

Phosphoinositide 3-Kinase Catalytic Subunit Deletion and Regulatory Subunit Deletion Have Opposite Effects on Insulin Sensitivity in Mice

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Studies ex vivo have shown that phosphoinositide 3-kinase (PI3K) activity is necessary but not sufficient for insulin-stimulated glucose uptake. Unexpectedly, mice lacking either of the PI3K regulatory subunits p85 α or p85 β exhibit increased insulin sensitivity. The insulin hypersensitivity is particularly unexpected in p85 α ^{-/-} p55 α ^{-/-} p50 α ^{-/-} mice, where a decrease in p110 α and p110 β catalytic subunits was observed in insulin-sensitive tissues. These results raised the possibility that decreasing total PI3K available for stimulation by insulin might circumvent negative feedback loops that ultimately shut off insulin-dependent glucose uptake in vivo. Here we present results arguing against this explanation. We show that p110 α ^{+/-} p110 β ^{+/-} mice exhibit mild glucose intolerance and hyperinsulinemia in the fasted state. Unexpectedly, p110 α ^{+/-} p110 β ^{+/-} mice showed a ~50% decrease in p85 expression in liver and muscle. Consistent with this in vivo observation, knockdown of p110 by RNA interference in mammalian cells resulted in loss of p85 proteins due to decreased protein stability. We propose that insulin sensitivity is regulated by a delicate balance between p85 and p110 subunits and that p85 subunits mediate a negative role in insulin signaling independent of their role as mediators of PI3K activation.

Type 2 diabetes is a serious public health problem that is growing rapidly in the developed world. The disease is characterized by insulin resistance of the peripheral organs and hyperglycemia. Initially, patients compensate by secreting elevated insulin, but over the course of the disease, the pancreatic beta cells fail to produce compensating levels of insulin. Based on ex vivo experiments, phosphoinositide 3-kinase (PI3K) is required for insulin-induced glucose uptake into muscle and fat and inhibition of glucose production in the liver (10, 30, 35, 38). Polymorphism in the human PI3K regulatory isoform p85 α gene has been associated with increased risk for developing type 2 diabetes (5). However, the consequences of the polymorphism on the expression level or function of p85 α have not yet been analyzed. Many of the metabolic effects of insulin require activation of the PI3K downstream target Akt. While constitutively active Akt induces translocation of the glucose transporter GLUT4 to the plasma membrane in adipocytes, dominant negative Akt inhibits it (13). Consistent with this, targeted disruption of Akt2 in the mouse resulted in insulin resistance and severe diabetes, and RNA interference (RNAi) for Akt2 inhibited insulin-dependent glucose uptake in 3T3L1 cells (12, 19, 28). An inactivating mutation in human AKT2 has been associated with severe insulin resistance and diabetes mellitus, demonstrating a pivotal role of AKT signaling in glucose homeostasis in humans (20).

Surprisingly, deletion of PI3K regulatory subunits in mice

improves insulin signaling. Despite the necessity of PI3K activity for insulin-induced glucose uptake, genetic ablation of PI3K regulatory isoforms p85 α or p85 β resulted in improved insulin signaling (15, 33, 39, 42, 43). Based on studies with mice lacking the first exon of p85 α (where p55 α and p50 α alternative splice forms are upregulated), Terauchi et al. hypothesized that the p55 α and p50 α isoforms were more efficient than p85 α in mediating insulin responses (39). However, mice deficient in all p85 α splice variants (p85 α ^{-/-} p55 α ^{-/-} p50 α ^{-/-}) and p85 β null mice also exhibited hypersensitivity to insulin (15, 43). More recently, mice lacking p55 α and p50 α isoforms but expressing p85 α were also shown to have increased insulin sensitivity (11).

There is evidence in some cell lines that p85 isoforms are in excess over p110 isoforms and can compete with p85/p110 enzyme for binding to IRS proteins (41). Although this model can explain how excess p85 impairs insulin signaling in many situations, it cannot explain why p85 α ^{-/-} p55 α ^{-/-} p50 α ^{-/-} mice display hypersensitivity to insulin despite a reduction in total PI3K and a 60% decrease of PI3K recruitment to IRS complexes after insulin treatment (15).

Recent studies point to a PI3K activity-dependent feedback inhibition of insulin signaling. The negative feedback loop results in an increase in IRS1 Ser-307 (Ser-312 in humans) phosphorylation, decreased kinase activity of the insulin receptor, decreased IR-IRS1 interaction, increased IRS1 cytosolic distribution, increased IRS1 degradation, and decreased association between IRS1 and SH2 domain-containing proteins, such as p85 (2, 22, 31, 47). Ser-307/Ser-312 phosphorylation is sensitive to inhibitors of the PI3K/Akt/aPKC/mTor pathway, as well as a c-jun kinase (JNK) small-molecule inhibitor and an

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IKK inhibitor (18, 22, 23, 31, 32). Obesity has been shown to result in insulin resistance (21). One mechanism by which obesity may cause insulin resistance is thought to occur via tumor necrosis factor (TNF)-induced, PI3K-dependent serine (Ser-307) phosphorylation on IRS proteins (27, 36, 45). Additionally, JNK has been implicated in obesity-induced insulin resistance, as obese mice lacking JNK1 ($JNK1^{-/-}$) exhibit decreased IRS1 Ser-307 phosphorylation and increased sensitivity to insulin in comparison to obese $JNK1^{+/+}$ mice (26). In addition, inhibition of PI3K or the downstream kinase mTOR has been shown to prevent downregulation of insulin/IRS-1 signaling (34, 37). Furthermore, a recent study has shown that deletion of $p70^{S6kinase}$ in the mouse increases insulin sensitivity (44). These results suggest the existence of a strictly controlled, complex, negative feedback regulation of insulin signaling downstream of PI3K and raise the possibility that deletion of PI3K genes (regulatory or catalytic) might improve insulin signaling by preventing insulin-responsive tissues from reaching the threshold of PI3K response that activates a negative feedback loop.

To test the model that reduction in total PI3K activity in insulin-sensitive tissues can improve insulin-dependent glucose disposal *in vivo*, we examined mice heterozygous for PI3K p110 α and/or p110 β . Heterozygous loss of p110 α or p110 β alone had no effect on insulin sensitivity as judged by insulin tolerance tests or glucose tolerance tests. However, mice with double heterozygous loss of p110 α and p110 β were slightly glucose intolerant and exhibited hyperinsulinemia in the fasting state. Interestingly, not only were the PI3K catalytic isoforms decreased by ~50% in tissues from p110 $\alpha^{+/-}$ p110 $\beta^{+/-}$ mice, but also p85 protein levels decreased by a similar amount. Downregulation of p110 α in cell lines using RNAi also resulted in a reduction of the p85 regulatory subunit, indicating a dynamic intracellular mechanism for control of p85/p110 ratios.

Thus, although heterozygous loss of p110 α and p110 β results in a similar reduction in total class Ia PI3K in muscle and liver to that observed in the p85 $\alpha^{+/-}$ p55 $\alpha^{+/-}$ p50 $\alpha^{+/-}$ mice, the effect on insulin-dependent glucose disposal is the opposite. These data argue against the model that loss of p85 isoforms improves insulin signaling by preventing a PI3K-dependent feedback shutoff of insulin signaling. The data imply that p85 has a negative role in insulin signaling independent of PI3K regulation and suggest that the ratio of p85 to p110 plays a critical role in setting *in vivo* insulin sensitivity.

MATERIAL AND METHODS

Mice. Mice on a 129 background with heterozygous loss of p110 α (p110 $\alpha^{+/-}$) or p110 α ($^{+/-}$) were intercrossed to produce wild-type, single heterozygous, and double heterozygous mice. All animals were housed in microisolator cages on a 12-h light–12-h dark cycle and were fed a standard rodent chow (Purina) or put on a high-fat diet (Teklad) after they reached 2 months of age. Animal maintenance and experimental procedures were according to the National Institutes of Health guidelines and were approved by the Animal Care Use Committee of Harvard Medical School.

