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Abstract

Protein kinase B (PKB)/Akt is considered to be a key target downstream of insulin receptor substrate 2 (IRS2) in the regulation of beta-cell mass. However, while deficiency of IRS2 in mice results in diabetes with insulin resistance and severe failure of beta-cell mass and function, only loss of the PKBbeta isoform leads to a mild metabolic phenotype with insulin resistance. Other isoforms were reported not to be required for metabolic regulation. To clarify the role of the three PKB isoforms in the regulation of islet mass and glucose homeostasis, we assessed the metabolic and pancreatic phenotypes of Pkbalpha, Pkbbeta or Pkbgamma -deficient mice. Our study uncovers a novel role for PKBalpa in the regulation of glucose homeostasis, whereas it confirms that Pkbbeta (-/-) mice are insulin resistant with compensatory increase of islet mass. Pkbalpha (-/-) mice displayed an opposite phenotype with improved insulin sensitivity, lower blood glucose and higher serum glucagon concentrations. Pkbgamma (-/-) mice did not show metabolic abnormalities. Additionally, our signalling analyses reveal that PKBalpa, but not PKBbeta or gamma, is specifically activated by overexpression of IRS2 in beta-cells and is required for IRS2 action in the islets.
Differential effects of PKB/Akt isoforms on glucose homeostasis and islet mass

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Word count: Materials and Methods: 1432 words
Introduction, Results and Discussion: 3620 words
Abstract

Protein kinase B (PKB)/Akt is considered to be a key target downstream of insulin receptor substrate 2 (IRS2) in the regulation of β-cell mass. However, while deficiency of IRS2 in mice results in diabetes with insulin resistance and severe failure of β-cell mass and function, only loss of the PKBβ isoform leads to a mild metabolic phenotype with insulin resistance. Other isoforms were reported not to be required for metabolic regulation. To clarify the role of the three PKB isoforms in the regulation of islet mass and glucose homeostasis, we assessed the metabolic and pancreatic phenotypes of Pkbα, Pkbβ or Pkbγ-deficient mice. Our study uncovers a novel role for PKBα in the regulation of glucose homeostasis, whereas it confirms that Pkbβ−/− mice are insulin resistant with compensatory increase of islet mass. Pkbα−/− mice displayed an opposite phenotype with improved insulin sensitivity, lower blood glucose and higher serum glucagon concentrations. Pkbγ−/− mice did not show metabolic abnormalities. Additionally, our signalling analyses reveal that PKBα, but not PKBβ or γ, is specifically activated by overexpression of IRS2 in β-cells and is required for IRS2 action in the islets.
Introduction

Adaptation of pancreatic islet mass and function relative to metabolic demand maintains glucose homeostasis and may prevent the development of type 2 diabetes. β-Cell proliferation, apoptosis, growth and function are tightly regulated by various extracellular factors and intracellular signalling pathways (23,24,34). In β-cells, insulin receptor substrate (IRS) 2 controls maintenance and expansion of islet mass (29,31,42). In fact, IRS2-deficient mice are insulin resistant, show β-cell failure, hyperglycaemia and finally develop diabetes (26,42). In contrast, deficiency for IRS1 only causes insulin resistance without the development of diabetes due to a compensatory increase in functional β-cell mass (1,38). These observations indicated that IRS2, but not IRS1, is necessary for maintenance and compensatory increase of β-cell mass. Furthermore, experiments with isolated islets revealed that overexpression of IRS2, but not of IRS1, can increase β-cell proliferation and protect cells against high glucose-induced apoptosis (29). Downstream of IRS2, phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB) signalling is considered to be the critical pathway for the regulation of β-cell mass and function (12,15,16,27). The serine-threonine kinase PKB, also known as Akt, is required for various cellular processes, from the regulation of cell cycle, survival and growth to glucose and protein metabolism. In mammals, three PKB/Akt isoforms have been characterised and named PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3. Although encoded by different genes on different chromosomes, the three isoforms display high homology at the protein level with 80-85% identical residues and the same structural organisation (43). However, they differ in terms of tissue-specific expression. PKBα is expressed in most tissues, PKBβ is highly expressed in insulin responsive tissues, whereas PKBγ expression is prominent in brain and testes (17). All three isoforms are expressed in β-cells (30,37). The role of PKB in different tissues has been studied in transgenic mouse
PKB isoforms in glucose homeostasis

While Pkbα−/− and Pkbγ−/− mice show impaired foetal growth and brain development, respectively, glucose homeostasis is unaffected in both models (9,11,14,39,46). In contrast, Pkbβ−/− mice are insulin resistant, mildly glucose-intolerant and have less adipose tissue. Depending on strain and gender, these mice show either late loss of β-cells followed by the development of diabetes and mild growth deficiency, or compensatory increase of β-cell mass without age-dependent progression into overt hyperglycaemia (10,17). These studies suggested that PKBβ is the only isoform playing a role in the regulation of energy homeostasis. On the other hand, constitutive activation of PKBα in β-cells is sufficient to increase growth and proliferation (5,40), and in INS1 cells prevents FFA-induced apoptosis (44). Furthermore, antagonization of total PKB activity in β-cells by ectopic expression of a kinase-dead mutant causes defects in insulin secretion (4), suggesting that in islets PKB is required mainly for normal function of the β-cells. Although these data support the notion that PKB must play a role in pancreatic β-cells, they are not in line with the stronger metabolic phenotype displayed by IRS2-deficient mice. In fact, PKBα and γ appear not at all to be required to regulate glucose homeostasis (9,11,39) and in the case of Pkbβ−/− mice, even though glucose homeostasis is impaired due to strong peripheral insulin resistance, overall metabolic phenotype is by far not as severe as in Irs2−/− mice (10), indicating that the capacity for β-cell compensation is retained in the absence of PKBβ.

