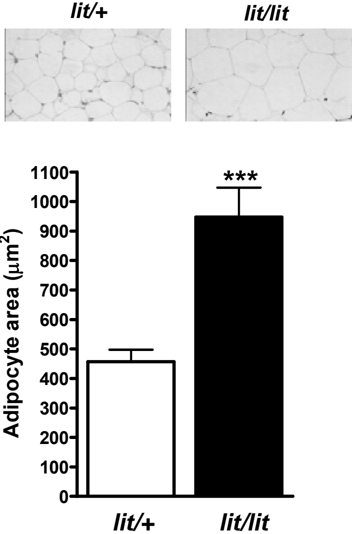

Figure 2F

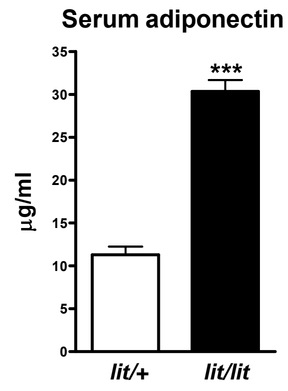

FIG. 3. Pik3r1 (A), p85α (B), p55α (C), p50α (D) and IGF-1 (E) mRNA levels in mesenteric from lit/lit and lit/+ mice, 4 h after treatment with either 120 ng/g body weight rrGH (+) or vehicle (-) (n=5/group). mRNA levels of total Pi3kr1 (amplifying a common region for the 3 splice variants), of each specific splice variant and of the IGF-1 gene were measured as described in Research Design and Methods. Percent values are expressed as means ± SEM, with vehicle-treated lit/+ mice as control group. *P<0.05; **P<0.01; ***P<0.001.

Figure 3A

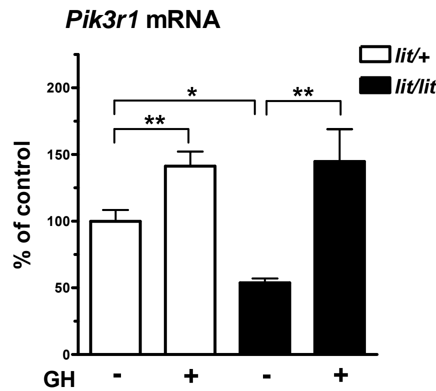

Figure 3B

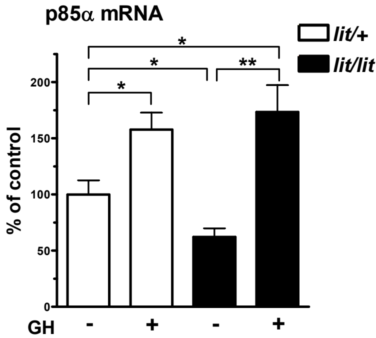

Figure 3B

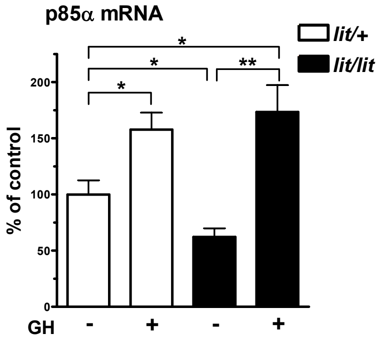

Figure 3C

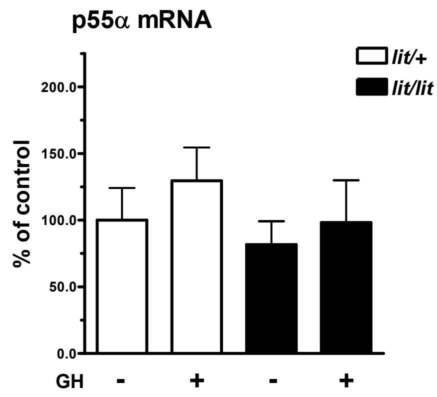

Figure 3D

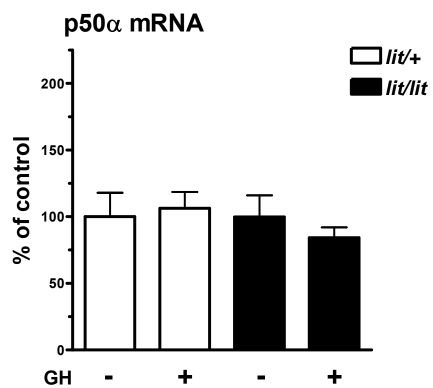

FIG. 4. In vitro regulation of Pik3r1 mRNA levels by GH. 3T3-F442A cells were induced to differentiate in DMEM, 10% FBS and 172 nM insulin. After 10 days, a serum-free media was used overnight, and cells were incubated with or without rhGH (200 ng/ml) for 3, 6 and 24 h. Percent values are expressed as means ± SEM (n=3/time point) of the time 0 reference control. *P<0.05; **P<0.01.