PCR. Mice were genotyped by PCR of crude tail genomic DNA (digested using modified Gitschier's buffer). In order to genotype p110 $\alpha^{+/-}$ mice, we used primers P α F (5'TGG CAT CCT CAA ATG ATA GTA ACA 3'), P α R (5'GAA TAA AAT AAA GAG GAG GCA TCA TAG 3'), and PNEO (5'GAA GGC TCT TTA CTA TTG CTT TAT 3'). In order to genotype p110 β mutant mice, we used primers P β F (5'GTA TTT GGA CCT GAT TTG ATG ATA G 3'), P β R

(5'GTT TTT ATT TGA AAT TAA ATC ACA TCG C 3'), and PNEO (5'GAA GGC TCT TTA CTA TTG CTT TAT 3').

TaqMan real-time PCR. CHO-IR cells were transiently transfected with bovine HA-p110 α , mouse Flag-p85 α , and either empty vector or short hairpin RNA (shRNA) for knockdown of expression of p110 α . After 48 h total RNA was isolated and analyzed by real-time PCR. Quantities were normalized to 18S rRNA quantities measured in the same samples. The following primers were used to amplify mouse p85 α : mp85a-539F (CCTTGTCGGGAGAGCAGTA), mp85a-604R (TTGACTTCGCCGTCTACCACT), and mp85a-561T (CAGGG CTGCTATGCCTGCTCCG) coupled to tetramethyl carboxyrhodamine.

Tissue lysate preparation and Western blotting. Mice were starved overnight, anesthetized with pentobarbital, and injected with 5 U of regular human insulin or phosphate-buffered saline into the inferior vena cava. After 5 min, liver and quadriceps muscles were removed and flash frozen in liquid nitrogen. Immunoprecipitation and immunoblot analysis of insulin-signaling molecules were performed on tissue homogenates extracted with buffer A containing 25 mM Tris-HCl (pH 7.4), 10 mM EDTA, 10 mM EGTA, 10 mM Na₃VO₄, 50 mM Na₄P₂O₇, 100 mM NaF, 5 μ g of leupeptin/ml, 5 μ g of aprotinin/ml, 2 mM phenylmethylsulfonyl fluoride, and 1% (vol/vol) Nonidet P-40, as described before (8).

Antibodies. Rabbit polyclonal anti-p85 antibody (anti-p85pan) was purchased from Upstate Biotechnology, Lake Placid, N.Y. Rabbit polyclonal anti-p110 α antibody and anti-p110 β antibody were purchased from Santa Cruz Biotechnology. Goat polyclonal anti-Akt antibody was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-Akt antibodies recognizing phosphorylated Ser-473 or Thr-308 of Akt1 were purchased from Cell Signaling Technology, Beverly, Mass. Rabbit polyclonal anti-IR antibody, anti-IRS-1 antibody, and anti-IRS-2 antibody were generated (8). Mouse monoclonal antiphosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. Mouse monoclonal anti-HA11 antibody was purchased from Covance/Babco, rabbit polyclonal anti-FLAG antibody was purchased from Sigma, and anti-FLAG M2 agarose was purchased from Sigma.

PI3K activity assays. The immunoprecipitates with anti-p110 α , anti-p110 β , anti-IRS-1, or anti-IRS-2 were washed twice with buffer A and twice with TNE (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA). The PI3K assay was performed by adding 20 μ l of HEPES (100 mM; pH 7), 20 μ l of lipids (67 μ M phosphoinositide (PI; Avanti Polar Lipids), 133 μ M phosphoserine (PS; Avanti Polar Lipids) and 10 μ l of ATP mix (70 mM HEPES [pH 7], 50 mM MgCl₂, 0.5 mM ATP, [γ -³²P]ATP [10 μ Ci/assay mixture]) to 50 μ l of immunoprecipitate. The reactions were performed at room temperature and stopped after 20 min by adding 25 μ l of HCl (5 M). The lipids were extracted with 160 μ l of CHCl₃-MeOH (1:1). The phosphorylated lipids were spotted on a thin-layer chromatography (TLC) and separated overnight with 1-propanol–2 M acetic acid (65:35). The radioactivity was visualized by using a PhosphorImager (Molecular Dynamics).

Akt activity assays. Tissue homogenates were immunoprecipitated with anti-Akt polyclonal antibody that recognizes both Akt1 and Akt2, and an Akt kinase assay with cross-tide as a substrate was performed as described elsewhere (40). Briefly, the immunoprecipitates were washed and resuspended in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 1 mM dithiothreitol (DTT) to which 20 μ M ATP, 5 μ Ci of [γ -³²P]ATP, and 5 μ g of cross-tide for AKT assay (Upstate Biotechnology) had been added. The reaction was performed at 30°C and after 20 min the reaction was stopped, and the aliquots were spotted onto P-81 paper, washed with 0.5% phosphoric acid, and counted for radioactivity.

High-fat diet. Mice were fed a high-fat diet (containing 55% fat and 24% carbohydrates by calories; catalog no. TD 93075; Harlan Tekland, Madison, Wis.) beginning at the time of weaning (21 days of age).

Weight measurements. Mice were weighed weekly between the ages of 2 and 6 months.

Glucose tolerance tests. Overnight-starved mice were injected intraperitoneally with 2 g of glucose per kg of body weight at 9 a.m. Blood glucose levels were measured with a One Touch Basic glucose meter at 0, 15, 30, 60, 120, and 180 min after injection.

Insulin tolerance tests. Mice were starved at 9:00 a.m. and injected intraperitoneally at 1:00 p.m. on the same day with regular human insulin at a dosage of 0.5 to 1.5 U per kg of body weight, dependent on age and gender of the animals. (In detail, female mice were injected with 0.5 U/kg at 2 months of age, 0.75 U/kg at 4 months of age, and 1 U/kg at 6 months of age. Male mice were injected with 0.75 U/kg at 2 months of age, 1 U/kg at 4 months of age, and 1.5 U/kg at 6 months of age.) Blood glucose levels were measured with a One Touch Basic glucose meter at 0, 15, 30, 45, 60, and 90 min after injection.

Insulin measurements. Serum insulin levels were measured in duplicate by using enzyme-linked immunosorbent assay reagents from Crystal Chem Inc. (catalog no. 90060).

Statistical analysis. Glucose and insulin tolerance tests were analyzed by repeated-measures analysis of variance. Weight growth curves, kinase assays, and glucose and insulin levels were analyzed by Student's *t* test.

RNA interference. Oligonucleotides designed to produce shRNAs targeting p110 α were cloned into a retroviral vector that utilizes a U6 promoter (14). The oligonucleotides were cloned into the vector after digestion of the vector with ApaI (Klenow)/EcoRI. The oligonucleotide sequences were **GGAGGAGAACCCTTATGTGACTTCTGAAGAGTACATAAGGGTCTCCTCCCTTTT** (top strand; nucleotides in bold are those targeting p110 α , human and bovine) and **AATCAAAAAGGGAGGAGAACCCTTATGTGACTTCTCAGAAGT CACATAAGGGTCTCCTCC** (bottom strand). Retroviral infection of HeLa cells was performed with the supernatants of 293T cells cotransfected with the p110 α shRNA retroviral vector and PCL Amphi packaging vector (a generous gift from William Hahn). Analysis of protein expression in the infected HeLa cells was conducted after 7 days of selection with puromycin.

Transient transfections. CHO cells were transfected with expression vectors for HA-p110 α (bovine), Flag-p85 α (mouse), Flag-p55 α (mouse), HA- Δ p85 α (human), and either empty vector or vector expressing shRNA for targeting of human p110 α . Two days later the cells were either lysed for Western blotting and immunoprecipitation or used for metabolic labeling. CHO cells were transfected with expression vectors for HA-Akt and increasing amounts of Flag-p85 α in pCMV6 and decreasing amounts of empty vector pCMV6. The cells were allowed to recover for 3 h in Dulbecco's modified Eagle's medium (DMEM)-F12-10% fetal calf serum (FCS). The cells were starved overnight in DMEM-F12-0% FCS. The next day cells were stimulated with 20 nM IGF-1 for 5 min, lysed, and immunoprecipitated with 5 μ l of anti-HA-11/500 μ g of total cell lysate. The lysate and immunoprecipitate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by immunoblotting with anti-p85pan, anti-AktT308P, and anti-HA-11 antibodies. All transfections were done according to the manufacturer's protocol (Lipofectamine Plus; Invitrogen).