The aim of this study was to clarify the role of PKB in the regulation of islet mass and to define the relevance of PKB isoforms for IRS2 action in β-cells. Although it had been shown that PKBα is dispensable for regulation of glucose homeostasis (9,11), we found lower blood glucose concentrations in Pkba−/− mice. Based on this observation, we assessed in more detail the metabolic and the endocrine pancreatic phenotypes of Pkba, Pkbβ or Pkbγ-deficient mice. In addition, glucose uptake into fat cells, insulin secretion and islet cell proliferation were investigated. Contrary to previous assumptions, implying that PKBβ is the only (or at least the
main) isoform playing a role in the regulation of glucose metabolism, we present evidence that both PKBα and β isoforms are required in the periphery for regulation of glucose homeostasis. While we confirmed that Pkbβ−/− mice are insulin resistant and glucose intolerant with compensatory increase of β-cell mass, Pkbα−/− mice showed lower blood glucose levels, were more insulin sensitive and revealed higher serum glucagon concentrations accompanied by a mild increase in α-cell mass and proliferation. Moreover, our in vitro experiments show that PKBα is specifically activated by IRS2 in β-cells and that its activation is required for IRS2-induced proliferation in islets.
Materials and methods

Animals

Mice deficient for the PKB isoforms (Pkbα−/−, Pkbβ−/− and Pkbγ−/− mice) were previously generated and described (13,39,46). Pkbα−/− and Pkbγ−/− mice were on a mixed 129/Ola and C57BL/6 genetic background, Pkbβ−/− on a mixed 129/SvJae and C57BL/6 background. Male and female mice at two different age points (two to three and five to six months old) have been investigated, comparing PKB-deficient mice with wild type littermates. Data presented are from five to six months old male mice, unless otherwise noted, since the younger age group and the females displayed a similar phenotype. Mice were housed according to the Swiss Animal Protection Laws in groups with 12-h dark-light cycles and with free access to food and water. All procedures were conducted with the relevant approval of the appropriate authorities.

Determination of glucose, insulin, glucagon and corticosterone

A blood sample was collected from the tail vein of mice, and D-glucose was measured using a glucose meter (FreeStyle, Disetronic Medical System AG, Burgdorf, Switzerland). After sacrificing the animals, blood was collected by cardiac puncture using an insulin syringe (BD Micro Fine, BD Consumer Healthcare, Le Pont de Claix, France) and supplemented with Aprotinin (Sigma-Aldrich, Saint Louis, Missouri, USA). After centrifugation, the serum was immediately frozen. Insulin and glucagon were measured with the Mouse Endocrine multiplex Kit from Linco (Linco Research, St.Charles, Missouri, USA). Alternatively, an ELISA Kit (Ultra Sensitive Rat Insulin ELISA Kit, Crystal Chem, Downers Grove IL, USA) was used to measure insulin. Corticosterone was measured with the Corticosterone Double Antibody RIA Kit (MP Biomedicals, Eschwege, Germany). For all experiments, blood
samples for metabolic parameters were drawn at the same time of the day, in the morning (9-
11 a.m.).

Oral glucose tolerance tests (GTT), intraperitoneal insulin tolerance tests (ITT) and
intraperitoneal glucagon challenge (GC)

For GTT and ITT, mice were fasted overnight. D-Glucose was orally administered at 2 g/kg
weight (D-(+)-glucose anhydrous, Fluka, Buchs, Switzerland). Insulin was administered by
injection (IP) at 0.75 U/kg weight (human recombinant insulin, Sigma-Aldrich, Saint Louis,
Missouri, USA). For GC, mice were fasted 8 hours and glucagon was administered by
injection (IP) at 30 µg/kg weight (GlucaGen, Novo Nordisk Pharma, Bagsværd, Denmark).
Tail blood was collected at the times indicated and D-glucose levels determined as described
above.

Morphometric analysis of the pancreatic islets

Pancreata were dissected, fixed in zinc formalin, embedded in paraffin and cut into 3 µm
sections. To assess the size and number of islets and of α-cells and β-cells three sections per
pancreas were stained for insulin (mouse monoclonal antibody, Sigma-Aldrich, Saint Louis,
Missouri, USA) and glucagon (rabbit polyclonal antibody, DAKO, Glostrup, Denmark).
Fluorescent secondary antibodies against mouse (FITC) and rabbit (Cy3) were purchased
from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA). Stained
sections were analysed using Axioplan 2 and Axio Vision software (Zeiss, Göttingen,
Germany). The area of the whole sections was assessed with Image J (National Institutes of
Health, Bethesda, Maryland, USA). To visualize proliferating β- and α-cells, Ki-67
immunostaining (goat polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz,
California, USA) was used (19) in combination with DAPI (Sigma-Aldrich, Saint Louis,
Missouri, USA) and insulin or glucagon staining, respectively. Apoptosis was assessed with ApopTag® Peroxidase in situ Detection Kits (Q-Biogene, Montreal, Quebec, Canada).

**Islet isolation and culture**

Islets were isolated from 5-months old wild type and PKB-deficient mice by collagenase (Worthington Biochemical Corporation, Lakewood, USA) digestion of the pancreas, as previously described (36). After a density gradient (Histopaque-1119, Sigma-Aldrich, Saint Louis, Missouri, USA) and handpicking for further purification, the islets were cultured in RPMI 1640 medium containing 11.1 mmol/l D-glucose (Invitrogen, Carlsbad, California), 10% FCS (Hyclone Laboratories Inc., Logan, Utah, USA), 100 units/ml penicillin, 100 μg/ml streptomycin and 40 g/ml gentamycin (Invitrogen, Carlsbad, California, USA). Islets were plated on plates coated with extracellular matrix (ECM) derived from bovine corneal cells (Novamed, Jerusalem, Israel) and allowed to attach and flatten for three days before the start of the experiments.

