Chapter 5

Glucose homeostasis in p110-deficient mice

ABSTRACT

Several studies have demonstrated the pivotal role of phosphoinositide 3-kinase (PI3K) in glucose homeostasis. PI3K is necessary but not sufficient for the insulin-induced translocation of the glucose transporter GLUT4 from an intracellular compartment to the plasma membrane and the subsequent glucose uptake from the blood into muscle and fat. Thus, it was surprising that mice deficient in p85α or p85β regulatory subunits (p85α-/- or p85β-/- or p85α+/-p55γ+/-p50α+/- or p85α-/-p55γ-/-p50α-/-) had increased insulin sensitivity as measured by insulin tolerance tests and glucose tolerance tests (Terauchi et al., 1999), (Fruman et al., 2000; Mauvais-Jarvis et al., 2002; Ueki et al., 2002b). The insulin hypersensitivity was particularly unexpected in p85α-/-p55γ-/-p50α-/- mice where a decrease in p110α and p110β 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 ultimate shut off insulin-dependent glucose uptake *in vivo*. Indeed, there is evidence that protein kinases downstream of PI3K, including Akt and mTOR negatively regulate insulin responses (Gual et al., 2003), (Li et al., 1999), (Ricort et al., 1995), (Rui et al., 2001), (Hartley and Cooper, 2002). To test this model, we examined insulin responses in mice deficient in PI3K p110 catalytic subunits. Homozygous deletion of either p110α or p110β results in early embryonic lethality (Bi et al., 1999), (Bi et al., 2002). Thus, we studied mice with heterozygous loss of these isoforms. In this chapter we show that mice heterozygous for p110α or p110β have normal insulin tolerance tests and glucose tolerance tests. However, mice that are heterozygous for both p110α and p110β have impaired glucose tolerance tests and slight hyperinsulinemia. At a molecular level, these mice showed a ~50% decrease in p110α, p110β and p85 expression in comparison to wild-type mice.

PI3K activity associated with IRS1 and IRS2 was reduced to ~42% and ~35% of wild-type in liver, and to ~61% and ~95% of wild-type in muscle, respectively. However, p110α+/-p110β+/- mice exhibited normal insulin induced Akt activity in liver and muscle. These data show that deletion of p110 catalytic genes has the opposite effect on glucose disposal as deletion of p85 regulatory genes, even though both result in a decrease in total and insulin-stimulated PI3K activity in liver and muscle. We propose that insulin signaling 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.

INTRODUCTION

Role of PI3K in glucose homeostasis

Insulin lowers blood glucose by promoting glucose transport into peripheral tissues. After a meal, pancreatic beta cells sense increasing blood glucose levels and secrete insulin into the blood. Myocytes, adipocytes and hepatocytes respond to insulin in various ways resulting in increased glucose uptake into muscle and fat, and inhibition of glucose production in the liver. Based on ex vivo experiments PI3K is required for both processes (Cheatham et al., 1994), (Kotani et al., 1995), (Okada et al., 1994), (Quon et al., 1995). Insulin- induced glucose uptake into skeletal and cardiac muscle and adipocytes is mediated via glucose transporters (GLUTs), predominantly GLUT4. Insulin causes translocation of GLUT4 containing vesicles from intracellular compartments to the plasma membrane where they fuse with the membrane and facilitate glucose uptake from the blood into the cell.

Skeletal muscle is thought to be the major organ for glucose disposal. After glucose infusion, up to 85% of glucose is taken up by skeletal muscle whereas this organ accounts for about 50% of glucose disposal after glucose ingestion (DeFronzo et al., 1981), (Basu et al., 2001). White adipose tissue accounts for less than 10% of whole body glucose disposal. However, endocrine factors secreted by this organ influence insulin sensitivity of the muscle (adipocytokines, such as leptin, resistin and adiponectin) (for review: (Jazet et al., 2003), (Yamauchi et al., 2002).

Insulin resistance is a major characteristic of type 2 diabetes mellitus. Two types of diabetes are described. Type 1 diabetes is characterized by decreased insulin production due to autoimmune destruction of pancreatic beta cells. In contrast, the primary defect in type 2 diabetes is believed to be decreased insulin sensitivity of the peripheral organs. 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. The resulting hyperglycaemia causes blindness due to retinopathy, loss of limbs due to peripheral neuropathy, loss of kidney function due to nephropathy and heart

disease. Type 2 diabetes is also linked to obesity, polycystic ovarian disease, hyperlipidaemia, hypertension and atherosclerosis.

Insulin signaling results in PI3K activation. Insulin binds to the alpha subunits of the heterotetrameric insulin receptor on the cell surface. This interaction leads to transautophosphorylation of the insulin receptor beta subunits on intracellular tyrosine residues. Insulin receptor substrates (IRS) bind to these sites via their phosphotyrosine binding (PTB) domains and are phosphorylated by the insulin receptor on multiple tyrosine residues. These sites in turn recruit various SH2 domain containing signaling molecules from the cytosol, including the PI3K regulatory subunit p85 (Backer et al., 1993). At the plasma membrane, PI3K has access to its lipid substrates PI-4-P and PI-4,5-P_2 and converts them into PI-3,4-P_2 and PIP_3.

Multiple downstream effectors of PI3K are believed to regulate glucose metabolism. Among the PI3K downstream targets, the role of the serine/threonine kinase Akt/PKB on glucose metabolism has been studied the most. Akt is recruited to the plasma membrane via the interaction of its pleckstrin homology (PH) domain with PI3K lipid products, as is the PH domain containing kinase phosphoinositide dependent kinase (PDK) -1. Two subsequent phosphorylation events cause full activation of Akt: first, PDK1 phosphorylates Akt in the activation loop in the kinase core (Thr-308). Full activation of Akt requires a second phosphorylation event near the carboxyl terminus (Ser-473) (Alessi et al., 1996; Alessi et al., 1997), (Toker and Newton, 2000). Many of the metabolic effects of insulin require activation of Akt. While constitutively active Akt induces GLUT4 translocation in adipocytes, dominant negative Akt inhibits it (Cong et al., 1997). Furthermore, Akt promotes glycogen synthesis in muscle via inhibition of glycogen synthase kinase (GSK)- 3 (Cross et al., 1995), blocks hepatic glucose production and lipolysis via phosphodiesterase (PDE) phosphorylation and promotes protein synthesis via the release of inhibition of the mammalian target of rapamycin (mTOR). Consistent with this, targeted disruption of Akt2 in the mouse resulted in insulin resistance and severe diabetes (Cho et al., 2001a), (Garofalo et al., 2003). In contrast, Akt1-/- mice were not insulin resistant suggesting that Akt2 is primarily responsible for insulin responses (Cho et al., 2001b). Protein kinase C (PKC) ζ and PKCλ are also activated by the PI3K downstream target PDK-1 (Akimoto et al., 1996;

Chou et al., 1998). PKCζ and PKCλ have also been shown to regulate GLUT4 translocation (Kotani et al., 1998), (Bandyopadhyay et al., 1997). Thus, multiple protein Ser/Thr kinases downstream of PI3K mediate insulin responses.

Deletion of PI3K regulatory subunits improves insulin signaling. Despite the necessity of PI3K activity for insulin- induced glucose uptake, genetic ablation of any PI3K regulatory isoform surprisingly resulted in improved insulin signaling (Terauchi et al., 1999), (Fruman et al., 2000), (Mauvais-Jarvis et al., 2002), (Ueki et al., 2002b). Based on studies with mice lacking the first exon of p85α (where p55α and p50α alternative splice forms are upregulated Terauchi et al. hypothesized that the p55α and p50α isoforms were more efficient than p85α in mediating insulin responses. However, this model was challenged when mice deficient in all p85α splice variants (p85α-/-p55α-/-p50α-/-) and p85α null mice also exhibited hypersensitivity to insulin (Fruman et al., 2000), (Ueki et al., 2002b). Furthermore, heterozygous loss of all p85α splice forms (p85α+/-p55α+/-p50α+/-) improved type 2 diabetes in mice with double heterozygous loss of IR and IRS-1 (p85α+/-p55α+/-p50α+/-IR+/-IRS-1+/-) (Mauvais-Jarvis et al., 2002). Polymorphism in the human p85α gene has been associated with increased risk for developing type 2 diabetes (Barroso et al., 2003). However, the consequences of the polymorphism on the expression level or function of p85α have not been analyzed yet.