Metabolic labeling experiments. CHO cells were transfected with expression vectors for HA-p110 α (bovine) or Flag-p85 α (mouse) in the presence or absence of an expression vector for shRNAi targeting p110 α . Two days after transfection, CHO cells were incubated for 1.5 h with methionine-cysteine-free medium containing 0.1 mCi of methionine-cysteine (Easy-Tag Express ³⁵S; NEG 772; NEN)/ml and then chased for the indicated times in regular growth medium (DMEM-F12-10% FCS). The cells were lysed and immunoprecipitated with 50 μ l of anti-Flag (M2) agarose beads/500 μ g of total cell lysate or with 250 μ l of anti-Flag (M2) agarose beads/5,000 μ g of total cell lysate, eluted with 0.5 mg of Flag peptide/ml for 1 h at room temperature, and subsequently immunoprecipitated with 25 μ l of anti-HA-11 antibody and 100 μ l of protein A/G mix/5,000 μ g of total cell lysate in a 3-ml total volume. The immunoprecipitate was separated by SDS-PAGE. The gel was dried, and the radiolabeled proteins were visualized by phosphorimager and autoradiography.

RESULTS

p110 α ^{+/-} p110 β ^{+/-} mice are slightly glucose intolerant. Since PI3K activity has been shown to be necessary for insulin-induced glucose uptake *in vitro* (10, 25), it was surprising that targeted disruption of any PI3K p85 regulatory isoform caused improved insulin sensitivity in mice even when total PI3K activity in tissues was reduced (11, 15, 39, 43). In order to understand if the increased insulin sensitivity was due to decreased PI3K available for downstream signaling, we examined mice in which catalytic subunits of PI3K were deleted. Since homozygous loss of either p110 isoform in mice is embryonic lethal (6, 7), mice with heterozygous loss of the isoforms were analyzed. At early ages, comparison between the different genotypes did not reveal any significant differences in blood glucose clearance during glucose or insulin tolerance tests (ATT or ITT) (data not shown). However, at 6 months of age, the male p110 α ^{+/-} p110 β ^{+/-} mice showed a mild but statistically significant glucose intolerance (Fig. 1a, upper left) and a small

(but not statistically significant) elevation in insulin during the GTT (Fig. 1a, upper right). No differences between p110 α ^{+/-} p110 β ^{+/-} and wild-type mice in glucose clearance during an insulin tolerance test were observed (Fig. 1b). Mice heterozygous for only p110 α or only p110 β were not statistically different from wild-type mice in the GTT or ITT but showed reproducibly an intermediate phenotype between p110 α ^{+/-} p110 β ^{+/-} and wild-type mice in glucose clearance during the GTT that did not achieve statistical significance (Fig. 1a, lower).

p110 α ^{+/-} p110 β ^{+/-} mice exhibit mild hyperinsulinemia in the fasted state. We assessed the blood insulin and glucose concentration in the fasted (after overnight food withdrawal) and fed (at 11 p.m.) state of male wild-type, p110 α ^{+/-}, p110 β ^{+/-}, and p110 α ^{+/-} p110 β ^{+/-} mice at 6 months of age. The p110 α ^{+/-} p110 β ^{+/-} but not the p110 α ^{+/-} or p110 β ^{+/-} mice exhibited significantly elevated insulin in the fasted state (Fig. 2a). In the fed state, the heterozygous mice exhibited normal insulin levels in comparison to wild-type mice (Fig. 2a). Also, the fasted and fed glucose levels of the heterozygous mice were not significantly different from those of wild-type mice (Fig. 2b). Since abnormalities in glucose homeostasis are often reflected in overall weight differences, we periodically weighed wild-type, p110 α ^{+/-}, p110 β ^{+/-}, and p110 α ^{+/-} p110 β ^{+/-} mice from weaning up to 6 months of age. The p110 α ^{+/-}, p110 β ^{+/-}, and p110 α ^{+/-} p110 β ^{+/-} mice exhibited a similar weight to wild-type mice (Fig. 2c).

Reduced PI3K catalytic and regulatory subunits in insulin-sensitive tissues of p110 α ^{+/-} p110 β ^{+/-} mice. We analyzed protein levels and activation states of enzymes in the insulin signaling pathway in liver and muscle of wild-type and p110 α ^{+/-} p110 β ^{+/-} mice. As expected, double heterozygous loss of p110 α and p110 β (p110 α ^{+/-} p110 β ^{+/-}) resulted in a ~50% reduction of PI3K activities associated with anti-p110 α or anti-p110 β immunoprecipitates from liver (Fig. 3a, left) and muscle (Fig. 3a, right) as well as a reduction of both p110 α and p110 β protein levels (Fig. 3b). In order to determine alterations in p85 protein levels, whole liver and muscle lysates were analyzed by Western blot analysis using an anti-p85pan antibody that recognizes all p85 isoforms. In both liver and muscle of p110 α ^{+/-} p110 β ^{+/-} mice, p85 protein levels were unexpectedly decreased to ~50% of levels in wild-type tissue. The smaller p85 isoform, p50 α , was also reduced in liver but not in muscle of p110 α ^{+/-} p110 β ^{+/-} mice in comparison to wild-type mice (Fig. 3b). It was difficult to visualize the p110 α protein from anti-p110 α immunoprecipitations in p110 α ^{+/-} p110 β ^{+/-} livers. Therefore, we performed an additional anti-p85 immunoprecipitation and anti-p110 α or anti-p110 β blotting. This approach also revealed approximately twice as much p110 α and p110 β in wild-type versus p110 α ^{+/-} p110 β ^{+/-} livers, consistent with our observation of twice as much PI3K activity.

Reduced PI3K activity associated with IRS proteins in insulin-sensitive tissues of p110 α ^{+/-} p110 β ^{+/-} mice upon insulin stimulation. No difference was observed between wild-type and p110 α ^{+/-} p110 β ^{+/-} mice in insulin receptor autophosphorylation. Although there was variability in the amount of insulin receptor and IRS protein phosphorylation from mouse to mouse in response to insulin injection, we saw no significant difference between wild-type and p110 α ^{+/-} p110 β ^{+/-} mice in

