Increased P85α Is a Potent Negative Regulator of Skeletal Muscle Insulin Signaling and Induces in Vivo Insulin Resistance Associated with Growth Hormone Excess

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Insulin resistance is a cardinal feature of normal pregnancy and excess growth hormone (GH) states, but its underlying mechanism remains enigmatic. We previously found a significant increase in the p85 regulatory subunit of phosphatidylinositol kinase (PI 3-kinase) and striking decrease in IRS-1-associated PI 3-kinase activity in the skeletal muscle of transgenic animals overexpressing human placental growth hormone. Herein, using transgenic mice bearing the p85 regulatory subunit of phosphatidylinositol kinase (PI 3-kinase) excess growth hormone (GH) states, but its underlying mechanism is unclear. Novel evidence suggesting that overexpression of p85α in skeletal muscle in response to GH. In contrast, p85α expression, reduction in insulin-stimulated IRS-1-associated PI 3-kinase activity in skeletal muscle in response to GH. In contrast, p85α expression and remained insulin-sensitive, whereas their wild type (WT) and p85α−/− counterparts responded to 3 days of GH administration with increases in p85α expression, reduction in insulin-stimulated IRS-1-associated PI 3-kinase activity and insulin resistance in response to GH. To confirm the role of p85α in the genesis of insulin resistance, we took advantage of insulin-sensitive mice with either heterozygous deletions of p85α or a complete knockout of p85β (5, 10, 11) and investigated the effect of GH injections on skeletal muscle p85α levels and IRS-1-associated PI 3-kinase activity. Animals with heterozygous disruption of p85α gene (p85α+−/− mice) were unable to increase p85α expression and remained insulin-sensitive, whereas their wild type (WT) and p85β+−/− counterparts responded to 3 days of GH administration with increases in p85α expression, reduction in insulin-stimulated IRS-1-associated PI 3-kinase activity and insulin resistance in response to GH. Together, these findings suggested that increased p85α is a potent negative regulator of insulin signaling in skeletal muscle and insulin resistance in vivo in response to growth hormone.
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EXPERIMENTAL PROCEDURES

Animals—TG mice that overexpress human placental growth hormone (TG-hPGH) driven by the metallothionine promoter have been described previously (1, 12). These mice were studied at 6–8 weeks of age (1). Mice overexpressing human placental growth hormone (TG-hPGH) have been characterized previously in the laboratories of Drs. Kahn and Cantley (5, 10, 11). All mice were of mixed genetic background consisting of 129S and C57BL/6; therefore, C57BL/6 mice were used as WT controls.

Skeletal muscle from mice with a LID was kindly provided by D. LeRoith, NIDDK, National Institutes of Health, Bethesda, MD and have been described previously (8, 9). These mice show a marked reduction in circulating IGF-1, elevated GH levels, and severe insulin resistance, primarily at the level of skeletal muscle. Treatment with a GH-releasing hormone agonist, MZ4-71 (GA), for 28 days effectively normalized GH levels and also restored insulin sensitivity (9).

Four to six mice in each group were used in each comparison. All mice were studied at ~14–18 weeks and were fed a normal mouse diet ad libitum. The guidelines for the care and treatment of the animals were approved by the Institutional Animal Care and Use Committee at the University of Colorado Health Sciences Center.

Materials—Regular insulin was purchased from Novo Nordisk, Princeton, NJ. Bovine serum albumin and protease inhibitors aprotinin and leupeptin were purchased from Roche Applied Science. Antibodies to IRS-1 and total (pan) p85, and specific to the α component of p85 (p85α), were purchased from Upstate Biotechnology, Lake Placid, NY. Antibodies to p110 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary horseradish peroxidase-conjugated antibody, protein A-Sepharose, and chemiluminescence reagents (ECL kit) were obtained from Amersham Biosciences. AG Resins, polyvinylidene difluoride membranes, PAGE gel equipment, and protein assay kits were from Bio-Rad. γ[32P]ATP was obtained from PerkinElmer Life Sciences. Recombinant rat growth hormone (rtGH) was purchased from the National Hormone and Pituitary Program, Harbor-UCLA Medical Center (Los Angeles, CA).