Free p85 might act as a competitor for recruitment of PI3K to the plasma membrane. Studies by Ueki et al. showed that there is more p85 protein than p110 protein in cells (Ueki et al., 2002a). Upon insulin stimulation the monomeric p85 might therefore compete with p85/p110 complexes for binding sites on IRS proteins. Although this model can explain how excess p85 impairs insulin signaling in many situations, it cannot explain why p85α-/-p55α-/-p50α-/- mice showed hypersensitivity to insulin despite a reduction in total PI3K and a 60% decrease of PI3K recruitment to IRS complexes after insulin treatment.

PI3K dependent serine phosphorylation of IRS proteins causes inhibition of insulin signaling. 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 human) 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 (Li et al., 1999), (Greene et al., 2003), (Aguirre et al., 2002), (Zick, 2001). 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 IKK inhibitor (Greene et al., 2003), (Li et al., 1999), (Gao et al., 2002) (Gual et al., 2003; Liu et al., 2001). Obesity has been shown to result in insulin resistance (Goldstein, 2002). One mechanism by which obesity causes insulin resistance is thought to occur via tumor necrosis factor (TNF)-induced, PI3K-dependent serine (Ser-307) phosphorylation on IRS proteins (Uysal et al., 1997), (Hotamisligil et al., 1993), (Ozes et al., 2001). Also, the c-jun kinase (JNK) has been implied in obesity-induced insulin resistance since obese mice lacking JNK1 (JNK1-/-) exhibit decreased IRS1 Ser-307 phosphorylation with increased sensitivity to insulin in comparison to obese JNK1+/+ mice (Hirosumi et al., 2002). These results indicate a tight negative feedback control of insulin signaling downstream of PI3K and raise the possibility that deletion of PI3K genes (regulatory or catalytic) might improve insulin signaling by preventing 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, we examined mice heterozygous for PI3K p110α and/or p110β. Heterozygous loss of p110α or p110β had no effect on insulin tolerance tests or glucose tolerance tests. However, mice with double heterozygous loss of p110α and p110β were slightly glucose intolerant and exhibit hyperinsulinemia at the fasting state. Interestingly, not only the PI3K catalytic isoforms are decreased by 50%, also p85 protein levels dropped by 50%. Given that genetic ablation of p85 isoforms generally leads to increased insulin sensitivity and glucose tolerance, these data imply a tight balance between a positive regulator (p110) and a negative regulator (p85).

Thus, although heterozygous loss of p110α and p110β results in a similar reduction in total class Ia PI3K in muscle and liver to that observed in the p85α+/-p55α+/-p50α+/- 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 shut off 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.

RESULTS

Mildly impaired glucose homeostasis upon combined heterozygous loss of p110α and p110β. Since PI3K activity has been shown to be necessary for insulin induced glucose uptake *in vitro* (Cheatham et al., 1994), (Hara et al., 1994), it was surprising that targeted disruption of any PI3K regulatory isoform caused improved insulin sensitivity in mice even when total PI3K activity in tissues was reduced (Fruman et al., 2000), (Ueki et al., 2002b). 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 (Bi et al., 1999), (Bi et al., 2002), mice with heterozygous loss of the isoforms were analyzed. To do so, female and male p110α+/-p110β+/+ (p110α+/-), p110α+/+p110β+/- (p110β+/-), p110α+/-p110β+/- and wild-type littermates at the ages of 2, 4 and 6 months were subjected to glucose and insulin tolerance tests (GTT and ITT, respectively). Mice were starved overnight and injected intraperitoneally with 2mg glucose per g of bodyweight (for GTT). Alternatively, to perform an ITT, they were starved for 4h in the morning and then injected with various amounts of insulin (0.5-1.5U insulin per kg of bodyweight). Then, the blood was collected from the tail and subjected to a glucometer to determine blood glucose levels. At early ages, there was no significant difference between genotypes detected in blood glucose clearance (data not shown). With increasing age (6 months), the male p110α+/-p110β+/- mice showed a mild defect in glucose clearance (Fig. 24). High fat diet induced obesity did not lead to abnormalities in glucose homeostasis in p110α+/- or p110β+/- mice in comparison to obese wild-type mice (data not shown). In order to measure insulin production during the GTT, blood from the tail was collected into heparinized tubes and the serum insulin levels were analyzed by enzyme linked immunoabsorbent assays (ELISA). Six -month -old male mice exhibited mild hyperinsulinemia during the GTT (the P values show that the difference is not statistical significant) (Fig. 24a, right panel).