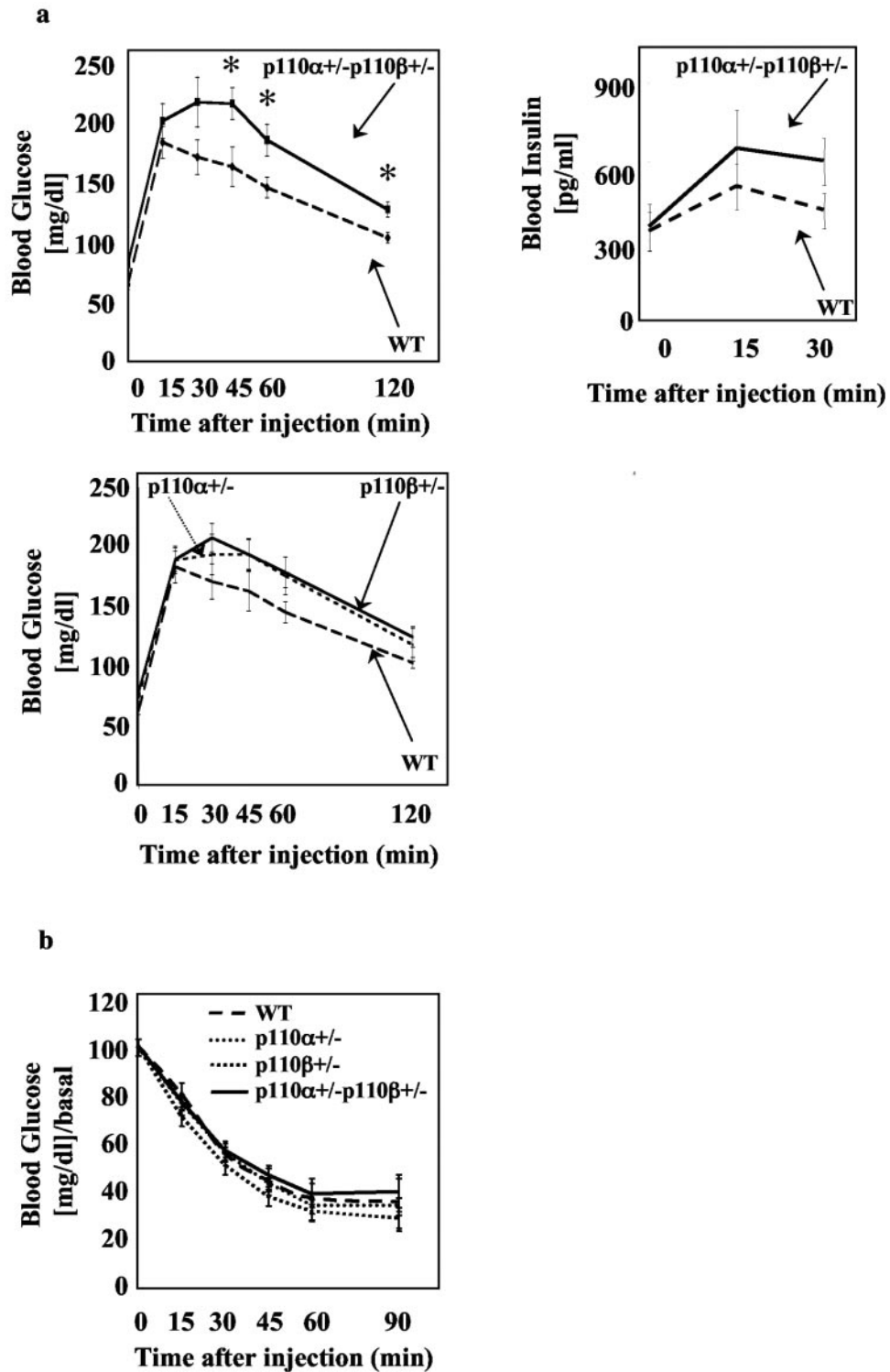


FIG. 1. p110 α ^{+/-} p110 β ^{+/-} mice are mildly glucose intolerant. (a) Blood glucose levels (p110 α ^{+/-} p110 β ^{+/-}, $n = 13$; wild type [WT], $n = 13$; p110 α ^{+/-}, $n = 11$; p110 β ^{+/-}, $n = 21$) and blood insulin levels (p110 α ^{+/-} p110 β ^{+/-}, $n = 10$; WT, $n = 5$) were measured in 6-month-old male mice subjected to glucose tolerance tests as described in Materials and Methods. (b) Insulin tolerance test (p110 α ^{+/-} p110 β ^{+/-}, $n = 10$; WT, $n = 5$; p110 α ^{+/-}, $n = 8$; p110 β ^{+/-}, $n = 12$) on 6-month-old male mice. Results are means \pm standard errors of the means of the blood glucose (GTT) or blood glucose/basal blood glucose ratio (ITT). *, $P < 0.05$, compared to WT.

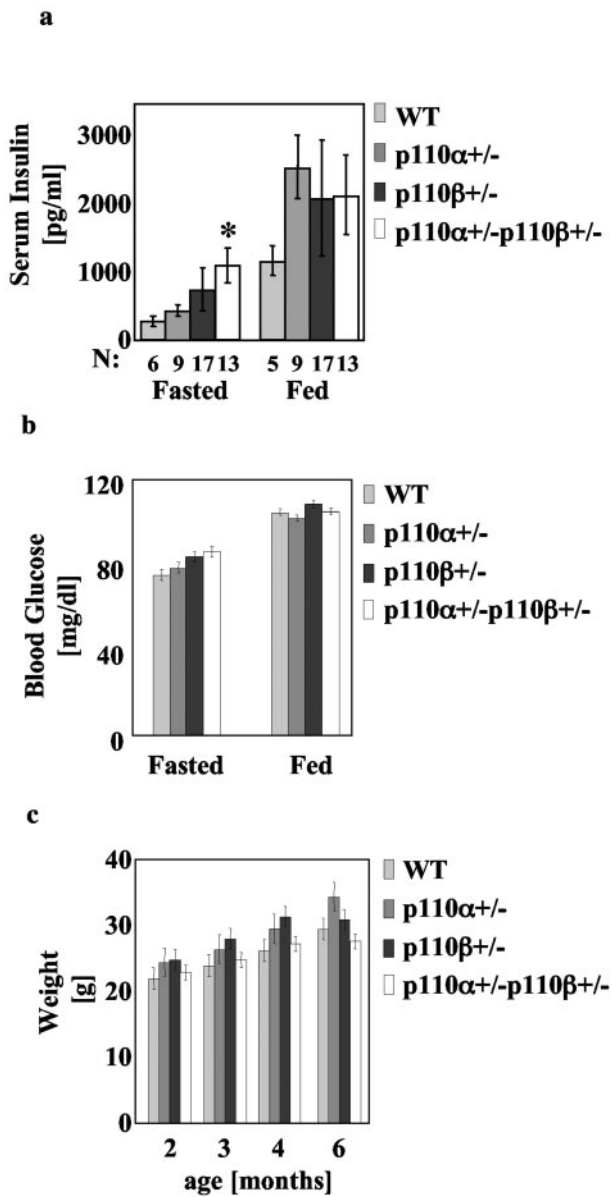


FIG. 2. Mild hyperinsulinemia in $p110\alpha^{+/-} p110\beta^{+/-}$ mice. (a) Plasma insulin concentrations in 6-month-old fasting and fed male mice. Results are means \pm standard deviations of the means. *, $P < 0.05$, compared to wild type (WT). (b) Weights for male mice with the indicated genotypes at different ages. The data express the means \pm standard deviations of the means (WT, $n = 6$; $p110\alpha^{+/-}$, $n = 8$; $p110\beta^{+/-}$, $n = 15$; $p110\alpha^{+/-} p110\beta^{+/-}$, $n = 12$). P values were calculated by using the Student t test. There was no statistically significant difference between genotypes. (c) Blood glucose levels of 6-month-old male fasting or fed mice with the indicated genotypes. The data express the means \pm standard deviations of the means (WT, $n = 6$; $p110\alpha^{+/-}$, $n = 8$; $p110\beta^{+/-}$, $n = 15$; $p110\alpha^{+/-} p110\beta^{+/-}$, $n = 12$). P values were calculated by using the Student t test. There was no statistically significant difference between genotypes.

the tyrosine phosphorylation of IRS1 and IRS2 (Fig. 4a). However, insulin-induced interaction between IRS1 and PI3K was reduced to $\sim 42\%$ in liver and $\sim 61\%$ in muscle of $p110\alpha^{+/-} p110\beta^{+/-}$ mice in comparison to levels in wild-type mice (Fig.

4b). Insulin-stimulated association between IRS2 and PI3K was normal in muscle ($\sim 95\%$) but reduced to $\sim 65\%$ in liver of $p110\alpha^{+/-} p110\beta^{+/-}$ mice (Fig. 4b).

Preserved insulin-dependent Akt activation in muscle and liver of $p110\alpha^{+/-} p110\beta^{+/-}$ mice. In vivo Akt activation in response to insulin injection was monitored using anti-phosphothreonine 308 antibody and anti-phospho-serine 473 antibody and by protein kinase activity in anti-Akt immunoprecipitates from liver and muscle. Despite the reduced total PI3K protein and anti-IRS-associated PI3K activity (Fig. 4), there did not appear to be a significantly reduced Akt activation in liver or muscle of the $p110\alpha^{+/-} p110\beta^{+/-}$ mice at the insulin dose used in these experiments (Fig. 5). This result is consistent with the normal insulin tolerance test observed for these mice (Fig. 1).

