Phosphatase and Tensin Homolog Regulation of Islet Growth and Glucose Homeostasis*

Received for publication, April 15, 2005, and in revised form, August 26, 2005

Published, JBC Papers in Press, September 6, 2005, DOI 10.1074/jbc.M504155200

Jake A. Kushner1, Laura Simpson3, Lynn M. Wartschow8, Shaodong Guo5, Matthew M. Rankin6, Ramon Parsons6, and Morris F. White6,2

From the 1Division of Endocrinology, Children’s Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania 19104, 2Institute for Cancer Genetics, College of Physicians and Surgeons, Columbia University, New York, New York 10032, and 3Howard Hughes Medical Institute, Division of Endocrinology, Children’s Hospital Boston, Harvard Medical School, Boston, Massachusetts 02115

The insulin and insulin-like growth factor (IGF)3 signaling cascades activate the phosphatidylinositol 3-kinase (PI3K). The 3’-lipid phosphate Pten ordinarily attenuates this cascade; however, its influence on insulin cell function or growth is unknown. To determine whether decreased Pten expression could restore insulin cell function and prevent diabetes in Irs2+/− mice, we generated wild type or Irs2 knock-out mice that were haploinsufficient for Pten (Irs2+/−::Pten+/−). Irs2+/− mice develop diabetes by 3 months of age as insulin cell mass declined progressively until insulin production was lost. Pten insufficiency increased peripheral insulin sensitivity in wild type and Irs2+/− mice and increased Akt and Foxo1 phosphorylation in the islets. Glucose tolerance improved in the Irs2+/−::Pten+/− mice, although insulin cell mass and circulating insulin levels decreased. Compared with Irs2+/− mice, the Irs2+/−::Pten+/− mice displayed normal glucose tolerance and survived without diabetes, because normal but small islets produced sufficient insulin until the mice died of lymphoproliferative disease at 12 months age. Thus, steps to enhance phosphatidylinositol 3-kinase signaling can promote insulin cell growth, function, and survival without the Irs2 branch of the insulin-insulin-like growth factor signaling cascade.

The insulin and insulin-like growth factor (IGF)3 signaling cascades promote nutrient homeostasis, tissue growth, and cell survival throughout life. The failure of these signaling pathways causes insulin resistance and metabolic disease, such as glucose intolerance, obesity, and dyslipidemia, that progress to diabetes when the β-cells fail to secrete sufficient insulin quickly enough to compensate for insulin resistance (1). The insulin receptor substrates Irs1 and Irs2 are especially important because they integrate signals from the insulin and IGF with heterologous cytokines and metabolites to coordinate systemic nutrient homeostasis (2). Irs2 is especially important for the regulation of glucose homeostasis because it promotes β-cell function, liver metabolism, and hypothalamic nutrient sensing (3). Thus, global dysregulation of Irs2 signaling causes peripheral insulin resistance that is exacerbated by concomitant pancreatic β-cell failure (4, 5).

Irs2 mediates many effects through activation of the PI 3-kinase cascade (6). The products of the PI 3-kinase, PI(3,4)P2 and PI(3,4,5)P3, create platforms to recruit Pdk1 and Akt to the inner face of the plasma membrane, where they interact to generate downstream signals (7). Akt plays a central role during the insulin response because it phosphorylates cellular substrates that control glucose metabolism, cell survival, and gene transcription (7). Pten is a potent negative regulator of insulin action and cellular proliferation and is one of the most frequently mutated genes in many forms of human cancer (8, 9). Pten attenuates downstream signaling by dephosphorylating the 3-phosphate on PI3P2 and PI(3,4,5)P3, which reduces the recruitment of Pdk1 and Akt to the membrane (9). Most interestingly, PTEN re-expression in a Pten null human breast cell line causes cell cycle arrest and apoptosis, along with increased expression of IRS2 (10). These findings suggested that Pten and Irs2 might coordinately regulate the PI 3-kinase → Akt cascade to influence both glucose homeostasis and cell growth.