**Insulin secretion and proliferation assays in isolated islets**

To assess glucose-stimulated insulin secretion, 24 islets of similar size per dish were incubated for 1 hour in the presence of 2.8 mM glucose and subsequently stimulated for 1 hour with 16.7 mM glucose. Overnight acid-alcohol extraction was used to collect total insulin and protein contents. Secreted insulin and total insulin content were measured using the Mouse Insulin ELISA Enzyme immunoassay (Mercodia, Uppsala, Sweden) and normalised by protein content, as measured using the Bicinchoninic Acid Assay (BCA) (Pierce, Rockford, USA). The BrdU Labeling and Detection Kit II (Roche, Basel, Switzerland) combined with insulin (guinea-pig antibody, DAKO, Glostrup, Denmark) and DAPI staining was used to assess proliferation in β-cells. Islets were incubated for two days in the presence of BrdU.
RNA extraction from isolated islets and Real Time PCR

Total RNA was extracted from 80 mouse islets using the Nucleospin RNAII Kit (Macherey-Nagel GmBH, Dueren, Germany) and reverse transcribed using SuperScriptTM II reverse transcriptase and random hexamers as primers (Invitrogen, Carlsbad, California, USA). Real Time PCR primers for actin, Pkbα, Pkbβ, Pkbγ and Glut2 were supplied by Applied Biosystems (Foster City, California, USA) and changes in mRNA expression were calculated using the difference of cycle threshold values, as previously described (36).

Fat cell isolation and glucose uptake

Isolation of white adipocytes from 5-months old wild type and Pkbα−/− mice and glucose incorporation experiments were performed as described previously (35,45). Adipocytes were incubated in Kreb's Ringer buffer containing 1% BSA in the presence of D-[U-14C]glucose with or without 100 nM insulin for 1 hour. The radioactivity in lysates was measured by liquid scintillation counting (Kontron Betamatic V, Kontron Instruments, Montigny-Le-Bretonneux, France). Glucose uptake was normalised by number of adipocytes.

Cell culture

Rat Insulinoma INS1 cells (2) clone 832/13 (21) were cultured in RPMI 1640 medium containing 11 mmol/l D-glucose, supplemented with 10% FCS (fetal calf serum), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax, 1 mM sodium pyruvate, 10 mM HEPES and 50 μM β-mercaptoethanol (Invitrogen, Carlsbad, California, USA).

Adenoviral gene transfer into islets and INS1 cells

Adenoviral vectors encoding myc-tagged IRS1, IRS2 or GFP have been described previously (29). Adenoviral vectors encoding HA-tagged PkBα, PkBβ or GFP were supplied by Vector
BioLabs (Philadelphia, Pennsylvania, USA). After three days of culture on ECM plates islets were exposed to viral particles for two days at a multiplicity of infection (MOI) of 1500-3000. INS1 cells were transfected at about 80% of confluence and an MOI of about 10. Viral particles were removed after six hours by changing medium. After two days, cells were pelleted in PBS by centrifugation and immediately frozen in liquid nitrogen.

**Immunoprecipitation and Western blotting**

INS1 cell pellets were lysed in a buffer containing 50 mM HEPES, 140 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 3 μg/ml aprotinin, 10 mM sodium fluoride, 1 mM disodium pyrophosphate and 1 mM sodium orthovanadate. The BCA assay was used to measure protein concentrations in the lysates. 500 μg of protein were incubated overnight with the respective antibody. Serum obtained from rabbits immunized with PKB isoform-specific peptides (13,39,46) was used for immunoprecipitation. After three hours incubation with Protein A beads (rpm Protein A Sepharose Fast Flow, GE Healthcare, Buckinghamshire, England), bound beads were washed several times with lysis buffer. Proteins bound to beads were finally dissociated by heating for 3 minutes at 70°C in NuPAGE LDS Sample Buffer with Sample Reducing Agent (Invitrogen, Carlsbad, California, USA). Eluates were frozen for later analysis. Equal amounts of lysates and eluates were loaded on NuPAGE Novex 4-12% Bis-Tris Gels (Invitrogen, Carlsbad, California, USA) and transferred onto Hybond-P PVDF membranes (Amersham, GE Healthcare, Buckinghamshire, England). The membranes were incubated overnight at 4°C with primary antibody and 1h at room temperature with secondary antibody. Signals were visualised with Lumi-Light Western Blotting Substrate (Roche, Basel, Switzerland) and quantified using LAS-3000 AIDA software (Fujifilm, Tokio, Japan). Antibodies against total PKB, phospho-PKB (Ser 473 mouse monoclonal / rabbit polyclonal), PKBβ and PKBγ were purchased from Cell Signalling Technology (Beverly, Massachusetts, USA). Additionally we
used antibodies against PKBα (BD Bioscience, Franklin Lakes, New Jersey, USA), actin (Millipore-Chemicon International, Billerica, Massachusetts, USA), IRS1 (Santa Cruz Biotechnology, Santa Cruz, California, USA), IRS2 (Millipore-Upstate, Billerica, Massachusetts, USA) and myc (self-produced). Secondary antibodies against mouse (Santa Cruz Biotechnology, Santa Cruz, California, USA) and rabbit (BioRad, Hercules, California, USA) were used.

Statistical analysis

Data are provided as means ± SEM. Unpaired Student's t-tests (two-tailed) were performed for comparison between data from wild type and PKB deficient mice with Welch’s correction in case of significantly different variances. ANOVA with Bonferroni’s post hoc test was used for multiple comparison analysis. Results with P values under 0.05 were considered statistically significant.
Results

PKBα is required for regulation of glucose homeostasis

To investigate whether PKBα plays a role in regulation of glucose homeostasis, we examined metabolic parameters of Pkba-deficient mice. Pkba−/− animals weighed less compared to control littermates (-14.5 ± 1.5%, Fig. 1A). In contrast to previously published data (9,11), we found significantly lower blood glucose (-21.2 ± 4.5%, Fig. 1B) and higher serum glucagon concentrations (+219 ± 97.3%, Fig. 1D) in Pkba−/− mice (random fed) compared to wild type littermates. Serum insulin (random fed, Fig. 1C) and serum corticosterone (Fig. 1E) concentrations were not significantly increased in Pkba−/− mice. Presented data refer to male mice, and females showed similar metabolic values (data not shown).