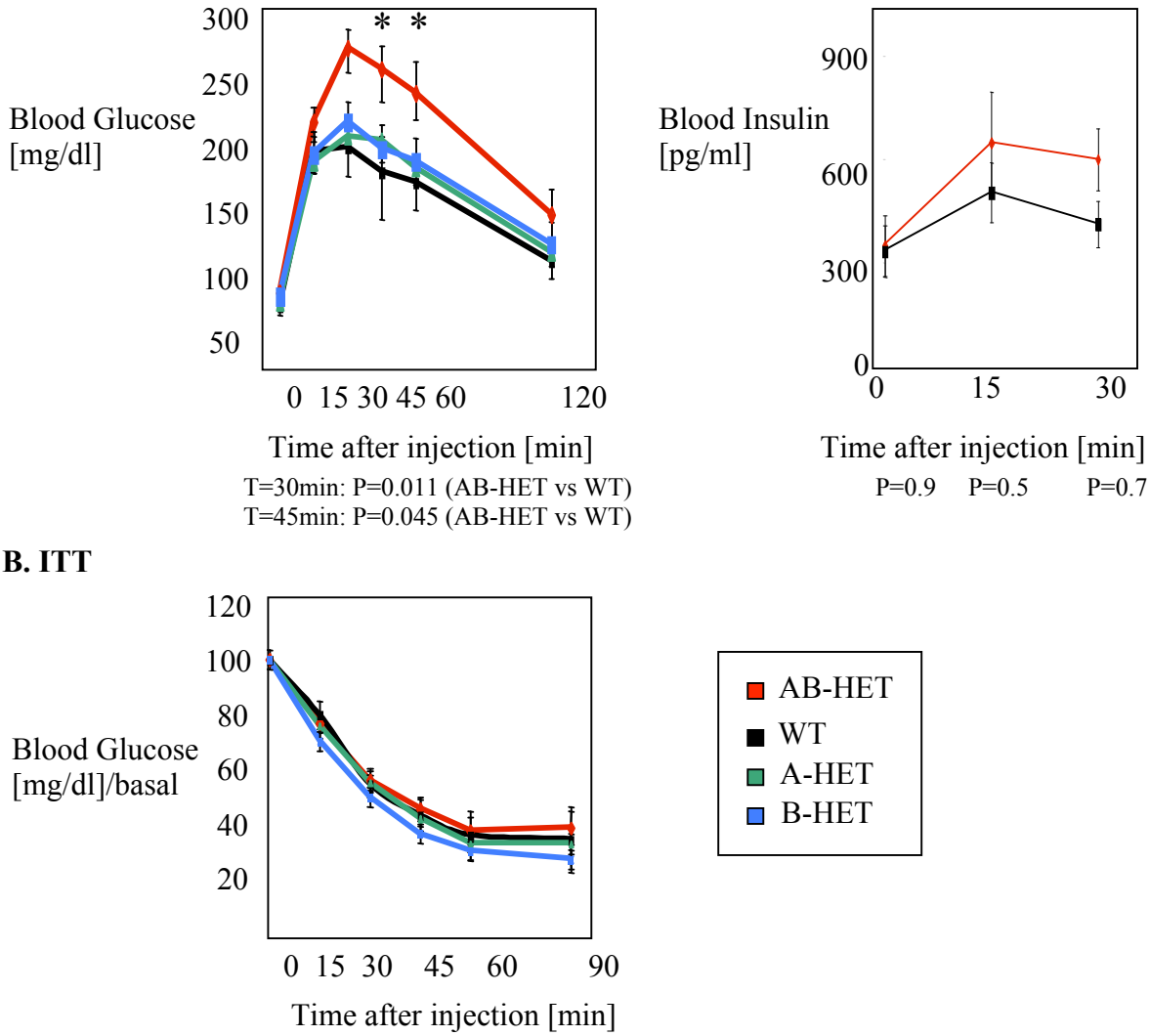

Fig. 24. Mice with heterozygous loss of p110□ and p110□ are slightly glucose intolerant. (A) Glucose tolerance test (GTT: AB-Het (p110□+/-p110□+/-): N=12, WT: N=6, A-Het: (p110□+/-) N=8, B-Het (p110□+/-): N=14, upper-left panel) and blood insulin levels during GTT (AB-Het: N=10, WT: N=5, upper-right panel) on six months old male mice with indicated genotypes. (B) Insulin tolerance test (ITT: AB-Het (p110□+/-p110□+/-): N=10, WT: N=5, A-Het (p110□+/-): N=8, B-Het: (p110□+/-) N=12, upper-right panel) on six months old male mice with indicated genotypes. Results are expressed as the mean +/- standard error of the blood glucose (GTT) or blood glucose/basal (ITT). P values were

Since abnormalities in glucose homeostasis are often reflected in overall weight differences, we frequently weighed wild-type, p110α+/-, p110β+/- and p110α+/-p110β+/- mice starting after weaning up to 6 months of age. The p110α+/-, p110β+/- and p110α+/-p110β+/- mice exhibited a similar weight to wild-type mice (Fig. 25a). On a high fat diet the mutant mice became obese to the same extent as wild-type mice (data not shown).

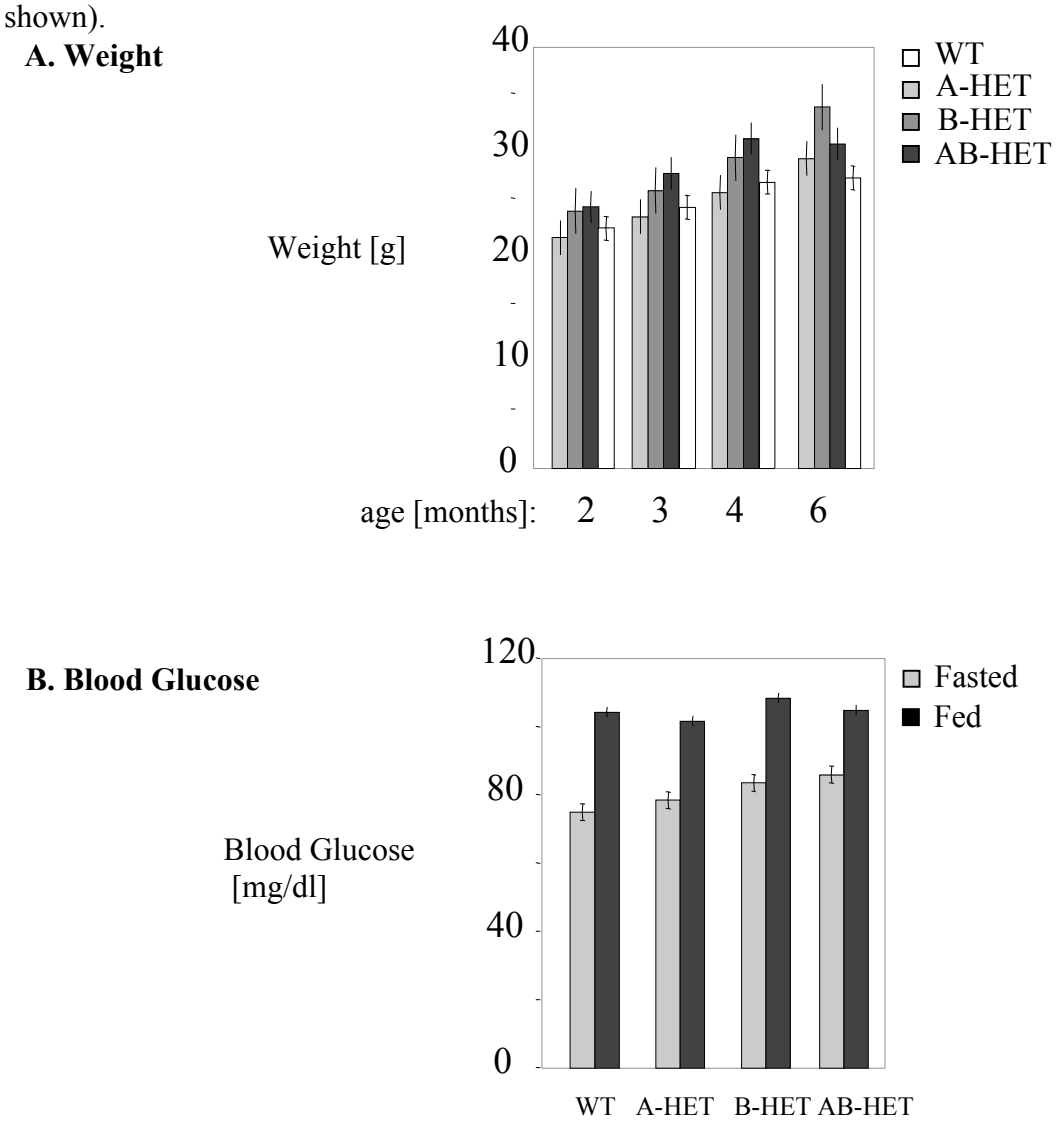

Fig. 25. Mice with heterozygous loss of p110α and/or p110β have normal weight and blood glucose levels. (A) Male mice with indicated genotypes (WT: wild-type, A-HET: p110α+/-, B-Het: p110β+/-, AB-HET: p110α+/-p110β+/-) were weighed at indicated times (upper panel). (B) Blood glucose levels of 6 -month -old male mice with indicated genotypes were assessed in the morning with or without over night fasting. The data express the mean bodyweight +/- standard deviation of the mean (AB-Het: N=12, WT: N=6, A-Het: N=8, B-Het: N=15). P values were calculated by Student T-Test. There was no statistical significant difference between heterozygous and wild-type mice.

Changes in insulin sensitivity are reflected in fasted and fed blood glucose and insulin levels: in the case of hypersensitivity to insulin, the glucose and insulin content in the blood are often lower, due to increased glucose clearance from the blood and compensating reduced insulin secretion by the pancreatic beta cells. In contrast, in the case of insulin resistance, the glucose uptake into peripheral tissues is decreased and therefore glucose is higher in the blood. Pancreatic beta cells secrete more insulin, hence also blood insulin levels are increased. We assessed the blood glucose and insulin concentration in the fasted (after overnight food withdrawl) and fed (at 11pm) state of male wild-type, p110α+/-, p110β+/- and p110α+/-p110β+/- mice at 6 months of age. P110α+/-, p110β+/- and p110α+/-p110β+/- mice had normal blood glucose levels (Fig. 25b), and only p110α+/-p110β+/- mice had mild basal hyperinsulinemia (Fig. 26).