Consistent with the role of Pten to attenuate PI 3-kinase signaling, short term antisense Pten knock-down causes a reversal of hyperglycemia in both db/db and ob/ob mice and at least partial amelioration of peripheral insulin resistance in db/db mice (11). Moreover, liver-specific Pten knock-out mice display enhanced insulin sensitivity and hepatic steatosis and glycogenosis (12). Pten may also have a role in fat. Pten expression negatively regulates insulin signaling in 3T3-L1 adipocytes (13), and adipose-specific Pten-deficient mice have enhanced peripheral insulin sensitivity and increased energy expenditure and as a result are lean (14). Similarly, muscle-specific Pten deletion results in increased glucose uptake and protection from dietary fat-induced diabetes (15). Although previous studies show that Irs2 has a critical tissue autonomous role to promote β-cell function in mice, it is not clear whether Pten significantly attenuates the Irs2-mediated PI 3-kinase signaling (16–18). In this report, we investigate whether Pten haploinsufficiency can restore sufficient β-cell function in Irs2+/− mice to prevent diabetes.

EXPERIMENTAL PROCEDURES

Mice—Irs2 and PTEN-deficient mice have been described previously (4, 19). To create mixed C57BL/6 × 129Sv genetic background Irs2+/−::Pten+/− intercross mice for glucose homeostasis studies, Irs2+/−::Pten+/− mice on a pure C57BL/6 genetic background were backcrossed for several generations (>3) with Irs2+/− mice on a mixed background.

* The abbreviations used are: IGF, insulin-like growth factor; PTEN, phosphatase and tensin homolog; IRS, insulin receptor substrate; BrdUrd, bromodeoxyuridine; PI, phosphatidylinositol.

1 Supported by National Institutes of Health Institutional Training Grant DK02024, National Institutes of Health Training Grant DK064101, a Charles H. Hood Foundation Child Health Research grant, a Lawson Wilkins Pediatric Endocrine Society clinical scholar award, and a March of Dimes Basil O’Connor career development award.

2 To whom correspondence should be addressed: Howard Hughes Medical Institute, Division of Endocrinology, Children’s Hospital Boston, Dept. of Medicine, Harvard Medical School, Karp Research Bldg., Rm. 04210, 300 Longwood Ave., Boston, MA 02115. Tel: 617-919-2846; Fax: 617-730-0244; E-mail: morris.white@childrens.harvard.edu.

3 The abbreviations used are: IGF, insulin-like growth factor; PTEN, phosphatase and tensin homolog; IRS, insulin receptor substrate; BrdUrd, bromodeoxyuridine; PI, phosphatidylinositol.
C57BL/6 × 129Sv genetic background. The subsequent Irs2<sup>−/−</sup>::Pten<sup>−/−</sup> mixed C57BL/6 × 129Sv genetic background mice were bred with Irs2<sup>−/−</sup> mixed C57BL/6 × 129Sv mice to produce the littermate animals of this study: wild type, Irs2<sup>−/−</sup>, Pten<sup>−/−</sup>, and Irs2<sup>−/−</sup>::Pten<sup>−/−</sup> mice. Mice were maintained in a barrier animal facility in the Harvard School of Public Health and fed Mouse Diet 5020 9F (9% fat calculated by weight, 21.6% fat calculated by kilocalories), (PMI Nutrition International, Richmond, IN). Genotyping of animals was done by PCR, as described previously (4, 19). Because male Irs2<sup>−/−</sup> mice have a more consistent diabetic phenotype, only male mice were used to characterize glucose homeostasis phenotypes and pancreatic pathology (20). Random-fed glucose and insulin measurements were performed as described previously (21). Intraportal glucose tolerance tests were performed on mice fasted for 15–16 h with 2 g of D-glucose per kg of body weight as described previously (21). Intraportal insulin tolerance tests were performed on fed mice with 1.5 units/kg, similar to methods described previously (21). Area under curve analysis was performed with trapezoid rule algorithms for individual glucose tolerance tests and reported as minmg/dl. Area above curve analysis was performed in a similar manner.