Next we assessed metabolic response in Pkba−/− and wild type littermates. During oral glucose tolerance tests (GTT) blood glucose levels remained consistently lower in Pkba−/− with a significant reduction in the area under the curve (-46.5 ± 12.3%, Fig. 2A). Intraperitoneal insulin tolerance tests (ITT) revealed lower levels of blood glucose in Pkba−/− mice after insulin administration with a significant reduction of the area under the curve (-54.2 ± 14.3%, Fig. 2B). During intraperitoneal glucagon challenge (GC) all animals showed a similar increase in blood glucose concentration after glucagon injection but a more rapid decrease was observed in Pkba−/− compared to control mice (Fig. 2C). We also assessed glucose incorporation into adipocytes isolated from peri-gonadal epididymal fat. As shown in Fig. 2D, insulin-induced glucose incorporation was significantly higher (+81.9 ± 31.0%) in adipocytes from Pkba−/− mice than from control littermates. No difference was found in adipocyte cell size (Fig. 2E). Additionally, we measured glucose-stimulated insulin secretion from isolated islets. Compared to islets from control littermates, insulin secretion from Pkba−/− islets was higher after stimulation with glucose (+202.0 ± 146.4%, Fig. 3A), whereas basal
values were similar. *Glut2* expression in islets was not changed and could therefore not explain this improvement (Fig. 3C). No significant increase in total insulin content was found between controls and *Pkbα*−/− islets (Fig. 3B).

Islet morphology was analysed by staining pancreatic sections for insulin, glucagon and the proliferation marker Ki-67. Pancreatic sections from *Pkbα*−/− mice consistently revealed a trend towards increased α-cell area (1.52 ± 0.3 fold, Fig. 4D) as well as proliferation (1.85 ± 0.6 fold, Fig. 4F-H). In contrast, islet area (Fig. 4A) and proliferation of β-cells (Fig. 4C) were unchanged compared to control animals (1.04 ± 0.14 and 0.92 ± 0.18 fold, respectively). Size of α-cells (Fig. 4E) and β-cells (Fig. 4B) was unchanged. In females, similar results were found but no increase in α-cell area (data not shown).

Expression of PKBβ and PKBγ was assessed in liver, skeletal muscle, fat and islets isolated from *Pkbα*-deficient mice and controls. No difference between controls and *Pkbα*−/− mice was observed (Supplementary fig. 1).

**Different role for PKBβ in regulation of glucose homeostasis and insulin sensitivity**

*Pkbβ*−/− mice weighed less compared to control littermates (-18.2 ± 1.6%, Fig. 5A). Analysis of metabolic parameters showed significantly higher blood glucose and serum insulin concentrations for *Pkbβ*−/− mice (random fed) compared to control littermates (+63.8 ± 4.2%, Fig. 5B and +306.0 ± 55.9%, Fig. 5C, respectively). Serum glucagon levels were not significantly changed (Fig. 5D). GTT and ITT have previously been performed with *Pkbβ*−/− mice from this strain and revealed glucose and insulin intolerance (13). Analysis of pancreas morphology in *Pkbβ*−/− mice revealed significantly increased islet area (2.4 ± 0.3 fold, Fig. 6A,G-H) as well as proliferation of β-cells (1.9 ± 0.2 fold, Fig. 6C,I-J), while α-cell area (Fig. 6D) and proliferation (Fig. 6F) were unchanged. No changes were found in α- (Fig. 6B) and β- (Fig. 6E) cell size. Similar phenotype was found for female mice, although increases in
blood glucose, serum insulin and glucagon were milder than in the males and not significant (data not shown). PKBα and PKBγ expression was analysed in islets of Pkbβ−/− and control mice and showed no significant differences (Supplementary fig. 2).

**PKBγ is dispensable for regulation of glucose homeostasis**

Pkby−/− male and female mice showed no differences in body weight (Fig. 7A) and blood glucose concentrations (random fed, Fig. 7B) compared to their wild type littermates, confirming previously published results (14,39). GTT and ITT for Pkbγ−/− mice have been previously performed and revealed no changes (13). Analysis of pancreas morphology of Pkbγ−/− males and females showed normal β- (Fig. 7C) and α- (Fig. 7E) cell area, as well as no changes in β- (Fig. 7D) and α- (Fig. 7F) cell size.

**PKBα acts downstream of IRS2 in the β-cells**

We wanted to analyse which isoforms of PKB are specifically activated by IRS2 in β-cells. In line with published data (28,32), we found that PKB was strongly activated after overexpression of IRS2 (5.1 ± 0.9 fold, Fig. 8A), while induction of PKB phosphorylation by IRS1 was only mild and restricted to certain conditions (i.e. starvation of the cells prior to virus transfection, data not shown). To analyse activation of the individual PKB isoforms we performed immunoprecipitations with isoform-specific antibodies and subsequently detected phosphorylation of PKB in immunoprecipitates by Western blotting. This procedure was necessary, as antibodies against specific phosphorylated PKB isoforms are not available. As shown in Fig. 8B and 8E, PKBα was specifically activated by overexpression of IRS2 (3.7 ± 0.7 fold increase relative to GFP). No phosphorylation was detected for PKBβ (Fig. 8C, E). However, we were able to detect specific activation of PKBβ but not PKBα after stimulation of adipocytes with insulin (data not shown) indicating that the antibody against PKBβ used in
our study is functional. Finally, PKBγ was activated by overexpression of IRS2 (1.7 ± 0.5 fold increase), but far less compared to PKBα and with higher variability between the experiments (Fig. 8D,E).