Serum Insulin

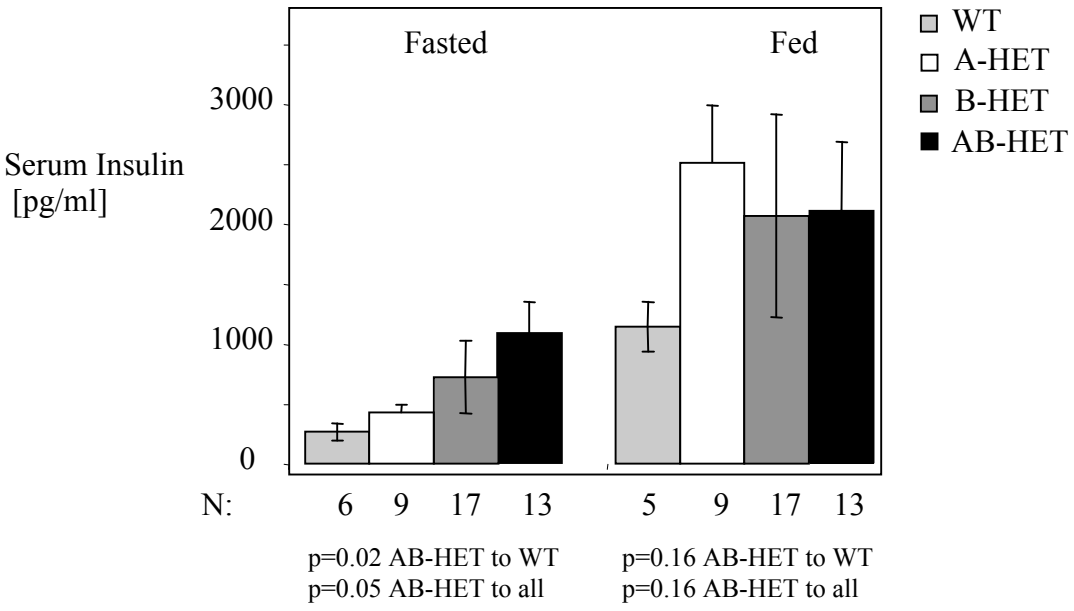

Fig. 26. Mice with heterozygous loss of p110α and p110β tend to have increased serum insulin levels. The fasted and fed serum insulin levels in 6 -month -old male mice with indicated genotypes (WT: wild-type, A-HET: p110α+/-, B-Het: p110β+/-, AB-HET: p110α+/-p110β+/-) were assessed in the morning after over night starvation (left panel) or in the evening (right panel). P values were calculated by Student T-Test.

Reduced PI3K regulatory subunits in insulin sensitive tissues of p110α+/-p110β+/- mice. Insulin induces glucose uptake in muscle and inhibits glucose production in the liver. Both of these effects are crucial for the regulation of glucose homeostasis. Therefore, we analyzed protein levels and activation state of enzymes in the insulin signaling pathway in liver and muscle of wild-type and p110α+/-p110β+/- mice. The mice were fasted overnight prior to vena cava injection with PBS or 10U/kg of bodyweight insulin. The tissues were dissected after 5min of treatment and lysates from liver and muscle were immunoprecipitated with anti-p110α or anti-p110β antibodies. In order to assess alterations in p110α and p110β protein levels, the immunoprecipitates were subjected to western blot analysis using either anti-p110α or anti-p110β antibodies. Alternatively, the immunoprecipitates were subjected to *in vitro* PI3K assays to analyze the p110α and p110β kinase activity. As expected, double heterozygous loss of p110α and p110β (p110α+/-p110β+/-) resulted in a ~50% reduction of both p110α and p110β protein levels as well as a ~50% reduction of PI3K activities associated with the catalytic isoforms in liver (Fig. 27, 28) and muscle (Fig. 29, 30). 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 p110α+/-p110β+/- mice p85 protein levels were unexpectedly decreased to ~50% of levels in wild-type tissue. The smaller p85 isoform p50α was also reduced in liver but not in muscle of p110α+/-p110β+/- mice in comparison to wild-type (Fig. 27, 29).

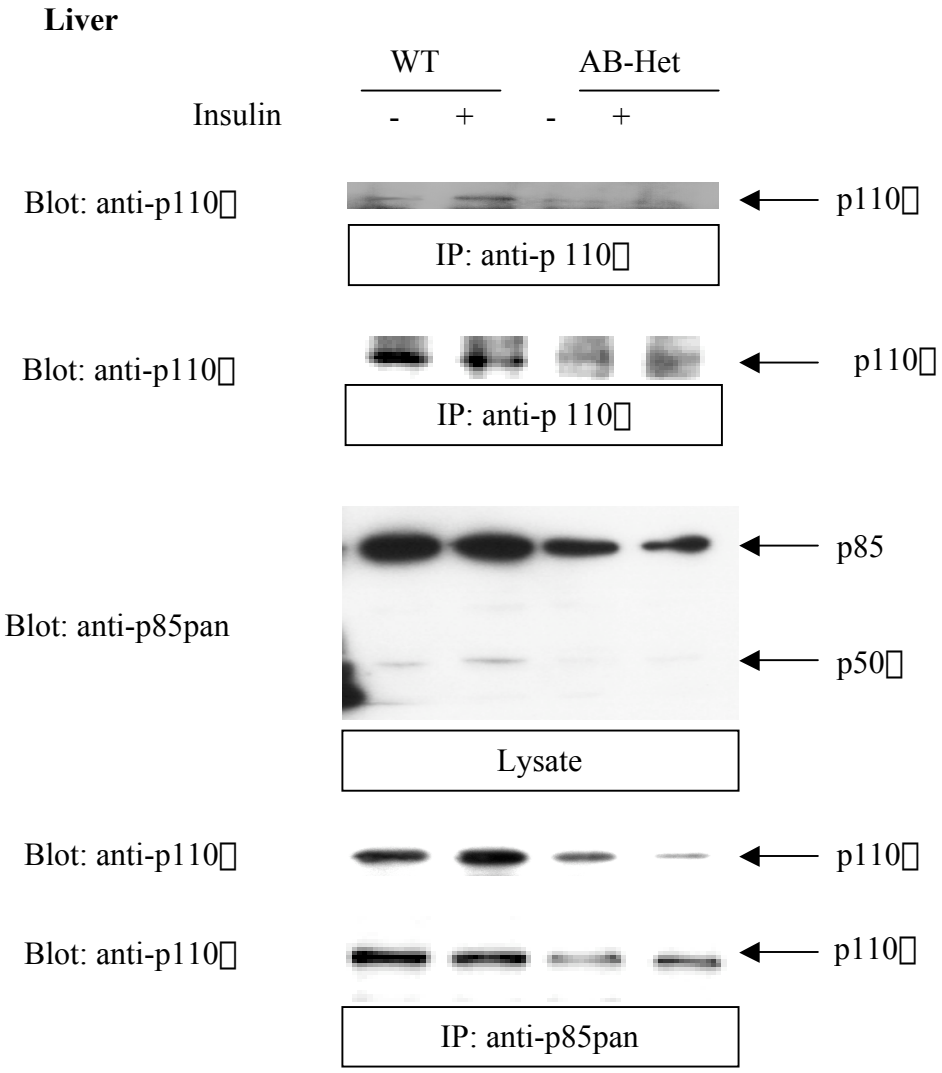

Fig. 27. Mice with heterozygous loss of p110 and p110 have decreased p110 and p85 levels in liver lysates. Wild-type or p110+/-p110+/- (AB-HET) 4 -month -old mice were starved overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of liver lysates were subjected to immunoprecipitation and proteins were resolved by SDS-PAGE and immunoblotted with anti-p110 and anti-p85 antibodies.