Immunohistochemistry—Immunohistochemical localization of antigens and double-label immunohistochemistry were performed similarly to methods described previously (21). Pancreas samples were dissected from fed mice and fixed with 4% paraformaldehyde/phosphate-buffered saline solution overnight. Five-micrometer longitudinal sections of paraffin blocks were rehydrated with xylene followed by decreasing concentrations of ethanol, microwaved in 0.01 M sodium citrate (pH 6.0) for 20 min, and permeabilized with 1% Triton X-100 in phosphate-buffered saline prior to primary antisera incubation. Guinea pig anti-insulin, rabbit anti-glucagon antibodies (Zymed Laboratories Inc.), and rat anti-BrdUrd (BU1/75, Accurate Chemical, Westbury, NY) were used as primary antibodies. Secondary antibodies were labeled with Cy2 or Cy3 (Jackson ImmunoResearch, West Grove, PA). Nuclear staining was performed with 4,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR).

Islet Morphometry—Adult β-cell area was measured by acquiring at least 20 adjacent nonoverlapping images with a ×10 objective of insulin-stained sections from 3- or 6–8-month-old male mice, at least five animals per genotype, with a Zeiss Axioskop microscope or a Zeiss Axioskop 2 plus mot microscope (Carl Zeiss MicroImaging, Thornwood, NY) and captured with a Hamamatsu Orca or Orca ER digital camera (Hamamatsu Photonics, K.K., Hamamatsu City, Japan). Images were analyzed for area with Improvision Open Lab software (Improvision Scientific Imaging, Lexington, MA) similar to studies published previously (21). Results of β-cell quantification are expressed as the percentage of the total surveyed area containing cells positive for insulin. Islet density and size were calculated from captured insulin-stained images. Results were compared with independent t tests (unpaired two-tailed) and are reported as p values.

Immunoprecipitation and Western Blotting—Mice were anesthetized, and the pancreas was removed, and islets were isolated as described previously (16). Islets were homogenized, and clarified supernatants containing 15 μg of total protein were immunoblotted with antibodies (Cell Signaling) against Akt, phospho-Akt, FoxO1, or phospho-FoxO1. The total amount of Akt and FoxO1 and the phosphorylated forms were detected by enhanced chemiluminescence (Calbiochem) and quantified using ImageQuant TL (16).

β-Cell Proliferation Studies—Three-month-old mice were injected with bromodeoxyuridine (BrdUrd, Roche Applied Science; 100 μg/g body weight) 6 h prior to sacrifice (21). 4,6-Diamidino-2-phenylindole/insulin/BrdUrd-stained pancreatic sections were examined to determine the total number of islets per section as well as the number of BrdUrd-positive β-cells per islet. BrdUrd-positive β-cells per islet were calculated by examining 5–6 sections per animal, 5–12 animals per genotype.

Statistics—All results are reported as means ± S.E. of at least eight mice per group, except Irs2<sup>−/−</sup> mice where only six mice survived to 3 months. **, p < 0.01, Irs2<sup>−/−</sup>::Pten<sup>−/−</sup> versus Irs2<sup>−/−</sup>.

RESULTS

Creation of Irs2 Knock-out Pten Haploinsufficient Mice—To examine the role of PTEN in glucose homeostasis, we intercrossed Irs2<sup>−/−</sup> mice with Pten<sup>−/−</sup> mice for several generations. Male Irs2<sup>−/−</sup>::Pten<sup>−/−</sup> mice crossed with female Irs2<sup>−/−</sup> mice produced most of the genotypes at an expected frequency; however, this cross consistently failed to produce pups without Pten, Pten<sup>−/−</sup>, Irs2<sup>−/−</sup>::Pten<sup>−/−</sup>, or Irs2<sup>−/−</sup>::Pten<sup>−/−</sup> mice.
Pten and β-Cell Function

These results indicated that a loss of Irs2 function did not rescue the embryonic lethal phenotype of Pten−/− mice that was reported previously (19, 22).

Irs2−/− mice ordinarily die with severe hyperglycemia between 10 and 15 weeks of age (4). By contrast, Irs2−/−:Pten−/− mice survived up to 1 year of age. At 3 months, Irs2−/−:Pten−/− mice weighed significantly more than Irs2−/− mice (p < 0.01), suggesting that decreased Pten prevented the pre-morbid weight loss characteristic of diabetic Irs2−/− mice (Fig. 1). Even by 6–8 months of age (when all the Irs2−/− mice were dead), the Irs2−/−:Pten−/− mice displayed a nearly normal body weight (Fig. 1). However, Irs2−/−:Pten+/− mice, like their Pten+/− littermates, succumbed to lymphoproliferative disease and renal failure around 12 months of age, which was attributed previously to the reduced levels of Pten (19, 22).