PKBα is required and sufficient for induction of proliferation in β-cells

To investigate if PKB isoforms are required for IRS2 action in β-cells, we overexpressed IRS2 in islets isolated from Pkb−/− mice and determined the proliferation rate. In control islets derived from Pkbα+/+ mice and overexpressing IRS2 we found a strong increase in cell proliferation (13.2 ± 0.6 fold, Fig. 9A left). In islets isolated from Pkbα−/− mice basal values were similar to control, but the increase in proliferation induced by IRS2 (10.4 ± 1.1 fold) was reduced by 27.1 ± 2.82% (Fig. 9A right). In islets of Pkbβ+/+ mice IRS2 induced an increase in proliferation of 3.3 ± 0.5 fold (Fig. 9B left) very similar to islets isolated from Pkbβ−/− mice (3.6 ± 1.2 fold, Fig. 9B right). To examine whether PKB isoforms are also sufficient to induce proliferation in islets, PKBα or PKBβ, respectively, were overexpressed in islets isolated from wild type C57BL/6 mice. Overexpression of PKBα, but not of PKBβ, resulted in a significant increase in cell proliferation (3.1 ± 0.36 fold, Fig. 9C).
Discussion

In β-cells PKB/Akt is considered to be an important downstream mediator of IRS2 in the regulation of islet mass, however, available data are contradictory. Our study was designed to re-evaluate the relevance of PKB in the control of blood glucose homeostasis and to clarify its role in the regulation of islet mass downstream of IRS2 with a focus on the single isoforms. This included an investigation of metabolic parameters and pancreas morphology in mice deficient for PKBα, β or γ isoforms, respectively, as well as in vitro signalling analyses. We discovered a novel role for PKBα in the regulation of insulin sensitivity. In addition PKBα was found to be the major isoform in β-cell signalling downstream of IRS2. While PKB isoforms are individually dispensable for regulation of maintenance of islet mass, PKBα may mediate IRS2-dependent compensation of functional β-cell mass.

Metabolic phenotypes differed in the three isoform-specific PKB-deficient mouse strains. Interestingly, we discovered a previously undescribed phenotype for Pkbα−/− mice in addition to growth retardation, which was (at least in part) ascribed to defects in placental development (46,48). We found that Pkbα−/− mice displayed significantly lower blood glucose and increased serum glucagon concentrations and responded better to insulin, indicating that they are more glucose tolerant and more insulin sensitive than their wild type littermates (Fig. 2). Improved insulin action was also reflected by increased insulin-stimulated glucose uptake into isolated adipocytes. Since the level of PKBβ in insulin-sensitive tissues as well as in islets did not differ between Pkbα−/− and control mice (Supplementary fig. 1), we can exclude that the observed metabolic phenotype is due to a compensatory increase of the PKBβ isoform. Our results contrast previous work suggesting normal glucose homeostasis in Pkbα−/− mice (9,11), although also in the study by Cho et al. a trend towards improved glucose tolerance and insulin sensitivity in Pkbα−/− mice was apparent. These differences might indicate that the
expressivity and penetrance of this phenotype is sensitive to genetic background and might
differ among the individuals, as was previously described for Pkbβ⁻/⁻ mice where high
variability was found not only between different strains but also among mice of the same
strain (17). The mice investigated in our study as well as by Cho et al. were on a mixed 129
Ola and C57BL/6 background (although belonging to separate strains), while data published
by Chen et al. refer to mice on a 50% 129 R1 and 50% C57BL/6 genetic background.
Whereas serum insulin, islet area and β-cell proliferation were similar in Pkbα⁻/⁻ mice relative
to control littermates, serum glucagon as well as α-cell area and α-cell proliferation were
increased in the majority of investigated animals. To the best of our knowledge, this is the
first study describing glucagon sensitivity and level in serum as well as the islet phenotype of
Pkbα⁻/⁻ mice. Improved insulin sensitivity could lead to the observed decrease in blood
glucose levels and compensatory rise in serum glucagon and corticosterone. In this case,
however, insulin secretion would be expected to be downregulated, while we observed normal
or even increased serum insulin concentration in Pkbα⁻/⁻ mice. This rather suggests a primary
β-cell defect and a deregulation of insulin secretion relative to metabolic needs. However,
lower blood glucose, increased serum glucagon, α-cell hyperplasia together with improved
glucose tolerance were also observed in glucagon receptor knock out mice (18,33). It could
therefore be that the phenotype of Pkbα⁻/⁻ mice is caused by defective glucagon signalling.
Nevertheless, after intraperitoneal administration of glucagon we observed a similar increase
in blood glucose concentration suggesting that Pkbα⁻/⁻ and control mice are similarly
sensitive to glucagon. The following more rapid decrease in blood glucose in Pkbα⁻/⁻ mice is
probably attributable to the improved insulin sensitivity. Since our mouse model is a whole
body knock out, we can not exclude that the observed phenotype both metabolically and in
terms of pancreas morphology is due to secondary effects. However, the fact that glucose
uptake was also improved in isolated fat cells and insulin secretion from isolated islets was
enhanced rather suggests a cell-autonomous effect of PKBα absence. To conclusively address this question, tissue-specific knock out for PKBα in β-cells or in specific insulin-target tissues (i.e. fat tissue, skeletal muscle and liver) would be required. 

*Pkbβ−/−* mice showed a distinct phenotype from *Pkbα−/−* mice. Despite being smaller than their control littermates, blood glucose and serum insulin concentrations were increased, consistent with previous studies showing that deficiency for PKBβ leads to insulin resistance (13). We also investigated in more detail pancreas morphology, as neither α-cell phenotype nor β-cell proliferation had been described yet. We found a compensatory increase in islet mass with concomitant increase in β-cell proliferation, while α-cell area and proliferation were unchanged relative to wild type littermates. In contrast to β-cell proliferation, β-cell size was unchanged indicating that the increase in β-cell mass is due to hyperplasia rather than hypertrophy. Expression of remaining PKB isoforms was not changed in islets of *Pkbβ−/−* mice indicating that the increase in islet mass is not due to a compensatory increase of PKBα expression (Supplementary fig. 2). Our results are in line with the findings by Cho *et al.* (10), though we also found a decrease in body mass, as described by Garofalo *et al.* (17), thus confirming that the β isoform of PKB is required for both glucose metabolism and growth. Although both PKBα and β are required to control growth, they seem to have opposite roles in the regulation of glucose homeostasis. While PKBβ improves insulin sensitivity in the periphery, PKBα appears to decrease it. Finally, we found no difference regarding metabolic parameters and pancreas morphology in *Pkbγ−/−* mice compared to control littermates, confirming published reports showing a dispensable role for PKBγ in glucose metabolism (14,39).