Liver

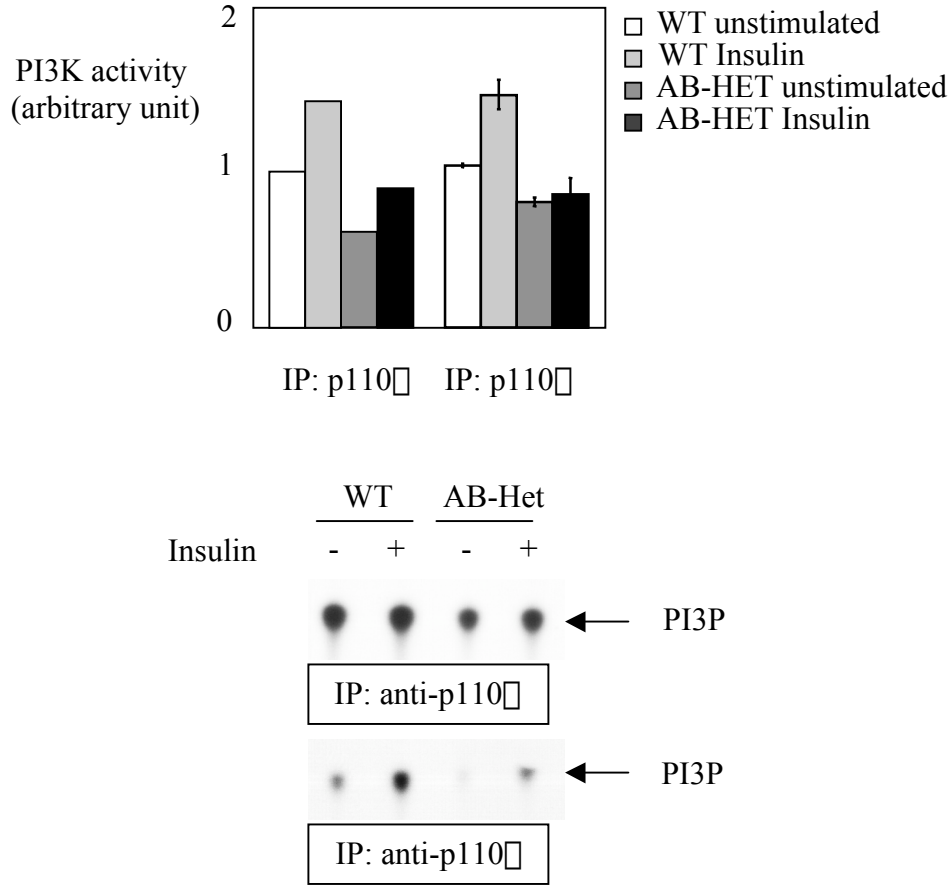

Fig. 28. Mice with heterozygous loss of p110 and p110 have decreased p110 activity in liver lysates. Wild-type or p110+/-p110+/- (AB-HET) 4 -month -old mice were starved overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of liver lysates were subjected to immunoprecipitation and subsequent in vitro PI3-kinase assay (WT unstimulated: N=2, WT Insulin: N=4, AB-HET unstimulated: N=4, AB-HET Insulin: N=6, results reported as mean +/- standard error). Lower panel shows a representative experiment.

Muscle

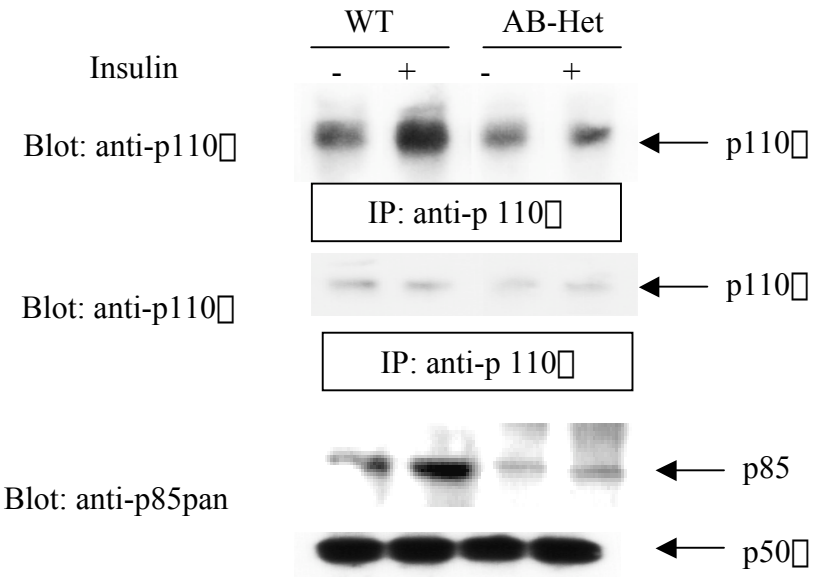

Fig. 29. Mice with heterozygous loss of p110α and p110b have decreased p110 and p85 levels in muscle lysates. Wild-type or p110α+/- p110β+/- (AB-HET) 4 -month -old mice were starved overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of muscle lysates were subjected to immunoprecipitation and proteins were resolved by SDS-PAGE and immunoblotted with anti-p110 and anti-p85 antibodies.

Muscle

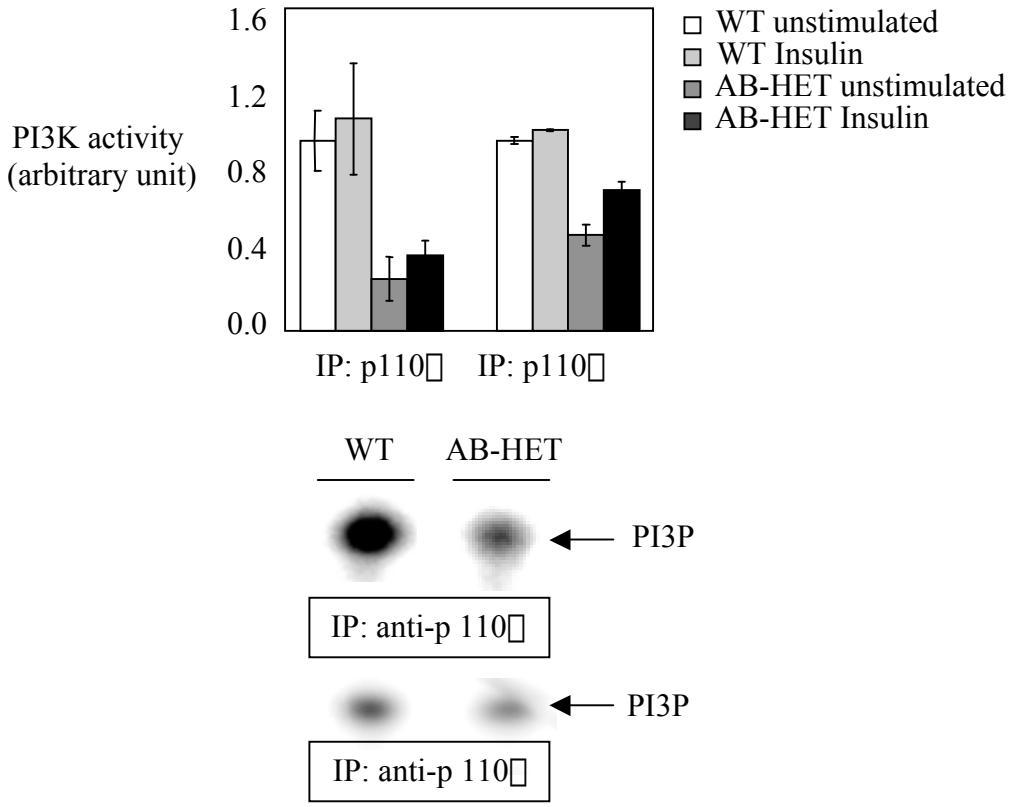

Fig. 30. Mice with heterozygous loss of p110α and p110β have decreased p110 activity in muscle lysates. Wild-type or p110α+/-p110β+/- (AB-HET) 4 -month -old mice were starved overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of muscle lysates were subjected to immunoprecipitation and subsequent in vitro PI3-kinase assay (WT unstimulated: N=2, WT Insulin: N=3, AB-HET unstimulated: N=3, AB-HET Insulin: N=3, results reported as mean +/- standard error). Lower panel shows a representative experiment of WT and AB-HET without stimulation.

The amount of PI3K activated upon insulin stimulation can be analyzed by measuring the recruitment of PI3K to insulin receptor substrates. Therefore, the liver and muscle lysates were immunoprecipitated with anti-IRS1 and anti-IRS-2 antibodies and then the immunoprecitipates were subjected to *in vitro* PI3K assays. Insulin-induced interaction between IRS1 and PI3K was reduced to ~42% in liver (Fig. 31) and ~61% in muscle (Fig. 32) of p110α+/-p110β+/- mice in comparison to wild-type. Insulin-stimulated association between IRS2 and PI3K was normal in muscle (~95%) (Fig. 32), but reduced to ~65% in liver (Fig. 31) of p110α+/-p110β+/- mice. The tyrosine phosphorylation of IR, IRS1 and IRS2 was assessed on lysates (IR) or anti-phosphotyrosine immunoprecipitates (IRS).