Glucose Homeostasis in Irs2−/−:Pten+/− Mice—Before Pten+/− or Irs2−/−:Pten+/− mice manifested evidence of lymphoproliferative disease and renal failure, we investigated the effect of reduced Pten expression on glucose homeostasis. At 3 months, Irs2−/−:Pten−/− mice were hyperglycemic during fasting and random-fed conditions (Fig. 2, a and b). As expected, most of these mice died at 3 months of age, and none survived beyond 4 months. Consistent with previous reports, young and old Pten−/− mice displayed normal fasting and random-fed blood glucose values (Fig. 2, a and b). Remarkably, fasting and random-fed glucose levels were largely normalized in the vast majority of Irs2−/−:Pten+/− mice at 3 and 6–8 months of age (Fig. 2, a and b); only 3 of more than 30 Irs2−/−:Pten+/− mice generated for this study developed diabetes at 3 months. Intraperitoneal glucose tolerance tests confirmed the significant improvement in the Irs2−/−:Pten+/− mice at 3 and 6–8 months of age (Fig. 2, c and d); however, the return to normal fasting glucose levels after 2 h was slightly retarded (Fig. 2, c and d). Thus, Pten haploinsufficiency cannot completely compensate for the absence of Irs2 in young and old mice, because the Pten+/− mice displayed normal glucose tolerance at both ages.

Insulin Sensitivity in Irs2−/−:Pten+/− Mice—Insulin tolerance tests confirmed that Pten haploinsufficiency normalized the insulin sensitivity of Irs2−/− mice (Fig. 3a). Integration of the insulin response curves revealed a 2-fold increased hypoglycemic response in the Irs2−/−:Pten+/− mice compared with Irs2−/− mice (p < 0.05) (Fig. 3b). By comparison, wild type mice and Pten+/− mice displayed identical hypoglycemic responses to exogenous insulin (Fig. 3, a and b). Thus, Pten haploinsufficiency per se was insufficient to cause gross alterations in glucose homeostasis, although it restored the systemic insulin response in the Irs2−/− mice.

Less insulin is required to maintain glucose homeostasis in Pten+/− mice. Consistent with this expectation, fasting and random-fed serum insulin levels were lower in the normal glycemic Pten+/− mice at 3 and 6–8 months of age (Fig. 3, c and d). Compared with the hypoinsulinemic Irs2−/− mice, circulating insulin was elevated in the young Irs2−/−:Pten+/− mice (Fig. 3, c and d). By 6–8 months of age, circulating insulin was about equal in Pten+/− and Irs2−/−:Pten+/− mice (Fig. 3, c and d).
heterozygous Pten disruption prevented diabetes in Irs2−/− mice by reducing the insulin requirement while promoting insulin production needed to maintain normal glucose tolerance.

**β-Cell Signaling and Replication of Irs2−/−::Pten+/−**—The loss of functional β-cells is an important component in the progression of Irs2−/− mice to diabetes (23). Increased insulin sensitivity in the Irs2−/−::Pten+/− mice could not restore glucose homeostasis without also restoring β-cell function (21, 23). Previous results show that β-cell mass can be restored in Irs2−/− islets by reducing the activity of nuclear Foxo1, which is associated with enhanced Pdx1 expression (24). These results suggest that activating the Akt → Foxo1 cascade might restore β-cell function in the Irs2−/− islets. To determine whether Pten insufficiency promotes Akt and Foxo1 phosphorylation, we isolated islets from wild type, Irs2−/−, Pten+/−, or Irs2−/−::Pten+/− mice and analyzed the tissue extracts by immunoblotting (Fig. 4a). Akt phosphorylation (Ser473) increased about 3-fold in both Pten+/− and Irs2−/−::Pten+/− islets (Fig. 4b). Similarly, Foxo1 phosphorylation (Ser253) normalized against its protein level increased in these mice compared with the wild type and Irs2−/− mice (Fig. 4c). Moreover, compared with the wild type, Foxo1 protein level normalized against Akt protein level decreased in both Pten+/− and Irs2−/−::Pten+/− islets (Fig. 4d). This decrease is consistent with previous reports that Akt mediated phosphorylation promotes degradation of Foxo1 (25, 26). By this analysis, Pten insufficiency promotes the Akt → Foxo1 signaling and reduces Foxo1 activity in wild type and Irs2−/− mice.