Figure 4

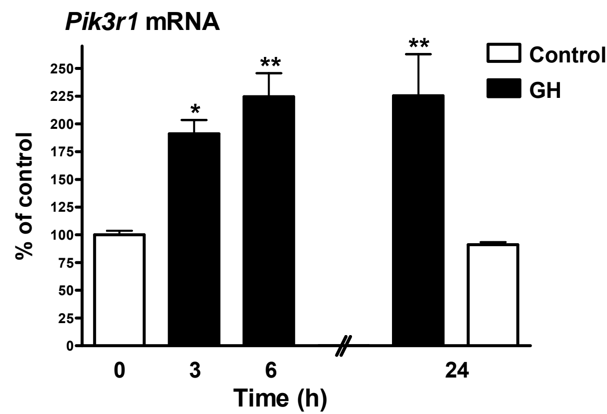

FIG. 5. Insulin-stimulated IRS-1-associated PI3K activity in epididymal fat from lit/lit and lit/+ mice (5A), bGH mice and their wild-type littermates (5B), and wild-type mice treated with rrGH (1 μg/g twice daily) or vehicle for 3 days (5C), was determined as described in Research Design and Methods, analyzing individually (unpooled samples) 4-7 mice/group. Densitometry values are expressed as means ± SEM in arbitrary units. PI-3P phosphatidylinositol-3,4,5. *P<0.05; **P<0.01; ***P<0.001. WT=wild-type.

Figure 5A

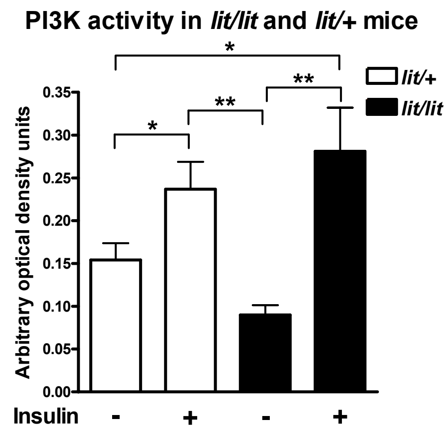

Figure 5B

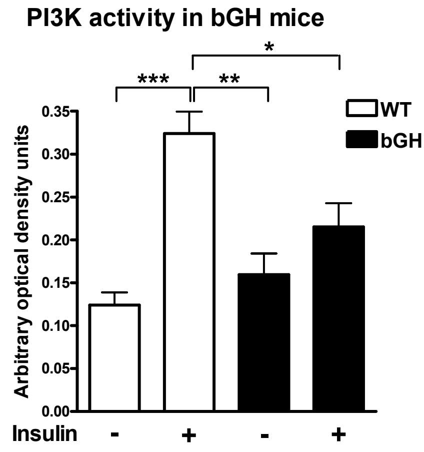

Figure 5C

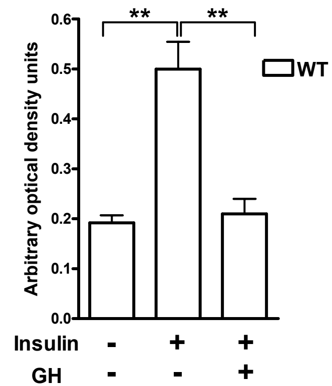

FIG. 6. Regulation of free p85α protein by GH action in vitro and in vivo. p110-p85α dimers were immunoprecipitated with anti-p110α and anti-p110β antibodies, and the remaining p85α in the supernatant was analyzed by Western blot. A: 3T3-F442A adipocytes treated with GH. Cells differentiated as described in Methods and Fig. 4 were incubated for 24 h with or without rrGH, 500 ng/ml (n=4-5 culture dishes/group). B: Perirenal fat from bGH mice and their wild-type littermates (n=6-9 mice/group). C: Epididymal fat from lit/+ and lit/lit mice (n=5-6 mice/group). Values in A, B, and C are means ± SEM expressed in percent from 3 independent experiments done with protein pools. Representative pictures are shown in the upper panels. ***P≤0.001. WT=wild-type.

Figure 6 A

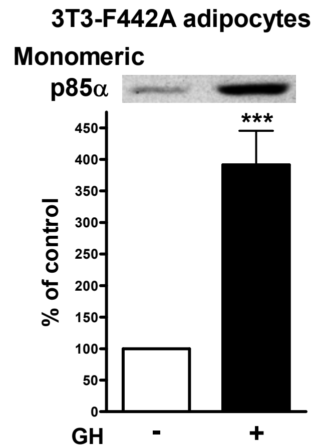

Figure 6 B

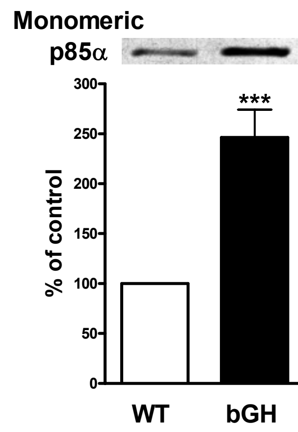

Figure 6 C

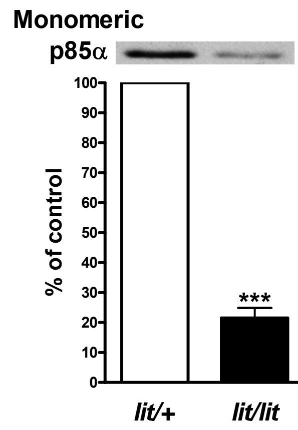

FIG. 7. Schematic representation of one proposed model illustrating modulatory effects of GH on insulin action through regulation of p85α expression in WAT. p85α has been extensively studied and shown to reciprocally regulate PI3K signaling (16; 18; 19; 27; 50). Increased levels of GH lead to increased expression of p85α, decreasing insulin-stimulated IRS-1-associated PI3K activity. The latter is a sine qua non of insulin resistance and may also lead to decreased lipid deposition and adiponectin secretion. GH deficiency leads to a decline in p85α expression, strong insulin-stimulated IRS-1-associated PI3K activity, increased insulin sensitivity (decreased insulin resistance), and may also lead to increased lipid deposition and enhanced adiponectin secretion. Thick arrows indicate either increase (continuous) or decrease (dashed).

Figure 7