To assess *in vivo* insulin signaling, we injected a large number of mice (up to 18 mice) at the same time and then analyzed only those that showed comparable IR tyrosine phosphorylation. This allowed us to control for variability in efficiency of insulin injections. The tyrosine phosphorylation of IRS1 and IRS2 proteins in response to insulin injections was comparable between mutant and wild-type mice (Fig. 31, 32).

Liver

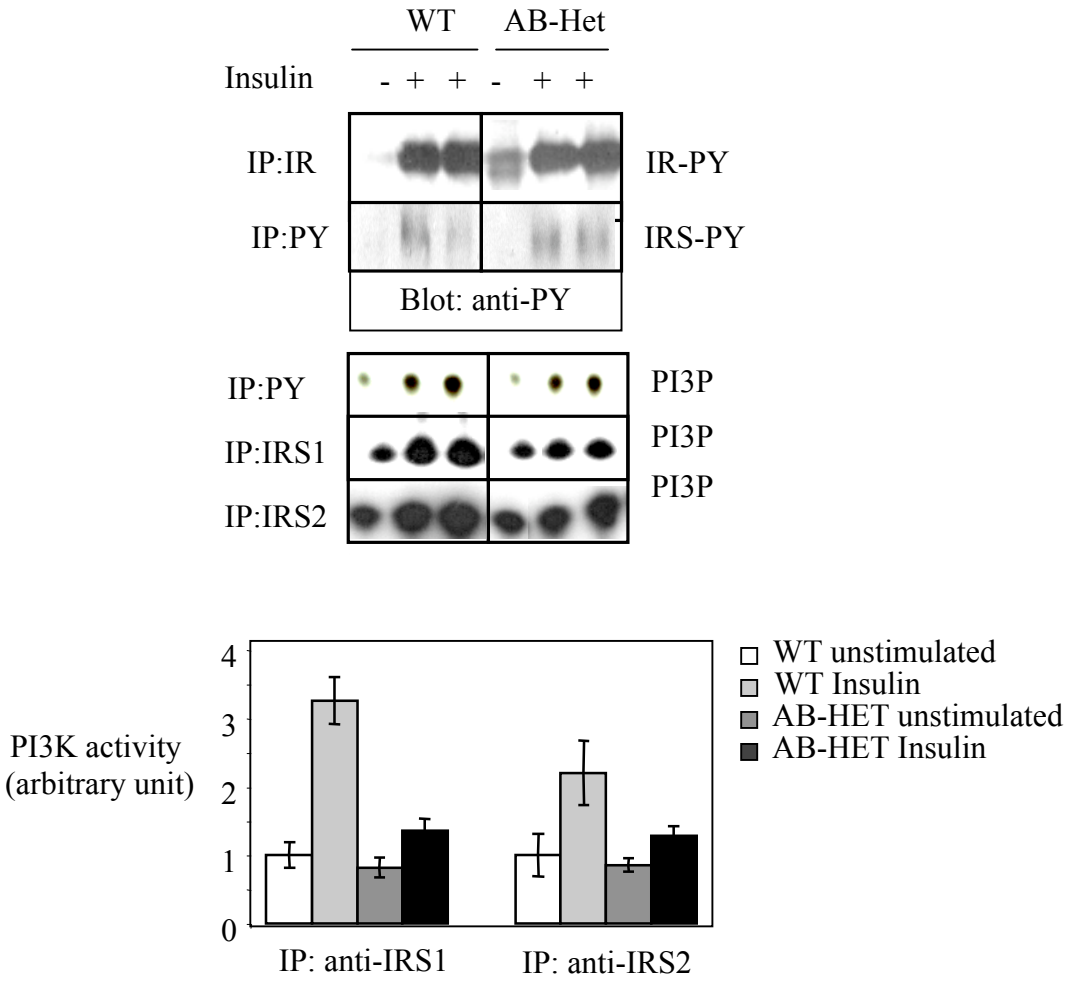

Fig. 31. Mice with heterozygous loss of p110 and p110 have decreased phosphotyrosine associated PI3-kinase activity in liver lysates. Wild-type or p110+/-p110+/- (AB-HET) 4 -month -old mice were starved overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of liver lysates were subjected to immunoprecipitation and subsequent in vitro PI3-kinase assay (WT unstimulated: N=2, WT Insulin: N=3, AB-HET unstimulated: N=4, AB-HET Insulin: N=4, results reported as mean +/- standard error) or the proteins were resolved on SDS-PAGE and probed with anti-phosphotyrosine. The samples were chosen for similar levels of phosphorylation of the insulin receptor from a total of 18 samples.

Muscle

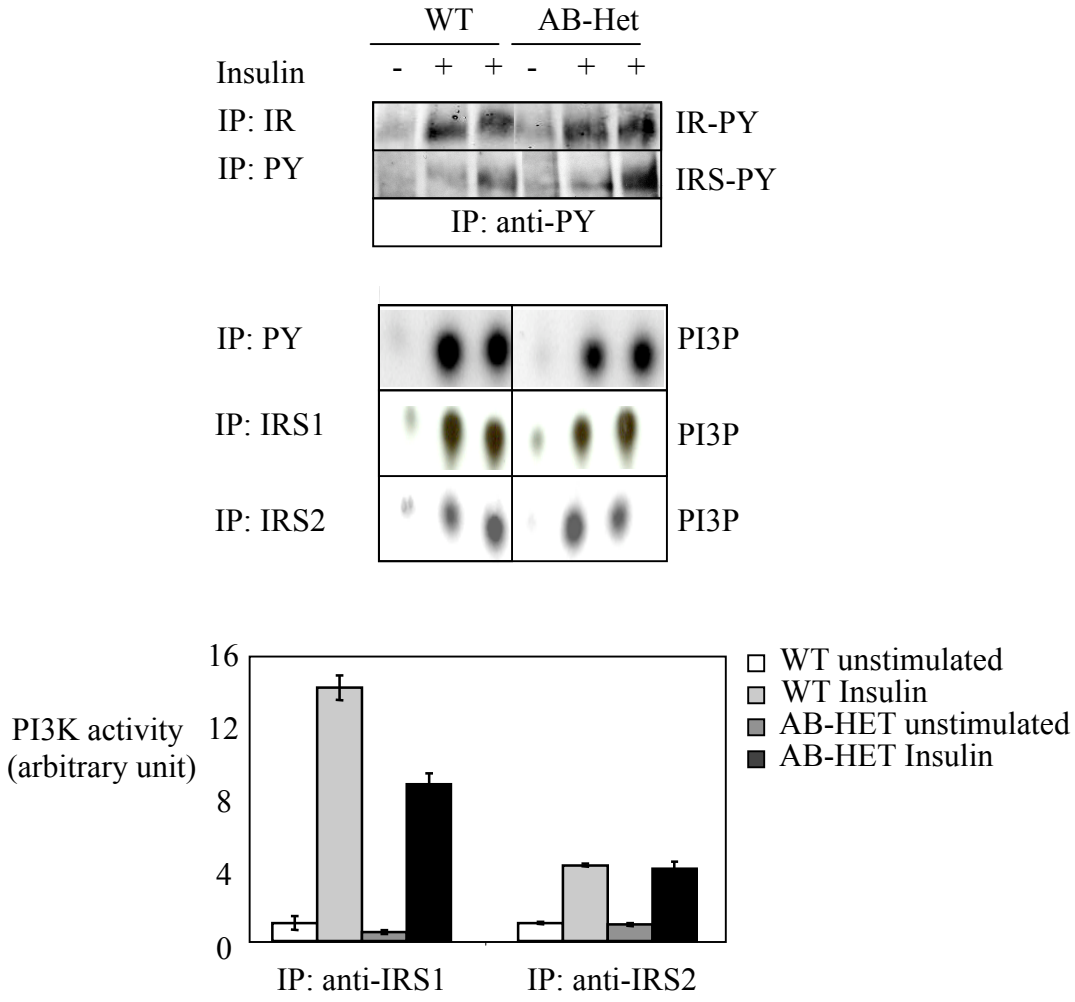

Fig. 32. Mice with heterozygous loss of p110□ and p110□ have decreased phosphotyrosine associated PI3-kinase activity in muscle lysates. Wild-type or p110□+/-p110□+/- (AB-HET) 4 -month -old mice were fasted overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of muscle lysates were subjected to immunoprecipitation and subsequently subjected to an in vitro PI3-kinase assay (WT unstimulated: N=2, WT Insulin: N=3, AB-HET unstimulated: N=3, AB-HET Insulin: N=4, results reported as mean +/- standard error) or the proteins were resolved on SDS-PAGE and probed with anti-phosphotyrosine. The samples were chosen for similar levels of phosphorylation of the insulin receptor from a total of 18 samples.