Because haploinsufficiency for Foxo1 promotes β-cell growth and function in Irs2−/− mice, we analyzed β-cell proliferation by measuring BrdUrd incorporation in the pancreas of 3-month-old mice (24). Compared with the wild type mice, BrdUrd incorporation was decreased in Pten+/− mice (Fig. 4e). This result was consistent with reduced insulin requirements because of increased peripheral insulin sensitivity of the Pten+/− mice. By contrast, BrdUrd incorporation was barely detected in the severely insulin-resistant Irs2−/− mice, which was consistent with the progressive loss of β-cell function during the progression to diabetes (Fig. 4e). However, analysis of the Irs2−/−::Pten+/− mice revealed increased BrdUrd incorporation that was significantly greater than Irs2−/− islets and equal to that in Pten+/− islets; however, the BrdUrd incorporation was substantially less than that measured in wild type mice (Fig. 4e). Thus, Pten haploinsufficiency (and increased activity of the Akt → Foxo1 cascade) facilitated Irs2−/− islet growth without disrupting the regulation of β-cell expansion because of systemic insulin requirements.

**Islet Histology and β-Cell Growth**—To confirm that Pten haploinsufficiency rescued islet function and growth, we compared pancreas histology in relevant Irs2−/−::Pten+/− intercross mice. Pten haploinsufficiency alone resulted in small but otherwise normal appearing islets (Fig. 5). Consistent with reduced insulin requirements, morphometric analysis confirmed that the total β-cell mass was decreased in Irs2−/− mice by ~50% at both 3 and 6–8 months of age (Fig. 5a). This decrease arose mainly from smaller islets of normal density (Fig. 6, b and c). Thus, increased Akt → Foxo1 signaling in the Pten−/− islets did not have a dominant effect on β-cell expansion in Pten−/− mice.

As reported previously, pre-morbid diabetic Irs2−/− mice displayed disorganized islet morphology with a few weakly insulin-staining β-cells and increased numbers of glucagon-containing α-cells (Fig. 5). By contrast, islets in Irs2−/−::Pten−/− mice were small but had a normal β-cell and α-cell organization and strong insulin immunostaining characteristic of wild type mice (Fig. 5). Except for the Irs2−/− mice that died, the
β-cell area and islet size increased in all experimental groups between 3 and 6–8 months of age; the increase was greatest in the wild type mice, reduced by 50% in the Pten+/− mice, and the least in Irs2−/−::Pten−/− mice (Fig. 6, a and b). Although Pten haploinsufficiency alone did not reduce islet density, it failed to restore islet density to normal in the Irs2−/−::Pten−/− mice at 6–8 months (Fig. 6c). Regardless, sufficient β-cell function was obtained to prevent severe diabetes in most of the Irs2−/−::Pten−/− mice. We attribute the infrequent (<10% incidence) diabetes in Irs2−/−::Pten−/− mice to this compensatory β-cell expansion because of the increased Akt → Foxo1 signaling.

DISCUSSION

We show that Pten haploinsufficiency in Irs2−/− mice (Irs2−/−::Pten+/−) restores sufficient β-cell growth and function to normalize glucose homeostasis and prevent diabetes. Despite many studies illustrating the importance of Irs2 → PI 3-kinase → Akt signaling for adult islet growth, little is known about the negative regulation of this pathway. Our results reveal that the Pten lipid phosphatase, which acts as a negative regulator of PI-3-kinase signals, inhibits the compensatory growth and function of β-cells in Irs2−/− mice. Although Pten haploinsufficiency is well known to reduce the life span of mice by 50% because of lymphoproliferative disease, it prolongs the life span of Irs2−/− mice by enhancing peripheral insulin sensitivity and promoting β-cell growth and function required for glucose tolerance (19, 22, 27).