Reduction of the PI3K catalytic subunit results in decreased protein stability of the PI3K regulatory subunit. The reduction in p85 regulatory subunits in the liver and muscle of the $p110\alpha^{+/-} p110\beta^{+/-}$ mice was unexpected and led us to further explore the effect of reducing p110 isoforms on p85 protein levels. We were able to dramatically reduce p110 α in HeLa cells by RNAi, and this resulted in a significant reduction in endogenous p85 proteins (Fig. 6a). In addition, coexpression of p110 α with p85 α in CHO-IR cells resulted in more p85 α protein than in cells expressing p85 α alone, and this effect was blocked by p110 α RNAi (Fig. 6b). Furthermore, knockdown of p110 α decreased both the protein levels of the smaller p85 α isoforms (p55 α and p50 α) lacking the N-terminal domains (SH3, proline-rich regions, and the Rho-GAP domain) but had no effect on the protein levels of a mutant form of p85 α lacking the p110-binding site ($\Delta p85\alpha$) (Fig. 6c). Consistent with previous observations that p85 binding to p110 α stabilizes p110 α transient overexpression of p85 α , p55 α or p50 α increased the p110 α protein levels whereas overexpression of $\Delta p85\alpha$ did not (Fig. 6b and c). To directly test whether protein stability of p85 α is altered upon binding to p110, we examined the half-life of p85 α complexed with p110 α versus free p85 α in metabolically labeled CHO-IR cells. Transiently overexpressed free p85 α turned over with a half-life of approximately 2.7 h, whereas p85 α complexed with p110 α had a half-life of approximately 9.0 h (Fig. 6d). In addition, p110 α turned over at approximately the same rate (7.3 h) as the subset of p85 bound to p110 α . This result is consistent with our previous observation that p85 and p110 form an essentially irreversible complex in vivo (9, 16). The mRNA levels of p85 α were not decreased upon knockdown of p110 α (Fig. 6e). Thus, the results indicate that the subset of p85 bound to p110 α turns over in concert with p110 α , while free p85 turns over approximately 2.5 to 3 times faster, explaining why loss of p110 results in loss of p85.

Free p85 blocks IGF-1-induced Akt activation. The effect of excess free p85 on IGF-1-mediated Akt activation was investigated by transient overexpression of p85 in CHO cells. Similar to insulin, IGF-1 causes activation of the PI3K-Akt pathway by tyrosine phosphorylation of IRS proteins mostly through the IGF receptor and to a minor degree through the insulin receptor. A twofold overexpression of p85 over endogenous p85 caused a dramatic decrease in IGF-1-induced Akt activation (Fig. 7). The IGF-1-induced Akt activation was completely abolished by higher levels of p85.

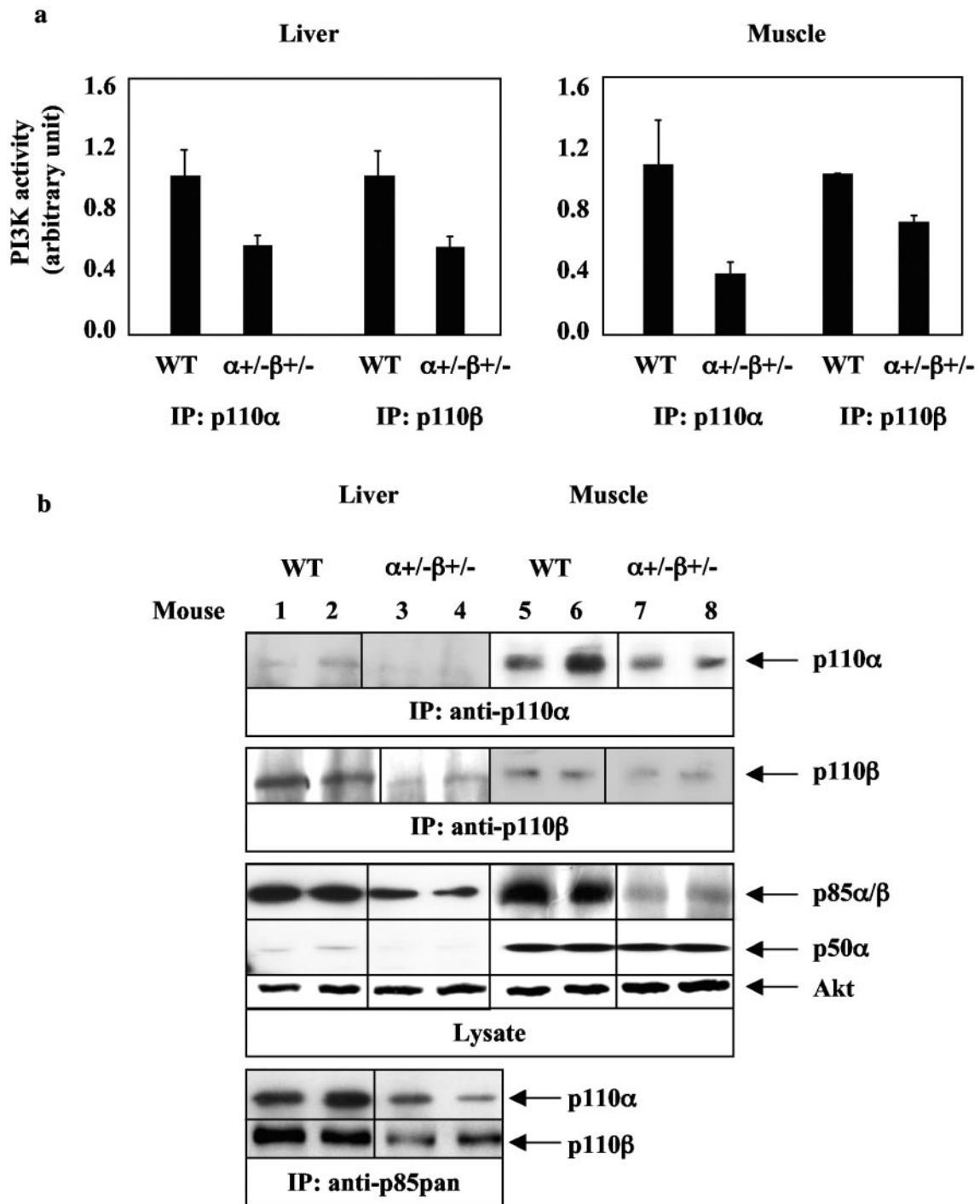


FIG. 3. p110α^{+/-} p110β^{+/-} mice have decreased p110 and p85 levels in liver and muscle. (a) PI3K assays in liver (for anti-p110α immunoprecipitation [IP]; wild type [WT], *n* = 3; p110α^{+/-} p110β^{+/-}, *n* = 3; for anti-p110β IP: WT, *n* = 3; p110α^{+/-} p110β^{+/-}, *n* = 6) and skeletal muscle (for anti-p110β and anti-p110α IP: WT, *n* = 3; p110α^{+/-} p110β^{+/-}, *n* = 3) from 4-month-old WT and p110α^{+/-} p110β^{+/-} mice. Results are means ± standard errors of the means and represent one of two experiments. (b) p110α, p110β, and p85 protein levels in liver (left) and skeletal muscle (right) from 4-month-old mice. IP was performed on 2 mg of muscle or 2 mg of liver from WT or p110α^{+/-} p110β^{+/-} mice. A representative experiment is shown. Each lane represents a single mouse.

DISCUSSION

Extensive studies have shown that PI3K activity is required for insulin-induced glucose uptake (10, 25, 35). It was therefore

surprising that loss of any p85 isoform resulted in increased insulin signaling even under conditions where total PI3K levels were reduced in insulin-sensitive tissues (11, 15, 33, 39, 43). We were interested to know whether reduction of PI3K by deletion