Insulin Tolerance Tests—Insulin tolerance tests were performed in p85α+/+, p85β−/−, and WT mice before and after GH injections (1 mg/kg subcutaneously twice daily for 3 days). Mice were fasted for 6 h and injected intraperitoneally with insulin (0.75 units/kg of body weight). Blood (5 μl) was collected from the tail vein at 0 and 60 min after insulin injection. Glucose measurements were performed with an AccuCheck Advantage glucose meter.

Acute Insulin Stimulation in Vivo and Tissue Collection—Mice were fasted for 6 h and anesthetized with ketamine (150 mg/kg) and acepromazine (5 mg/kg), and abdominal cavities were opened, and the inferior vena cava was exposed. Approximately 300 mg of gastrocnemius muscle from one hind limb was rapidly removed and frozen immediately. The samples were stored at −80 °C until analysis.

Western Blotting of Total p85, p85α, and p110—Muscle tissue was homogenized, and protein was assayed as described previously (1). Membranes were blocked with 5% nonfat milk (Bio-Rad,) in TBS-T for 1 h at room temperature. The membrane was washed three times with TBS-T and probed with a polyclonal total p85 antibody (1:500 dilution), monoclonal p85α antibody (1:500 dilution), or polyclonal p110 antibody (1:250 dilution). Transfer and washing conditions were performed as described previously (1). The bands were visualized with enhanced chemiluminescence (ECL) and exposed to Kodak BIOMAX films (Eastman Kodak Co.). The specific bands were quantitated using a GELDOC density scanner and Quantity One software (Bio-Rad).

Association of p85α with p110—Immunoprecipitation of p110 was carried out as described previously using polyclonal anti-p110 antibody (Santa Cruz Biotechnology) (1). Immunoblotting with either anti-p85α or anti-p110 antibodies was performed in the immunoprecipitates and supernatants.

IRS-1-associated PI 3-Kinase Activity—The level of IRS-1-associated PI 3-kinase activity was determined in muscle extracts after immunoprecipitation with IRS-1 antibody overnight at 4 °C (400 μg of muscle protein/4 μg of antibody) followed by incubation with protein A-Sepharose overnight, as described previously (1). The lipids were resolved by thin layer chromatography in CHCl3, MeOH, H2O, NH4OH (60:47:11:1). The lower band was quantitated using a Kodak Dynamic phosphorimaging device.

Cell Culture Experiments—3T3-L1 preadipocytes were grown to 60% confluence in growth medium (Dulbecco’s modified Eagle’s medium containing 5% glucose, 10% fetal calf serum, 50 μg/ml gentamicin, and 0.5 mM glutamine). After 24 h incubation with 1% fetal calf serum, cells were exposed to rtGH, either 500 or 2500 ng/ml for an additional 24 h. Cells were lysed, and expression of p85α was determined by Western blotting as described above.

Statistics—Results are expressed as mean ± S.E. and compared using either paired or unpaired t test as indicated. p values of < 0.05 are considered statistically significant.

RESULTS

In our initial experiments, we previously reported increases in the amounts of total p85 expressed in skeletal muscle of transgenic mice overexpressing hPGH (1). Further analyses revealed that the increases in p85 levels were predominantly accounted for by significant increases in p85α-specific isoform (p < 0.005; Fig. 1), whereas the levels of p110 were unchanged (data not shown). We confirmed this 2–3-fold increase in the p85α isoform by immunoprecipitating and immunoblotting with a p85α-specific antibody (data not shown).