Our results highlight the balance that needs to be achieved between cell growth and metabolism that is essential throughout life. In the wrong context, reduced PTEN function can facilitate the dysregulated growth of transformed cells to the detriment of the organism; Pten is one of the most frequently mutated genes in many forms of human cancer. However, the converse is not necessarily beneficial because Pten attenuates peripheral insulin action in normal mice and prevents compensatory β-cell expansion in Irs2−/− mice. Although these results highlight Pten as an interesting target for diabetes treatment, the side effects loom large, and whether safe PTEN inhibitors can ever be developed and targeted to the tissues of interest, i.e. liver, muscle, adipose, and β-cells, is an important question that deserves attention.

Increased peripheral insulin sensitivity resulting from Pten haploinsufficiency was far greater in the absence of Irs2 than in wild type mice, suggesting that Pten potently antagonizes Irs2-related signals to promote peripheral insulin action. Previous work shows that Pten is a negative regulator of peripheral insulin action in liver, adipocytes, and muscle (12-15). Given that these same tissues are effected by disruption of Irs2, it seems likely that diminished hydrolysis of PI(3,4)P2 and PI(3,4,5)P3 produced largely through Irs1 signaling in the Irs2−/− mice, plays an important role in the enhanced insulin sensitivity in Irs2−/−::Pten−/− mice.

β-Cell dysfunction is a central component in the pathogenesis of human type 2 diabetes, with abnormal islet growth that is inadequate for the degree of peripheral insulin resistance (3, 28, 29). Indeed, our previous studies show that islet function is rate-limiting in the development of diabetes in Irs2−/− mice (16, 21, 30). Although mean β-cell area and islet size was equivalent in Irs2−/−::Pten+/− mice and Irs2−/− mice at 3 months, it was significantly increased in Irs2−/−::Pten−/− mice by 6–8 months. Rescue of β-cell area and islet size was associated with a significant increase in β-cell proliferation in Irs2−/−::Pten−/− mice compared with Irs2−/− mice at 3 months. Thus, our findings of improved peripheral insulin sensitivity, rescued islet growth, and sustained islet function in Irs2−/− mice with Pten haploinsufficiency reinforces the importance of PTEN in glucose homeostasis. Moreover, our results suggest that pharmaceutical strategies to selectively increase Irs2-related signaling in human diabetes could be beneficial.

Whereas Pten haploinsufficiency promotes glucose homeostasis, negative regulation of PTEN might not be a viable strategy as a therapy for human type 2 diabetes. PTEN is a powerful negative regulator of cellular proliferation and one of the most frequently mutated genes in many forms of human cancer (8, 9). Although homozygous Pten disruption is embryonic lethal in mice, Pten−/− mice survive into adulthood.
but develop tumors of breast, prostate, lymphoid system, and other soft tissues, upon loss of heterozygosity of the remaining Pten allele in the cancerous tissue (19, 22, 27). Whereas our studies of Irs2−/−::Pten+/+ mice illustrate that Pten is an important negative regulator of peripheral insulin sensitivity and islet growth and function, loss of Irs2 did not rescue the lethal lymphoproliferative disease that occurs with Pten haploinsufficiency (19, 27). The tumor phenotypes observed in Irs2−/−::Pten+/+ mice are complex, and careful analysis is required to determine whether Irs2 slows the progression of disease. This could be the case for mammary tumors, where Irs2 signaling was shown to play a role in metastasis (31).

The mechanisms governing replication of terminally differentiated β-cells and neogenesis from progenitor cells are unclear. Understanding these pathways is important because peripheral insulin resistance can be compensated by β-cell mass expansion to sustain glucose homeostasis. However, this normal response fails in systemic cancerous tissue (19, 22, 27). Whereas our studies of H9252 mice illustrate that Pten is an important negative regulator of peripheral β-cell function in the nucleus. It is important to understand how decreased nuclear Foxo1 signaling during Pten haploinsufficiency increases the phosphorylation of Akt and Foxo1, which can reduce the accumulation of Foxo1 in the nucleus. It is important to understand how decreased nuclear Foxo1 signaling during Pten haploinsufficiency might reveal new strategies to prevent or cure diabetes.

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