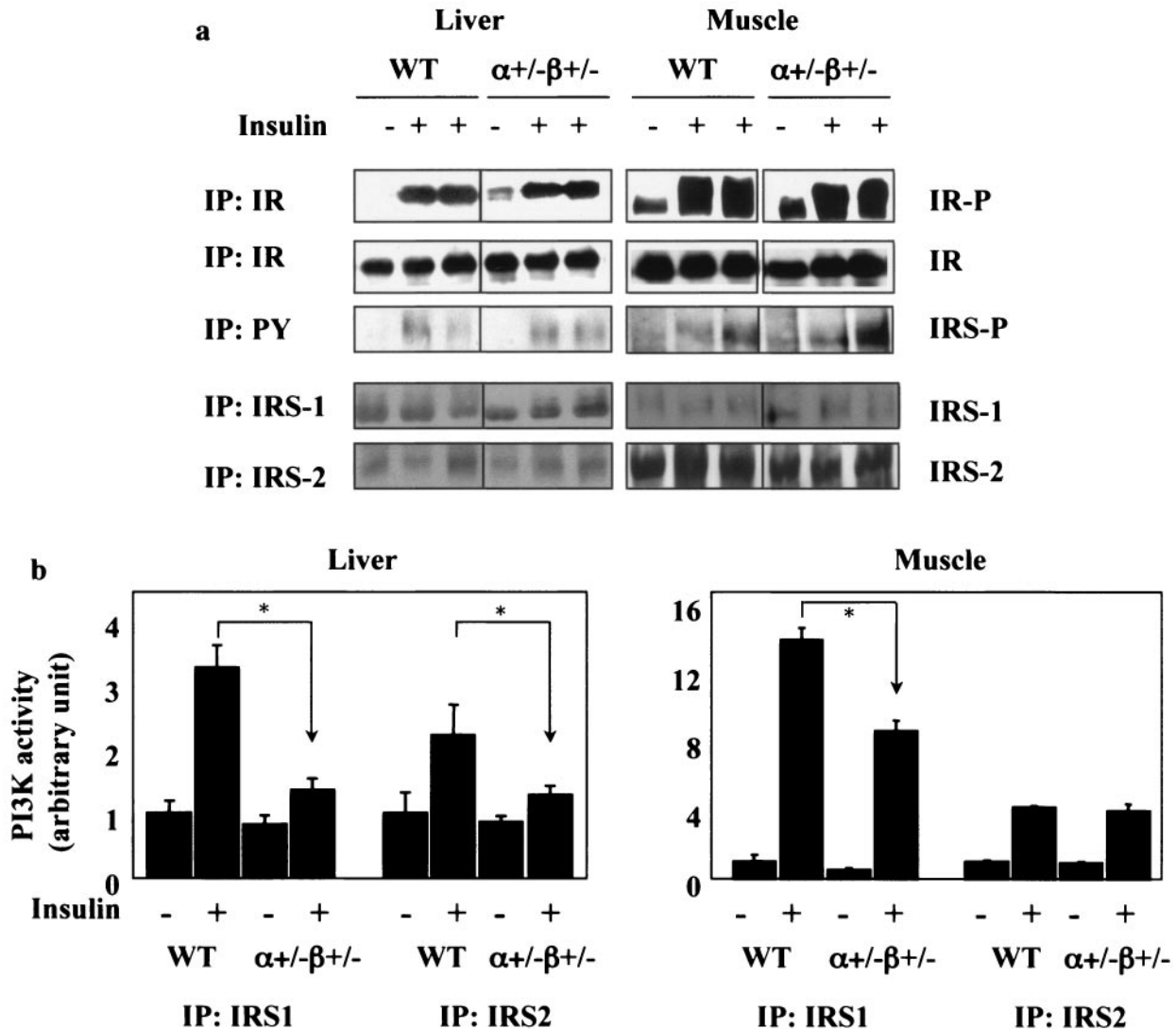


FIG. 4. $p110\alpha^{+/-} p110\beta^{+/-}$ mice have decreased IRS-associated PI3K activity in liver and muscle. (a) Phosphorylation of insulin receptor (upper) and insulin receptor substrates (lower) in liver (left) and skeletal muscle (right) from 4-month-old wild-type (WT) and $p110\alpha^{+/-} p110\beta^{+/-}$ mice injected with saline or 5 U of insulin. Each lane represents a single mouse. (b) PI3K activity associated with IRS1 or IRS2 in liver (WT, $n = 2$ [-insulin] or $n = 3$ [+insulin]; $p110\alpha^{+/-} p110\beta^{+/-}$, $n = 4$ [-insulin] or $n = 4$ [+insulin]) and skeletal muscle (WT, $n = 2$ [-insulin] or $n = 3$ [+insulin]; $p110\alpha^{+/-} p110\beta^{+/-}$, $n = 3$ [-insulin] or $n = 4$ [+insulin]) from 4-month-old WT and $p110\alpha^{+/-} p110\beta^{+/-}$ mice injected with saline or 5 U of insulin. Results are means \pm standard errors of the means and represent one of two experiments. *, $P < 0.05$ compared to WT. The samples were chosen for similar levels of phosphorylation of the insulin receptor from a total of 18 samples.

of p110 subunits might also improve insulin sensitivity. We show here that combined heterozygous loss of p110 α and p110 β results in a ~50% decrease of class Ia PI3K without improved insulin signaling. Indeed, mutant mice have a mild glucose intolerance and exhibit mild hyperinsulinemia in the fasting state. Elevated insulin levels point to insulin resistance similar to the early stages of diabetes, in which pancreatic β cells secrete more insulin in order to compensate for insulin resistance in the peripheral tissues. We therefore conclude that reduction in total PI3K activity does not improve insulin-dependent glucose disposal and that increased insulin sensitivity observed in $p85\alpha^{-/-} p55\alpha^{-/-} p50\alpha^{-/-}$ mice is due to elimination of a negative role played by p85 isoforms rather than due to a general effect of reducing PI3K signaling.

The mechanism by which deletion of p85 causes increased insulin sensitivity in mice is still not clear but could involve multiple components. Insulin-dependent Akt activation is enhanced in both $p85\alpha^{+/-} p55\alpha^{+/-} p50\alpha^{+/-}$ mice and $p85\beta^{-/-}$ mice without increased PI3K activity associated with IRS proteins (33, 43). These findings raise the possibility that p85 isoforms unbound to p110 might activate a PIP₃ phosphatase or an Akt phosphatase and thereby contribute to a shutoff of Akt signaling (24). Another possibility is that unbound p85 contributes to JNK activation. JNK activity causes phosphorylation on serine 307 of IRS-1, a site implicated in inhibition of insulin signaling (1, 26). In $p85\alpha^{-/-} p55\alpha^{-/-} p50\alpha^{-/-}$ brown adipocytes, insulin-dependent JNK activation is impaired in comparison to brown adipocytes from wild-type mice and the