As PKB function is thought to be required for IRS2-dependent regulation of islet mass (12,15,16,27), we assessed activation/phosphorylation of the PKB isoforms downstream of IRS2. As shown by Lingohr *et al.* (28), we confirmed a strong (five-fold) induction of PKB
phosphorylation in β-cells overexpressing IRS2, but not IRS1. Analysis of activation of the single PKB isoforms revealed that PKBα was specifically activated by IRS2, whereas activation of PKBγ was weak and inconsistent. Interestingly, PKBβ was not activated in β-cells after overexpression of IRS2 (Fig. 8). This implies that in β-cells IRS2 signalling occurs mainly via PKBα, whereas PKBβ does not play a major role downstream of IRS2. Such specific activation suggests distinct non-redundant roles for PKB isoforms in β-cell signalling, in line with previous findings showing isoform-specific activation and functions for PKB downstream of IRS in other tissues (3,7,22,47).

As described previously, IRS2, but not IRS1, can induce a strong increase in cell proliferation in isolated islets (29). To address the question whether the observed specific activation of PKBα is required for IRS2 action in β-cells, we analysed the enhancement of cell proliferation in isolated islets overexpressing IRS2 and deficient for PKBα or β. Our results indicate that PKBα is required downstream of IRS2 in islets, as the IRS2-induced increase in cell proliferation was significantly reduced in islets deficient for PKBα (Fig. 9). Induction of proliferation was, however, only 27% abrogated, indicating that other signalling targets in addition to PKBα must be required for full induction of proliferation by IRS2. Proliferation was similar in control islets and Pkbα−/− islets after transfection with AdV-GFP, implying that the proliferation rate under basal conditions does not depend on PKBα. Requirement for PKBα agrees well with our observation that PKBα is specifically activated by overexpression of IRS2 but not of IRS1. In contrast, we observed a similar IRS2-induced increase of proliferation in control and Pkbβ−/− islets suggesting that PKBβ is not required downstream of IRS2, which is in line with the finding that overexpression of IRS2 does not activate PKBβ. Although IRS2-induced proliferation does not reach the same extent as in Pkbα−/− islets, implying a potential role of PKBβ in this action, basal proliferation is also reduced compared to the controls and could thus explain this difference. The very low proliferation rates found...
in isolated $Pkb\beta^{-/-}$ islets cultured on ECM plates suggests that $Pkb\beta^{-/-}$ islets might suffer from a growth disadvantage on this matrix. In fact, even higher $\beta$-cell proliferation was found in pancreatic sections of $Pkb\beta^{-/-}$ mice. PKB was shown to be required for growth of pancreatic islets on ECM (6,20,25,41) and this observation suggests that PKB$\beta$ could be the responsible isoform. This hypothesis is supported by the finding that proliferation in $Pkb\beta^{-/-}$ islets can be significantly increased after adenoviral re-expression of the PKB$\beta$ isoform (data not shown).

We observed differences in basal proliferation rates between $Pkb\alpha^{+/+}$ and $Pkb\beta^{+/+}$ islets. Because the levels of basal proliferation also varied within strains between experiments these differences were probably due to experimental variations such as islet isolation. Nevertheless, the relative differences and induction rates were consistently maintained within the strains.

Finally, the prominent role played by PKB$\alpha$ in the regulation of $\beta$-cell proliferation is supported by our finding that overexpression of this isoform, but not of PKB$\beta$, in isolated wild type islets is sufficient to significantly increase cell proliferation (Fig. 9C).

We recently proposed that maintenance and compensatory expansion of islet mass may be regulated by separate pathways downstream of IRS2 (31). It was predicted that PKB is dispensable for maintenance of $\beta$-cell mass, whereas PKB$\alpha$ might mediate expansion of islet mass. The results of the present study are fully compatible with this model and support the idea that none of the three PKB isoforms are required to maintain islet mass, as in none of the isoform-specific PKB-deficient strains islet mass was decreased, but in the case of $Pkb\beta^{-/-}$ mice even increased. As others have shown previously, islet mass was not affected even after 80% of PKB activity was removed in $\beta$-cells (4), indicating that redundancy between isoforms is an unlikely explanation for the mild phenotype found in isoform-specific PKB-deficient mice. Consistently, absence of one isoform does not lead to compensatory upregulation of the remaining two isoforms in any of the tissues analysed by us (Supplementary fig. 1 and 2) as well as in previous studies (11,17,39,46). In combination with
previous data, showing that constitutive activation of the α isoform is sufficient to increase β-cell growth and proliferation (5,40), our results support the notion that PKBα contributes to compensatory increase of functional islet mass. The recent discovery that heterozygosity for PKBα converts the mild metabolic phenotype of $Pkb\beta^{-/-}$ mice into overt type 2 diabetes with prominent β-cell dysfunction supports our findings implying an important role played by PKBα in glucose homeostasis and in particular in β-cell function (8).
Acknowledgements

We thank Heidi Seiler and Dora Schmid for excellent technical assistance, and Stephan Wuest and Reto Rapold for their help in isolation and handling of primary adipocytes. We are thankful to Prof. Michal Neeman and Katrien Vandoorne for kind support during the revision of the manuscript, and to Helga Ellingsgaard and Jan Ehses for discussions and various other contributions. This study was supported by the Takeda Foundation and the Julius Klaus Stiftung. This project was carried out in the framework of COST action BM0602. The FMI is part of the Novartis Research Foundation. O.T. was supported by the Gebert Rüf Stiftung (GRS - 027/06). Work performed in Dr. Yang's laboratory was supported by National Basic Research Program of China (2006CB503900 and 2006CB943503).

References


PKB isoforms in glucose homeostasis


**Figure legends**

**Figure 1. PKBα is required for metabolic regulation.** Body weight (A), blood glucose (B), serum insulin (C), serum glucagon (D) and serum corticosterone (E) concentrations were examined in random fed Pkbα−/− mice (filled bars) and control littermates (open bars). Data are presented as mean ± SEM with n=9-15. Results are from 5-6 months old male mice. Similar results were obtained in younger mice (2-3 months old) and in females (data not shown). *, P<0.05; ***, P<0.001.