The catalytic p110 subunit was depleted by triple immunoprecipitation from the muscle lysate (4–6), and the levels of p110 and p85α were analyzed by immunoblotting the immunoprecipitate and the supernatant. The amount of p85α recovered in the immunoprecipitate reflects
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p85α bound to p110, whereas the amount of p85α in the supernatant denotes free p85α subunit, existing in excess of p110.

The levels of p85α in the p110 immunoprecipitate were similar between the two groups of mice. However, the amount of free p85α (recovered in the supernatant) was significantly increased (p < 0.02; in the TG-hPGH mice (Fig. 2), confirming a substantially greater expression of p85α in the insulin-resistant TG-hPGH animals.

Because TG-hPGH animals have elevated IGF-1 levels (8, 9), which could potentially contribute to the p85α excess, we measured the expression of p85α in a different model of insulin resistance and GH excess. LID mice demonstrate profound insulin resistance, primarily at the level of skeletal muscle (8). These animals display low circulating levels of IGF-1 and compensatory elevations of GH (8, 9). Furthermore, treatment of these animals with a GA for 28 days reduced their levels of GH and completely reversed their insulin resistance (9).

We found that expression of p85α in skeletal muscle of LID mice was significantly higher (p < 0.01) than in WT mice (Fig. 3), suggesting a direct influence of GH. Moreover, treatment of LID mice with GA resulted in normalization of the p85α expression (Fig. 3) concomitant with a reversal of insulin resistance (9).

Lastly, we performed a series of definitive experiments in animals lacking either p85α (heterozygotes p85α+/−) or p85β (homozygotes p85β−/−). These mice were studied before and after two daily injections of GH for 3 days. Both p85α+/− and p85β−/− mice are more sensitive to insulin than the WT mice, as described previously (2, 5, 10, 11). Here we demonstrated that GH failed to induce insulin resistance in the p85α+/− mice while inducing insulin resistance in the WT and p85β−/− animals. Blood glucose levels were measured following an insulin challenge test before and after GH administration to document the development of insulin resistance, shown in Fig. 4. Although the WT and the p85β−/− mice became resistant to insulin after 3 days of GH injections, as noted by blunted blood glucose responses to insulin at 60 min (p < 0.005 versus pre-GH treatment), the p85α+/− mice remained highly sensitive to insulin.

The levels of p85α in skeletal muscle from the p85α+/− mice were ~50% of those in the WT mice (Fig. 5). Although GH administration significantly increased p85α in the WT and p85β−/− animals (p < 0.05 and <0.01, respectively), it had no effect on p85α in the p85α+/− mice. As expected, insulin increased IRS-1-associated PI 3-kinase activity in all animals not exposed to GH (Fig. 6A, p < 0.01). Insulin-stimulated PI 3-kinase activity was somewhat greater in the p85α+/− mice than in two other groups. Moreover, whereas administration of GH blunted insulin-stimulated IRS-1-associated PI 3-kinase activity in the WT and p85β−/− animals, it failed to affect PI 3-kinase activity in the p85α+/− mice. In addition, we measured the amount of p110 associated with IRS-1 in p85α+/− and p85β−/− mice versus controls treated with and without GH (Fig. 6B). As expected, we observed a reduced amount of p110 in the IRS-1 immunoprecipitates after GH treatment, confirming that p85α competes with the p85α-p110 heterodimer for the IRS-1 binding sites in GH-treated animals. Notably, in p85α+/− mice, the GH effect is absent, mirroring the PI 3-kinase activity results, demonstrating that reducing the p85α levels restores insulin-stimulated p110 association with IRS-1.

To confirm that the effect of GH on p85α levels is direct, we examined the effect of rat recombinant GH on p85α levels in cultured 3T3-L1 preadipocytes (Fig. 7). GH treatment increased p85α protein expression in 3T3-L1 preadipocytes, demonstrating that GH directly increases p85α levels.