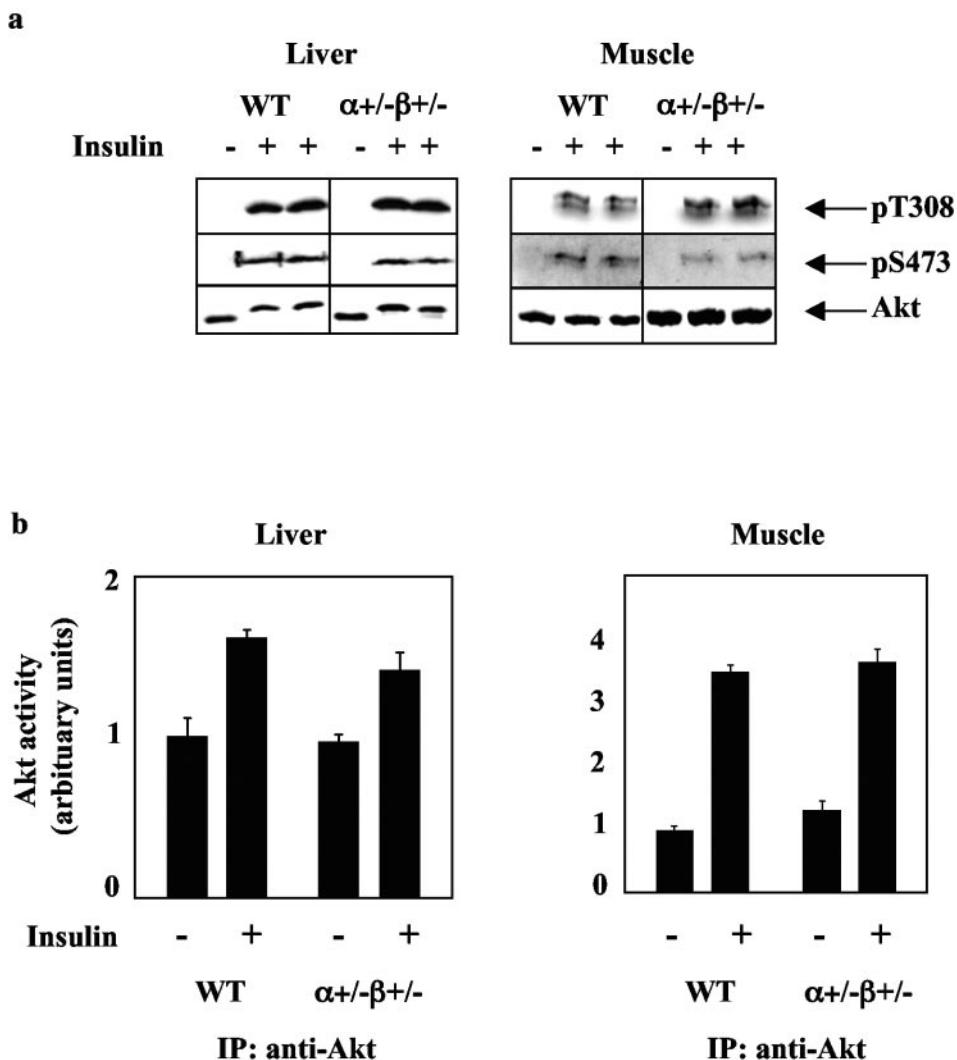


FIG. 5. p110 $\alpha^{+/-}$ p110 $\beta^{+/-}$ mice have normal insulin-induced Akt activation in liver and muscle. (a) Akt phosphorylation at threonine 308 (pT308) and serine 473 (pS473) and total Akt protein levels in liver and skeletal muscle from 4-month-old wild-type (WT) and p110 $\alpha^{+/-}$ p110 $\beta^{+/-}$ mice injected with saline or insulin. (b) Akt activity assays in liver (WT, $n = 3$ [-insulin] or $n = 3$ [+insulin]; p110 $\alpha^{+/-}$ p110 $\beta^{+/-}$, $n = 3$ [-insulin] or $n = 6$ [+insulin]) and skeletal muscle (WT, $n = 2$ [-insulin] and $n = 3$ [+Ins]; p110 $\alpha^{+/-}$ p110 $\beta^{+/-}$, $n = 3$ [-insulin] or $n = 4$ [+insulin]) from 4-month-old WT and p110 $\alpha^{+/-}$ p110 $\beta^{+/-}$ mice injected with saline or insulin. Results are means \pm standard errors of the means and represent one of two experiments. *, $P < 0.05$ compared to WT. The samples were chosen for similar levels of phosphorylation of the insulin receptor from a total of 18 samples.

defect can be rescued by reintroduction of p85 isoforms as well as by a p85 mutant (delta-p85) that lacks the binding site for p110 (42). Finally, unbound p85 can compete for p85/p110 complexes for binding to IRS proteins and thus limit signaling.

We found that heterozygous loss of either p110 α or p110 β alone does not lead to significant abnormalities in the GTT or ITT, while loss of both proteins causes a significant impairment in the GTT. These results suggest that p110 α and p110 β both contribute to insulin signaling in vivo. Asano et al. suggested that p110 β specifically mediates insulin-induced GLUT4 translocation: microinjection of inhibitory antibodies against p110 β blocked insulin-induced translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes, whereas inhibitory antibodies against p110 α had only a minor effect (3). The difference between our results and those of Asano et al. may be explained

by differences in expression levels of p110 isoforms in insulin-sensitive tissues in vivo versus expression levels in 3T3-L1 adipocytes. Mice with combined heterozygous loss of p110 α and p110 β have a mild glucose intolerance, especially at the later time points of the GTT, and exhibit mild hyperinsulinemia in the fasting state. These findings point to a defect in liver function of the mutant mice.

Previous studies have shown that monomeric p110 is thermally unstable, while heterodimerization with p85 causes stabilization of p110 protein (17, 46). We show here that the stabilization of p110 by p85 isoforms requires the direct association between the two proteins, since all splice variants of p85 α (p85 α , p55 α , and p50 α) can augment p110 α protein levels, whereas the Δ p85 α mutant which is lacking the p110 interaction domain cannot. The studies presented here further

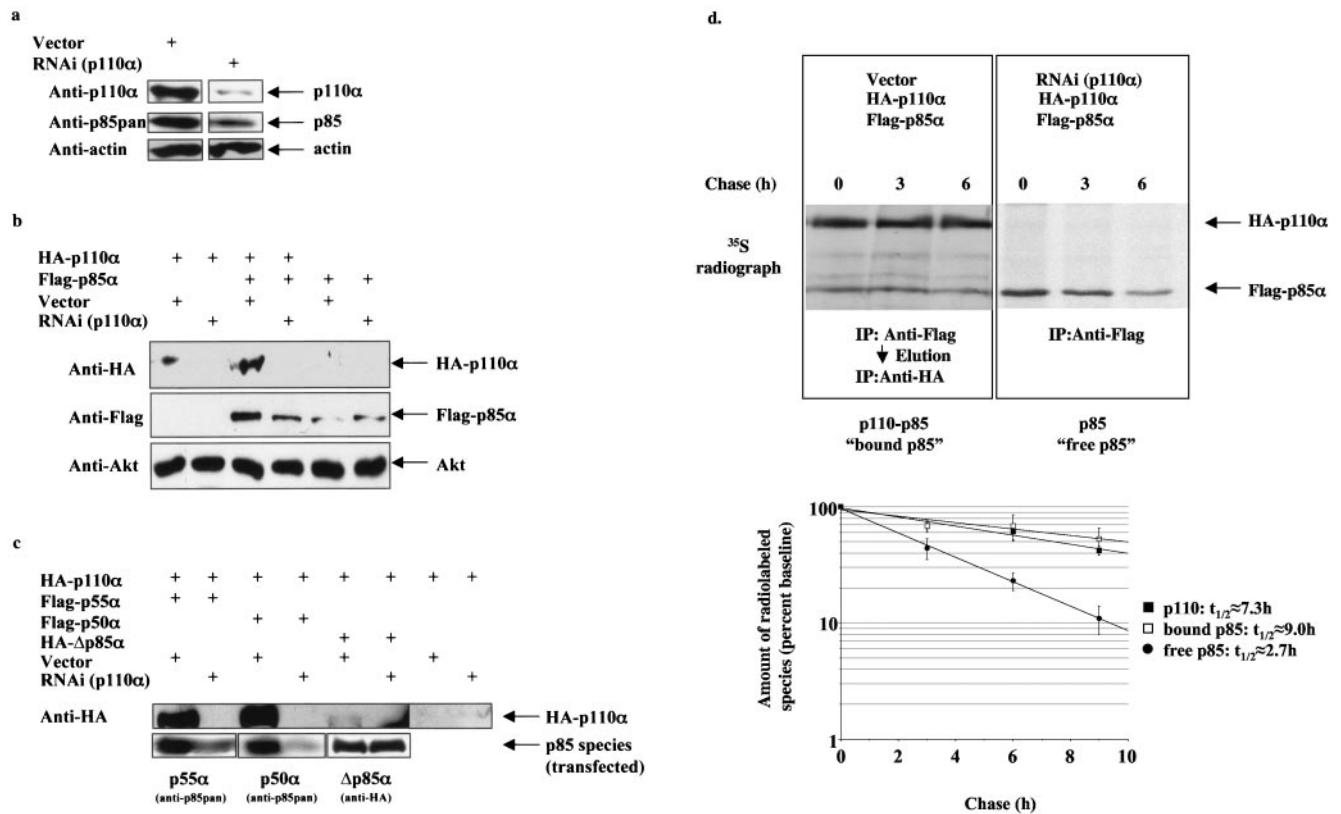


FIG. 6. Loss of p110 protein by RNAi causes instability of the p85 protein. (a) Endogenous p110 α and p85 protein levels in HeLa cells infected with either empty vector or shRNA to knock down expression of p110 α and selected for 7 days with puromycin. (b) p110 α and p85 α protein levels in CHO cells transiently transfected with bovine HA-p110 α or mouse Flag-p85 α and either empty vector or shRNA to knock down expression of p110 α . (c) p110 α , p55 α , p50 α , and Δ p85 α protein levels in CHO cells transiently transfected with bovine HA-p110 α , mouse Flag-p55 α , mouse Flag-p50 α , or human HA- Δ p85 α (p85 α lacking the inter-SH2 domain that binds p110) and either empty vector or shRNA to knock down expression of p110 α . (d) Pulse-chase experiment of CHO cells transiently transfected with bovine HA-p110 α , mouse Flag-p85 α , and either empty vector or shRNA for knockdown expression of p110 α . Graph shows the amount of radiolabeled p110, p85 bound to p110, and free p85 relative to baseline ($t = 0$ h). Data express means \pm standard errors of the means (p110, $n = 5$; bound p85, $n = 3$; free p85, $n = 5$). (e) Protein and relative mRNA levels of mouse Flag-p85 α from CHO cells transiently transfected with bovine HA-p110 α , mouse Flag-p85 α , and either empty vector or shRNA to knock down expression of p110 α . Relative mRNA levels were determined by real-time PCR and normalized to the expression level of 18S rRNA. Results are means of relative mRNA levels \pm standard deviations of the means.

indicate that p85 protein stability is positively regulated by the p110 protein level both in vivo and in cells in culture. Also, this mechanism seems to be dependent on the direct association between p110 and p85, since Δ p85 α protein levels are not affected by knockdown of p110. The decrease of p85 upon loss of p110 could be a compensatory mechanism to ensure that a system with decreased PI3K activity is not additionally hampered by the resulting excess of monomeric p85. These results indicate that the ratio of p85 to p110 is tightly controlled in the

cell. The results suggest that free p85 is preferentially degraded compared to p85 complexed with p110. However, the mechanism for this discriminatory degradation is not yet clear.