**Figure 2. Pkbα−/− mice are more insulin sensitive.** (A-B) Curves represent blood glucose concentration during 180 min oral glucose tolerance test (GTT) (A) and intraperitoneal insulin tolerance test (ITT) (B) in Pkbα−/− mice (closed squares) and control animals (open squares). Results are mean ± SEM of n=3-7 and are from 5-6 months old male mice. Same trends were found in younger animals (2-3 months old) and in females. Bar graphs to the right show the respective area under the curve (AUC) of GTT and ITT, respectively. (C) Blood glucose concentration during 60 min intraperitoneal glucagon challenge (GC) in Pkbα−/− (closed squares) and control male mice (open squares). Data are mean ± SEM of n=4-5. Female mice showed similar results (data not shown). (D) Glucose uptake measured after insulin stimulation (100 nM) of perigonadal fat cells isolated from Pkbα−/− female mice (filled bars) and control littermates (open bars). Adipocyte size is shown in (E). Data are presented as mean ± SEM of n=5-6. *, P<0.05; **, P<0.01.

**Figure 3. Insulin secretion in islets isolated from Pkbα−/− mice.** Glucose-stimulated insulin secretion (A) and total insulin content (B) in islets isolated from Pkbα−/− female mice (filled bars) and control littermates (open bars). Data are presented as mean ± SEM of n=3-8. Glut2
mRNA expression (C) in islets isolated from Pkbα⁻/⁻ female mice (filled bars) and control littermates (open bars). Data are means ± SEM of n=5.

**Figure 4. Pancreatic phenotype of Pkbα⁻/⁻ mice.** Pancreatic sections of Pkbα⁻/⁻ mice were immuno-stained for insulin and glucagon and islet morphology was analysed. β-Cell area (A) and size (B) as well as α-cell area (D) and size (E) in sections of Pkbα⁻/⁻ mice (filled bars) are shown compared to the respective controls (open bars). Expression of Ki-67 was assessed to visualize proliferating cells. Ki-67-positive β-cells (C) and α-cells (F) (insulin and glucagon positive, respectively) are normalized by β- and α-cell area, respectively. (G-H) Representative pancreatic sections of Pkbα⁺/⁺ and Pkbα⁻/⁻ mice co-stained for glucagon (dark red cells) and Ki-67 (brown nuclei, arrow). Data are means ± SEM of n=6 and results are from 5-6 months old male mice. Females showed similar results but no increase in α-cell area (data not shown).

**Figure 5. Metabolic parameters in Pkbβ⁻/⁻ mice.** Analysis of body weight (A), blood glucose (B), serum insulin (C) and serum glucagon (D) concentrations in random fed Pkbβ⁻/⁻ mice (hatched bars) and control littermates (open bars). Data are means ± SEM of n=11-22. Results are from 5-6 months old male mice. Similar results were obtained with younger mice (2-3 months old), whereas females showed milder and not significant increase in blood glucose and serum insulin (data not shown). ***, P<0.001.

**Figure 6. Pancreatic phenotype of Pkbβ⁻/⁻ mice.** β-Cell area (A) and size (B), α-cell area (D) and size (E), Ki-67 positive β-cells (C) and α-cells (F) in pancreatic sections of Pkbβ⁻/⁻ mice (hatched bars) compared to controls (open bars). (G-H) Representative sections of Pkbβ⁺/⁺ and Pkbβ⁻/⁻ mice stained for insulin (green), glucagon (red) and DNA (DAPI, blue).
(I–J) Representative sections co-stained for insulin (red) and Ki-67 (brown nuclei, arrows).
Data are presented as mean ± SEM of n=6-7. Results are from 5-6 months old male mice. Same results were obtained with younger mice (2-3 months old) and in females (data not shown). *, P<0.05; **, P<0.01.

Figure 7. PKBγ is dispensable for regulation of glucose homeostasis. Body weight (A) and blood glucose concentration (B) in random fed Pkbγ−/− mice (checked bars) compared to littermate controls (open bars). β-Cell area (C) and size (D), and α-cell area (E) and size (F) in pancreatic sections of Pkbγ−/− mice (checked bars) compared to controls (open bars). Data are presented as mean ± SEM of n=24 for metabolic parameters and n=6 for morphologic analysis. Results are from 2-3 months old male mice. Same results were obtained with females (data not shown).

Figure 8. PKBα acts downstream of IRS2 in β-cells. (A) Western blot analysis showing phosphorylation of PKB after adenoviral overexpression of myc-tagged IRS1 and IRS2, respectively, in INS1 cells. Endogenous expression of PKB was assessed for normalization. As control, cells were transfected with AdV-GFP. Signals of 7 independent experiments were quantified and the mean increase in phosphorylation in % of control is presented as a bar graph on the right. As the antibody against phosphorylated PKB detects all three isoforms, signals in the lysates are not isoform-specific. (B–E) To analyse activation of PKB isoforms, lysates of INS1 cells overexpressing IRS2 were immunoprecipitated with isoform-specific antibodies. Representative Western blots for PKBα (B), PKBβ (C) and PKBγ (D), respectively, are shown. Signals for phosphorylated PKB were normalized to signals of the respective non phosphorylated PKBα, PKBβ or PKBγ isoform. For detection of PKBβ and PKBγ all available antibodies were raised in rabbit, thus it was not possible to avoid signal of
the antibody on the blot. Bar graph (E) shows mean ± SEM of 6-7 experiments for PKBα (left) and PKBγ (right) after overexpression of GFP (light grey) or IRS2 (dark grey). Data are represented as % of control. **, P<0.01; ***, P<0.001.