Preserved Akt activation in insulin sensitive tissues despite reduced PI3K activity. The activities of the PI3K downstream targets, Akt and Erk were analyzed by western blot analysis of liver and muscle lysates using phospho-specific antibodies. Furthermore, anti-Akt immunoprecitipates were subjected to *in vitro* kinase assays in order to determine the activity of Akt in liver and muscle lysates. Both Akt activity and Erk activity were preserved upon stimulation with saturating amounts of insulin in liver and muscle of p110α+/-p110β+/- mice (Fig. 33, 34). In summary, targeted disruption of PI3K catalytic isoforms results, unexpectedly, in reduced expression of PI3K regulatory isoforms. Insulin-induced recruitment of PI3K activity to IRS proteins is reduced in liver and muscle tissues. Nonetheless, treatment of p110α+/-p110β+/- mice with saturating levels of insulin results in normal Akt and Erk activation in these tissues. However, p110α+/-p110β+/- mice exhibit mild signs of insulin resistance, such as mild hyperinsulinemia at a basal state and mildly delayed glucose uptake in the GTT, indicating that subsaturating insulin responses are impaired.

Liver

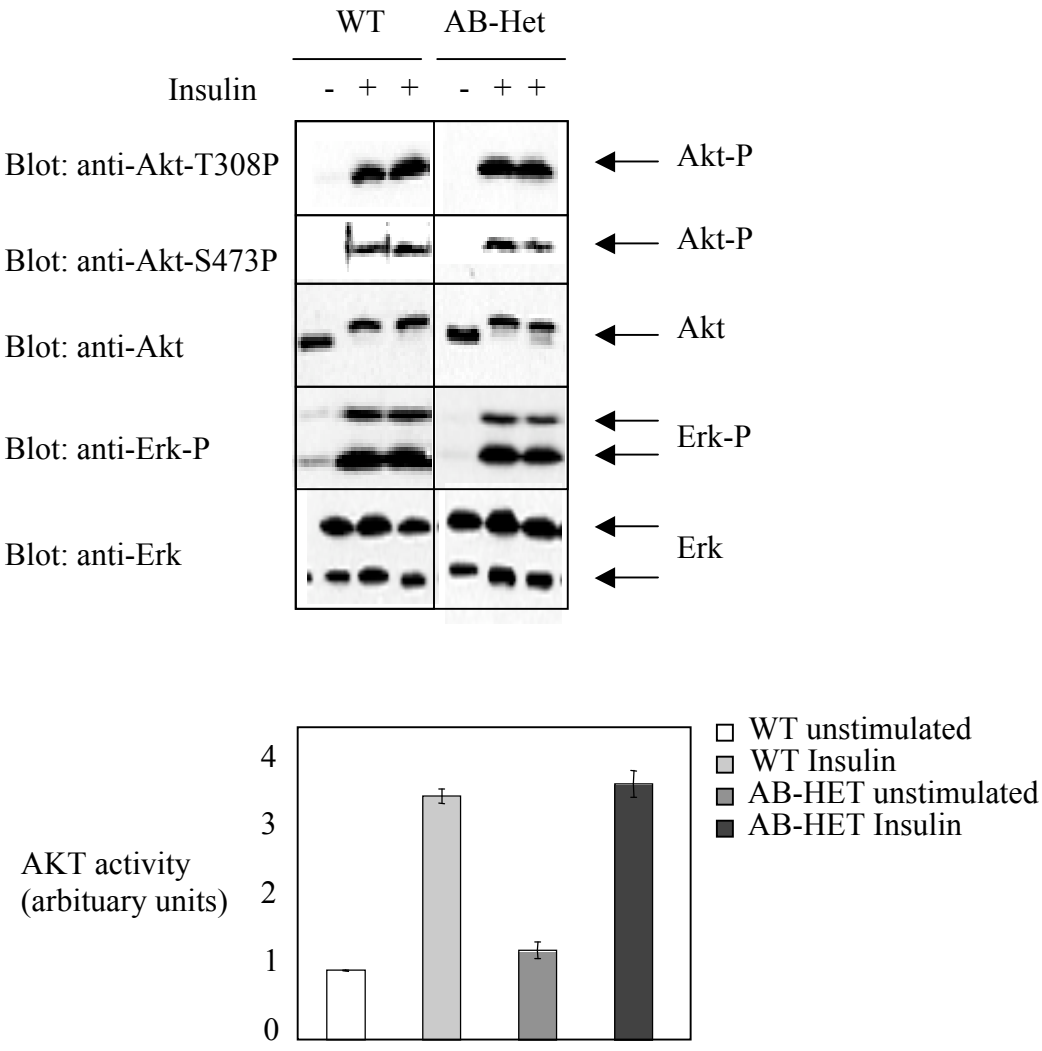

Fig. 33. Mice with heterozygous loss of p110α and p110β have normal Akt and Erk activity in liver lysates. Wild-type or p110α+/-p110β+/- (AB-HET) 4 -month -old mice were fasted overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of liver lysates were subjected to immunoprecipitation and subsequent in vitro Akt kinase assay (WT unstimulated: N=3, WT Insulin: N=3, AB-HET unstimulated: N=3, AB-HET Insulin: N=6, results reported as mean +/- standard error) or the proteins were resolved on SDS-PAGE and probed with indicated antibodies. The samples were chosen for similar levels of phosphorylation of the insulin receptor from a total of 18 samples.