DISCUSSION

The seminal finding of this investigation was that GH, whether endogenous or administered exogenously, induces in vivo insulin resistance by increasing the amount of skeletal muscle p85α monomers, unbound to its catalytic p110 subunit. Confirmation of this mechanism was illustrated in this study by critical observations in knock-out mice of opposing insulin sensitivity. Liver-specific IGF-1 knock-out mice, known to be insulin-resistant and to manifest high GH levels (8, 9), demonstrated p85α levels that were approximately twice as high as WT mice. Definitive support for this direct GH effect was achieved by treating these IGF-1-deficient animals with a GH-releasing hormone antagonist for 28 days, which normalized their p85α levels and reversed their insulin resistance (9). Consistent with the hypothesis tested in this study, animals with heterozygous disruption of p85α gene (p85α+/− mice) were unable to increase p85α expression and remained insulin-
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sensitive, whereas their WT and p85β−/− counterparts responded to GH with increases in p85α expression (Fig. 5), reduction in insulin-stimulated IRS-1-associated PI 3-kinase activity (Fig. 6), and insensitivity to insulin (Fig. 4).

Although the clinical correlates of excess GH in promoting insulin resistance have been well studied, the cellular mechanisms underlying this form of insulin resistance remain enigmatic. Studies of the effects of short term GH exposure in humans (13) and rodents (14) have shown insulin resistance combined with a reduced activity in the downstream target of insulin action, glycogen synthase. These findings have been supported by animal studies of long term GH exposure in which a reduced activity in the IRS-1-dependent insulin-signaling cascade in skeletal muscle was found. The effects of GH on insulin receptor tyrosine kinase activity appeared to be indirect and to result from hyperinsulinemia that develops after chronic exposure to excessive GH levels. This is supported by our previous findings in TG-hPGH mice (1) and results from studies in cultured cells showing that exposure to GH did not change insulin receptor tyrosine phosphorylation (15). A common finding in studies with animals treated with excess chronic GH excess is a diminished response to insulin injection in terms of IRS-1 tyrosine phosphorylation in skeletal muscle (15–18), accompanied by increased basal phosphorylation of IRS-1. At the same time, transgenic mice over-expressing pituitary GH have been found to have elevated p85 levels and reduced PI 3-kinase activity (16). Given our similar findings on the effect of p85α levels and IRS-1-associated PI 3-kinase activity, human placental growth hormone appears to mediate its insulin-resistant effect through the same mechanism. Thus, impaired insulin-stimulated PI 3-kinase activity in skeletal muscle (15–17) suggests that the signals initiated by GH excess converge on the PI 3-kinase pathway. 

Recently, a hypothesis that an imbalance between the subunits of the PI 3-kinase (the amounts of free p85 monomer and the p85-p110 heterodimer of the PI 3-kinase) in favor of the free p85α may promote insulin resistance has been put forth by a number of investigators (2–6).
Results of the present study strongly supported this hypothesis in response to growth hormone. PI 3-kinase belongs to the class 1a 3-kinases (7) that exist as heterodimers consisting of a regulatory subunit (p85), which is tightly associated with its p110 catalytic subunit (19–21). The regulatory subunits, p85, are encoded by at least three genes that generate highly homologous products. Two isoforms are termed p85α and p85β (products of the two genes). Three splice variants of p85α have been reported, including p85α itself, p55α, and p50α, and a third gene product is p55γ. The p85α, however, appears to be the most abundant isoform (7).

One of the first indications that an imbalance between the abundance of p85 and p110 can alter PI 3-kinase activity came from the work of Giorgino et al. (22). These authors treated L6 cultured skeletal muscle cells with dexamethasone and observed a decrease in PI 3-kinase activity. Surprisingly, however, they found almost a 4-fold increase in expression of p85α (no change in p85β) and only a minimal increase in p110. They concluded that p85α might compete with p85-p110 heterodimer, thus reducing the PI 3-kinase activity.