Figure 9. PKBα is required for induction of proliferation by IRS2 in β-cells. Cell proliferation was assessed in isolated islets after adenoviral transfection with IRS2-, PKBα-, PKBβ- or GFP-encoding vectors. As the islets were counterstained for insulin, the positive cells represent β-cells (although occasionally other islet cells may have been counted). (A-B) Graphs show the percentage of BrdU-positive cells of total number of cells in islets isolated from Pkbα−/− (A) and Pkbβ−/− (B) female mice and respective controls after overexpression of GFP (light grey) and IRS2 (dark grey). Values are mean ± SEM of n=3-5. (C) Graph shows the percentage of BrdU-positive cells in islets isolated from wild type C57BL/6 male mice after overexpression of GFP (light grey), PKBα (dark dotted) and PKBβ (light dotted). Values are mean ± SEM of n=6. Equal adenoviral expression was tested in parallel experiments in INS1 cells (data not shown). *, P<0.05; ***, P<0.001.
Figure 1

A. Body weight [g]

B. Blood glucose [mmol/l]

C. Serum insulin [ng/ml]

D. Serum glucagon [pg/ml]

E. Serum corticosterone [ng/ml]

***

*
Figure 2

A. Blood glucose concentration over time.[mmol/L]

B. Blood glucose concentration over time.[mmol/L]

C. Blood glucose concentration over time.[mmol/L]

D. D-Glucose incorporation [pmol/2x10^4 cells * hour].

E. Adipocyte size [µm^2].

Legend:

- **Pkbα^+/+**
- **Pkbα^-/-**
Figure 3

A

In insulin secretion (pmol/g * hour) vs. glucose (mM) for Pkbα+/+ and Pkbα-/- mice.

B

Total insulin content (pmol/µg) for Pkbα+/+ and Pkbα-/- mice.

C

Glut2 mRNA expression in islets (% of control) for Pkbα+/+ and Pkbα-/- mice.
Figure 5

A: Body weight [g]

B: Blood glucose [mmol/l]

C: Serum insulin [ng/ml]

D: Serum glucagon [pg/ml]

- **Pkbβ +/-**
- **Pkbβ +/-**
Figure 7

A. Body weight [g]

B. Blood glucose [mmol/L]

C. β-Cell area [% of pancreas area]

D. β-Cell size [μm²]

E. α-Cell area [% of pancreas area]

F. α-Cell size [μm²]

- *Pkbγ*+/+
- *Pkbγ*−/−
Figure 8

A. GFP IRS1 IRS2
myc
phospho-Ser473 PKB
PKB

B. lysates IP eluates
GFP IRS2 GFP IRS2
phospho-Ser473 PKB
PKBα

C. lysates IP eluates
GFP IRS2 GFP IRS2
phospho-Ser473 PKB
PKBβ

D. lysates IP eluates
GFP IRS2 GFP IRS2
phospho-Ser473 PKB
PKBγ

E. Phospho-Ser473 increase in % of control
PKBα
PKBγ
Figure 9

A

Proliferating cells [% of total cells]

0 2 4 6 8

GFP IRS2

Pkbα⁺/⁺ Pkbα⁻/⁻

* P

B

Proliferating cells [% of total cells]

0 2 4 6 8 10 12

GFP IRS2

Pkbβ⁺/⁺ Pkbβ⁻/⁻

C

Proliferating cells [% of total cells]

0.0 0.5 1.0 1.5 2.0 2.5

GFP PKBα PKBβ

*** ***
**Supplementary Methods**

**Organ lysates and Western blotting**

Liver, femoral muscle and perigonadal fat pads were dissected and homogenised in a buffer containing 50 mM Tris HCl, 40 mM beta-glycerolphosphate, 120 mM NaCl, 1% NP40, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 3 μg/ml aprotenin, 10 mM sodium fluoride, 1 mM disodium pyrophosphate and 1 mM sodium orthovanadate. Protein concentrations in the lysates were determined by the BCA assay and Western blotting was performed as described in the Methods section.

**Supplementary Figure Legend**

**Supplementary figure 1. Expression of PKB isoforms in Pkbα−/− tissues.**

(A) Western blot analysis showing expression of PKBβ in skeletal muscle, fat and liver from Pkbα−/− and wild type mice. Actin was detected for normalization. To control for the isoform specificity of the antibody, protein expression was analysed in lysates of brain extracted from Pkbβ−/− (C1) and Pkbβ+/+ (C2) mice. Six mice per group were analysed. Signals were quantified and the mean expression in % of control is presented as a bar graph below. PKBγ could not be detected by immunoblotting (data not shown) except in the brain controls, confirming the low expression of this isoform in metabolic tissues. (B) Real Time PCR analysis showing mRNA expression of Pkbβ and Pkbγ in islets isolated from Pkbα−/− (filled bars) and control mice (open bars). The results were normalised to actin mRNA levels. Data are presented in % of control (mean ± SEM of n=5-9).
Supplementary figure 2. Expression of PKB isoforms in $Pkb\beta^{-/-}$ islets.

Real Time PCR analysis showing mRNA expression of $Pkb\alpha$ and $Pkb\gamma$ in islets isolated from $Pkb\beta^{-/-}$ (hatched bars) and control mice (open bars). The results were normalised to actin mRNA levels. Data are presented in % of control (mean ± SEM of n=6-9).
Supplementary figure 1

A

![Supplementary figure A](image)

- *Pkbα*+/+
- *Pkbα*−/−

PKBβ

- muscle
- fat
- liver

PKBβ protein expression [% of control]

<table>
<thead>
<tr>
<th>Tissue</th>
<th><em>Pkbα</em>+/+</th>
<th><em>Pkbα</em>−/−</th>
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<tr>
<td>muscle</td>
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<td>liver</td>
<td>[Bar Graph]</td>
<td>[Bar Graph]</td>
</tr>
</tbody>
</table>

B

![Supplementary figure B](image)

Pkb mRNA expression in islets [% of control]

- *Pkbβ*
- *Pkbγ*

- *Pkbα*+/+
- *Pkbα*−/−
Supplementary figure 2

![Bar graph showing Pkb mRNA expression in islets as a percentage of control. The graph compares Pkbα and Pkbγ between Pkbβ^+/+ and Pkbβ^−/− genotypes.](image-url)