Figure 8 presents a pictorial representation of how deletion of p85 versus p110 subunits can have opposing effects on insulin sensitivity. Our previous studies have shown that p85 can exist in vivo, not only as a dimer complexed to p110 (PI3K) but also in an unbound form (41). While the p110-p85 complex acts as a mediator in insulin signaling by producing PI-3,4,5-P₃

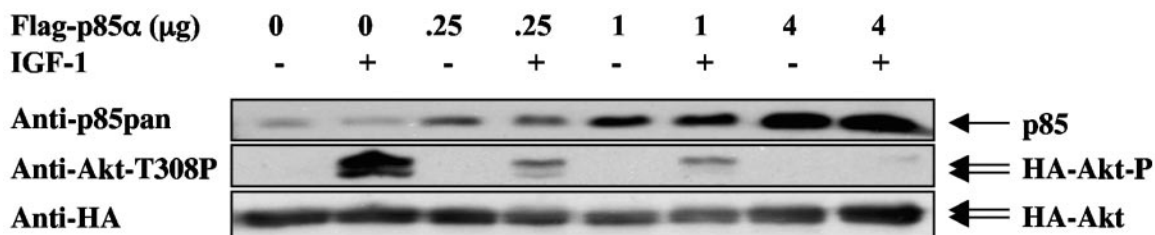


FIG. 7. Overexpression of p85 results in decreased insulin sensitivity. HA-Akt and increasing amounts of p85α were cotransfected into CHO cells. The cells were starved overnight, and one set of cells was stimulated with 20 nM IGF-1 for 5 min. The cells were lysed and HA-Akt was immunoprecipitated and blotted with anti-AktT308P antisera.

for Akt activation, free p85 provides a negative signal, as shown in Fig. 7, where a twofold overexpression of p85 over endogenous p85 potently blocked IGF-1-induced Akt activation. The ratio between p110-p85 and p85 might therefore define the normal state of insulin sensitivity in a wild-type setting. In the case of a mild reduction of total p85 levels in mice without loss of p110 proteins (e.g., p85α^{+/-} or p85β^{-/-} mice), increased insulin sensitivity is observed (33, 43). These findings argue that a negative regulator of insulin sensitivity is lost. Since wild-type p110 can only exist as a dimer bound to p85, but p110 levels are not reduced in p85α^{+/-} or p85β^{-/-} mice, only the free p85 pool can be reduced in these mutant mice. Therefore, we argue that free p85 is a negative regulator in insulin signaling and that the pool of free p85 (which has a shorter half-life and is not protected by p110) is preferably decreased in p85α^{+/-} or p85β^{-/-} mice. This is in agreement with the findings of Ueki et al. (41), showing that in p85α^{+/-} and p85α^{-/-} mouse embryonic fibroblasts the ratio between p110-p85 and free p85 is shifted towards p110-p85. The shorter

half-life for free p85 that we report here explains why free p85 is preferentially lost in the p85 knockout mice.

In the case of p110α^{+/-} p110β^{+/-} mice, we observe decreased insulin sensitivity. This finding now argues that the balance is shifted toward the negative regulator, meaning that preferably p110-p85 complexes are decreased over free p85. We suggest that upon reduction of p110 the p85 pool normally bound to p110 is less stable and therefore more rapidly degraded and hence preferentially reduced, while the pool of free p85 is not affected upon loss of p110. The proposed model fits best the present data but requires further investigation. Importantly, evidence is emerging that under physiological or pathological conditions levels of p85 are altered in insulin-sensitive tissues. Treatment of female mice with placental growth hormone to mimic pregnancy-induced insulin resistance resulted in an increase in p85 proteins but not p110 proteins in skeletal muscle (4). In addition, p85 levels decrease in skeletal muscle of postpartum women in parallel with increased insulin sensitivity (29).

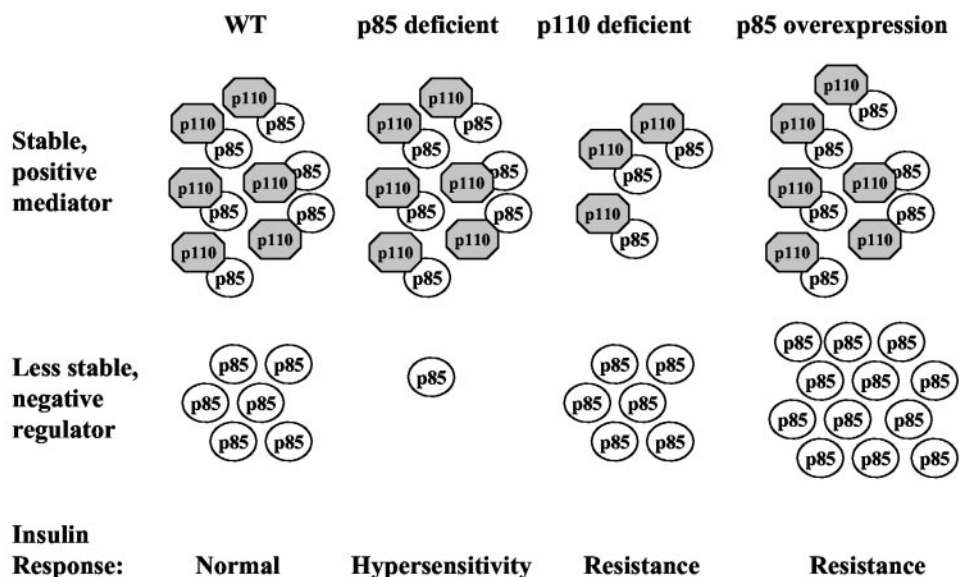


FIG. 8. Model. Insulin sensitivity is tightly regulated by the ratio of the positive mediator, p110-p85, and the negative regulator, unbound p85. A given ratio between p110-p85 and p85 might define the normal state of insulin sensitivity in a wild-type setting. In p85α^{+/-} or p85β^{-/-} mice, free p85 is decreased preferentially. Therefore, the balance between p110-p85 and free p85 is shifted towards the positive mediator, p110-p85, causing increased insulin sensitivity in the mutant mice. In contrast, in p110α^{+/-} p110β^{+/-} mice the p110-p85 pool is preferentially decreased over free p85. Hence, the balance is shifted towards the negative regulator, free p85, resulting in decreased insulin sensitivity. Overexpression of p85 causes a substantial increase in free p85 and therefore shifts the balance towards the negative regulator, resulting in decreased insulin sensitivity.

In summary, we report that double heterozygous loss of PI3K catalytic subunits p110 α and p110 β results in mice with slight glucose intolerance and with hyperinsulinemia in the fasting state. Besides the ~50% decrease in PI3K catalytic isoforms, also p85 protein levels were reduced in p110 α ^{+/-} p110 β ^{+/-} mice, indicating a surprising role of p110 subunits in controlling p85 levels. Given that genetic ablation of p85 isoforms leads to increased insulin sensitivity and glucose tolerance, we conclude that p85 plays a negative role in insulin signaling independent of its role in mediating activation of the p110 catalytic subunit. Therefore, pharmacological intervention into p85 expression in insulin-sensitive tissues might improve insulin responses in diabetic patients.

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