A string of several publications including the laboratories of Drs. Kahn and Cantley expanded on this observation (2–6, 10, 11). These investigators have generated p85α knock-out mice and p85α+/− heterozygous mice to address this question. Initially, they found that animals with a targeted disruption of p85α are hypersensitive to insulin. They identified that mice with a higher ratio of p85α-p110 dimer to free p85 are more insulin-sensitive. To determine this ratio, they immunodepleted p110 and blotted both the immunoprecipitates and the supernatant with p85 antibody. The amounts of p85 in the p110 immunoprecipitates denote p85 bound to p110, whereas the amount of p85 in the supernatant represents free (excess) p85. The greater the ratio of bound to free, the greater insulin sensitivity mice display. Furthermore, Cantley and Kahn (10, 11) have reported that mice with homozygous disruption of both genes encoding p85β (p85β−/−) are also more insulin-sensitive and display an enhanced insulin signaling in skeletal muscle. The p85β null mice were leaner and remained resistant to weight gain while on a high fat diet. Calculations of the expression of various isoforms of p85 revealed that 70–80% of skeletal muscle p85 is represented by p85α with p85β representing the majority of the remaining 20–30%. The same group of authors has then overexpressed p85α in cultured cells. This overexpression significantly inhibited the PI 3-kinase activity (4). Overexpression of p50α or p55α produced a lesser effect. These experiments were in concert with the competition hypothesis.

We took advantage of these insulin-sensitive p85α+/− and p85β−/− insulin-sensitive mice to test our hypothesis that GH induces insulin resistance by increasing expression of p85. We reasoned that mice lacking p85α would be unable to develop insulin resistance due to their genetic constraints in the expression of the p85α isoform. Furthermore, we suspected that the p85β−/− mice would be able to increase gene expression of the p85α isoform in response to GH and become insulin-resistant. Both of these hypotheses were confirmed in these experiments (Figs. 4 and 5). The inability to increase p85α in response to GH maintained insulin-stimulated IRS-1-associated PI 3-kinase activity and insulin sensitivity in the p85α+/− mice. In contrast, the p85β−/− mice, which were able to effectively increase p85α expression, were rendered vulnerable to the insulin-resistant effects of GH (Fig. 6). Together, these data indicated that insulin resistance is significantly increased in skeletal muscle as a result of expressing a greater amount of p85α protein. Recent data in human pregnancy support a causal role for an increase in p85α to mediate the insulin resistance of normal pregnancy. Friedman and colleagues (23–25) demonstrated that p85α levels in rectus abdominis muscle of pregnant women are twice as high as non-pregnant controls (23) and return to normal in the vastus lateralis of women 1 year postpartum (24). Very recent findings in the vastus lateralis muscle of obese pregnant women who were biopsied in pregnancy and again within 3 months postpartum, when insulin sensitivity normalizes, also demonstrate a 2-fold elevation of p85α in the third trimester when compared with postpartum (25). This postpartum decline in the levels of p85α did not occur in women with gestational diabetes who failed to normalized their insulin sensitivity. The most recent data of Del Rincón et al. (26) suggest that the administration of GH to normal volunteers augments p85α mRNA levels in their skeletal muscle.

In summary, the current study fully supported the hypothesis that insulin resistance can be caused by an overabundance of the p85α monomer, which competes with the p85-p110 dimer for binding to IRS-1 and activation of PI 3-kinase. We have also demonstrated that when p85α is underexpressed, as in p85α+/− mice, GH is incapable of causing insulin resistance. Our data identify p85α overexpression as a potent negative regulator of skeletal muscle insulin sensitivity in vivo associated with growth hormone excess (Fig. 8). This mechanism of insulin resistance has direct clinical implications for states of GH excess such as the insulin resistance of normal pregnancy and acromegaly but could have

3 J. E. Friedman, unpublished data.
implications for other states of glucose intolerance as well. Thus, p85α remains a potential therapeutic target for the treatment of insulin resistance and a possible factor underlying susceptibility to Type 2 Diabetes.

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