Muscle

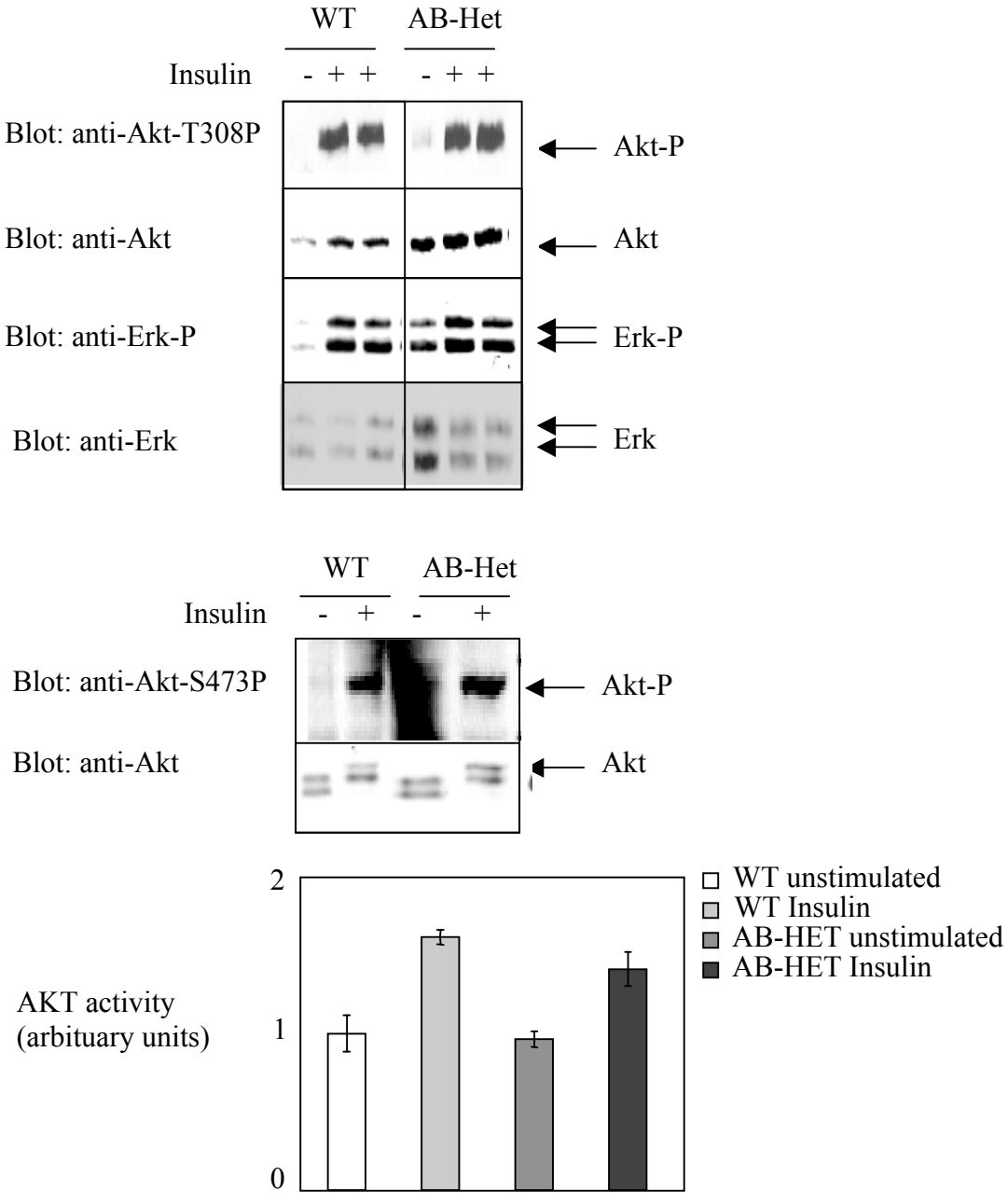

Fig. 34. Mice with heterozygous loss of p110α and p110β have normal Akt and Erk activity in muscle lysates. Wild-type or p110α+/-p110β+/- (AB-HET) 4 -month -old mice were fasted overnight and then injected for 5min with either insulin or PBS into their vena cava. Equal amounts of muscle lysates were subjected to immunoprecipitation and subsequent in vitro Akt kinase assay (WT unstimulated: N=2, WT Insulin: N=3, AB-HET unstimulated: N=3, AB-HET Insulin: N=4, results reported as mean +/- standard error) or the proteins were resolved on SDS-PAGE and probed with indicated antibodies.The samples were chosen for similar levels of phosphorylation of the insulin receptor from a total of 18 samples.

DISCUSSION

Extensive studies have shown that PI3K activity is required for insulin induced glucose uptake (Cheatham et al., 1994), (Hara et al., 1994), (Okada et al., 1994). 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 (Terauchi et al., 1999), (Fruman et al., 2000), (Mauvais-Jarvis et al., 2002), (Ueki et al., 2002b). We were interested to know whether reduction of PI3K by deletion of p110 subunits might also improve insulin sensitivity. We show here that combined heterozygous loss of p110α and p110β results in a ~50% decrease of class Ia PI3K (Fig. 27, 29) without improved insulin signaling (Fig. 24). Indeed, mutant mice have a mild glucose intolerance and exhibit mild hyperinsulinemia in the fasting state (Fig. 24a and 26). 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 p85α-/-p55α-/-p50α-/- 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 question arises how does p85 inhibit insulin signaling independently of p110. Loss of p85 increases PIP_3 levels independently of PI3K activity (Mauvais-Jarvis et al., 2002), (Ueki et al., 2002a). In agreement with this model, Akt activity is increased in both p85α+/-p55α+/-p50α+/- mice and p85α-/- mice without increased PI3K activity associated with IRS proteins (Mauvais-Jarvis et al., 2002), (Ueki et al., 2002b). These findings raise the possibility that p85 isoforms might activate a PIP_3 phosphatase and thereby contribute to a shut-off of PI3K signaling (Gupta et al., 1999). Another possibility is that p85 contributes to JNK activation independent of PI3K regulation. JNK activity causes phosphorylation on serine 307 on IRS-1 (Aguirre et al., 2000). There is a growing understanding that serine phosphorylation on IRS proteins results in decreased insulin signaling (Hirosumi et al., 2002). In p85α-/-p55α-/-p50α-/- brown adipocytes

JNK activity is decreased in comparison to wild-type and the defect can be restored by reintroduction of p85 isoforms as well as a p85 mutant (delta-p85) that lacks the binding site for p110 (Ueki et al., 2003).

Single heterozygous loss of either p110α or p110β does not lead to abnormalities in the GTT or ITT (Fig. 24). These mutant mice behave like wild-type, even when they are challenged with high fat induced obesity (fat secretes factors that cause insulin resistance). The most likely explanation for their unaltered glucose homeostasis is that the remaining p110 is sufficient to mediate proper insulin signaling. Asano et al. suggested that p110β specifically mediates insulin induced GLUT4 translocation: microinjection of inhibitory antibodies specifically blocking p110β activity blocked insulin induced translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes whereas inhibitory antibodies towards p110α had only a minor effect (Asano et al., 2000). We found that single heterozygous loss of p110α or p110β does not point to isoform specificity. It is possible that the remaining p110α or p110β is sufficient for normal signaling in each case. Complete loss of either isoform results in early embryonic lethality (Bi et al., 1999), (Bi et al., 2002). Therefore, it would be interesting to compare the glucose homeostasis of mice with homozygous loss of either p110α or p110β in distinct tissues, such as liver, muscle, fat, brain or the pancreas.

Many studies showed that monomeric p110 is thermally unstable while heterodimerization with p85 causes stabilization of p110 protein (Fruman et al., 1999), (Yu et al., 1998). Therefore, it was not surprising that fibroblasts derived from p85α-/-p55γ-/-p50α-/-p85β-/- cells have substantially decreased p110 protein levels (described in the previous chapter). However, the studies presented in this chapter indicate that the p85 protein level is regulated by the p110 protein level. 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 (which acts as a dominant negative). One could assume that the excess p85 over p110 is tightly controlled in the cell. We have preliminary evidence by northern blot analysis of liver lysates that the loss of p85 protein is not reflected by loss of mRNA (data not shown). It is therefore likely to be controlled on the posttranslational level. Furthermore, in contrast to the 85kD isoforms, the loss of the smaller p50α isoform was only detected in liver of

p110α+/-p110β+/- but not in muscle (Fig 27 and 29). This finding argues for a p110-dependent, tissue-specific regulation of p85 levels.

Upon insulin treatment of p110α+/-p110β+/- mice, we detected a significantly decreased PI3K activity associated with IRS proteins in liver and muscle despite normal IRS tyrosine phosphorylation (Fig. 31 and 32). This is probably due to a 50% decrease of both the catalytic as well as regulatory subunits of PI3K. However, despite the decreased PI3K activity we detected normal Akt activity in liver and muscle after insulin treatment (10U/kg) (Fig. 33 and 34). However, also the Akt activity in p85α-/-p55γ-/-p50α-/- mice has been shown to be similar to wild-type despite a 60% decrease of PI3K activity associated with IRS proteins (Fruman et al., 2000). We believe that the signal amplification downstream of activated PI3K causes maximum Akt activation in both mutant mouse models. It is possible that defects in *in vivo* Akt activation in the p110α+/-p110β+/- mice would have been detected at lower levels of insulin injection.

In summary, we report that double heterozyogous loss of PI3K catalytic subunit p110α and p110β 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 by ~50% in p110α+/-p110β+/- 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.