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Tissue-Specific Targeting of the Insulin Receptor Gene

Rohit N. Kulkarni and Terumasa Okada

Research Division, Joslin Diabetes Center, Department of Medicine, Harvard Medical School, One Joslin Place, Boston MA 02215

The techniques to study the mechanisms that underlie the pathogenesis of disease processes have been revolutionized by the development of methods that allow spatiotemporal control of gene deletion or gene expression in transgenic and knockout animals. The ability to interfere with the function of a single protein in a specific tissue allows unprecedented flexibility for exploring gene function in both health and disease. The present review will summarize some of the different knockouts and transgenics generated recently to study type 2 diabetes and critically evaluate the techniques used to examine the function of the insulin receptor in two nonclassical insulin target tissues—the pancreatic islet and the central nervous system.

Key Words: Cre/lox; insulin receptor; islet β-cells; brain; knockout.

Introduction

Type 2 diabetes is caused by a combination of defects in insulin action and insulin secretion ultimately leading to uncontrolled hyperglycemia. It is generally accepted that both the defects should be present for a full manifestation of the disease. Although considerable progress has been made over the last few decades in understanding the pathophysiology underlying the defects in insulin action and the potential abnormalities in insulin secretion, the precise mechanisms that underlie these defects are still unclear.

The study of defects in insulin action has focused largely around understanding the pathways used by the hormones insulin and IGF-I. The receptors for insulin and IGF-I are expressed ubiquitously and are important for the growth and metabolism of virtually every tissue in the body *(1–3)*. Insulin and IGF-I bind to distinct receptors that in turn transmit signals by phosphorylating insulin receptor substrates (IRS) including the four IRS proteins, Shc, Gab-1, FAK, Cbl, and potentially other substrates *(1,4–8)*. These insulin receptor substrates play different but crucial roles in cellular processes that are important for the metabolism and growth of tissues including glucose transport and utilization, protein synthesis, cell growth, proliferation and antiapoptosis. Several reviews provide an excellent update on these signaling networks *(6–8)*. Over the last decade, several laboratories have created global and tissue-specific knockouts of genes that code for protein(s), which are considered potentially important in regulating the effects of insulin and/or IGF-I. In this review we will summarize some of the conventional and tissue-specific transgenic and knockout models that have recently been created and highlight the factors derived from these studies that contradict the current concepts in understanding the pathogenesis of type 2 diabetes. To maintain the perspective of this issue and to complement the contributions by other authors, we will focus on the techniques adopted for the tissue-specific disruption of the insulin receptor gene to examine insulin action in the islet β-cell and the central nervous system (CNS).

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Author to whom all correspondence and reprint requests should be addressed: Rohit N. Kulkarni, MD, PhD, Rm 602, Joslin Diabetes Center, One Joslin Place, Boston MA 02215. E-mail: Rohit. Kulkarni@joslin. harvard. edu

Global Transgenic and Knockout Models

The techniques used to generate conventional knockouts using homologous recombination have been described elsewhere *(9,10)*. Global knockout of the genes coding for insulin in mice leads to growth retardation and death due to diabetes mellitus with ketoacidosis and liver steatosis *(11)*. IGF-I null mice show growth defects similar to IGF-II null mutants *(12,13)*, and, depending on the genetic background, some of the IGF-I knockouts die, while others survive into adulthood *(13)*. Mice lacking the IGF-I gene exhibit postnatal lethality, growth retardation, infertility, and defective development of bone and muscle *(13,14)*. Similar findings were reported in a human with homozygous partial deletion of the IGF-I gene *(15)*.

Mice homozygous for a null mutation of the insulin receptor show normal intrauterine growth but die within 48 to 72 hr after birth due to severe hyperglycemia and diabetic ketoacidosis *(16,17)*. Humans with leprechaunism, however, manifest quite a different phenotype with intrauterine growth retardation and only mild hyperglycemia *(18,19)*. On the other hand, IGF-I receptor null mutants show severe growth deficiency and die at birth due to respiratory failure *(13)*. Taken together these global knockouts underscore the crucial importance of insulin and IGF-I and their cognate receptors in the overlapping regulatory functions of metabolism and growth in mice and humans.

Gene deletion of proteins downstream to the insulin and IGF-I receptor, including the IRS proteins, leads to different phenotypes. Thus, IRS-1 knockouts exhibit postnatal growth retardation and hyperinsulinemia but a relatively normal lifespan *(20,21)* and, interestingly, the IRS-1 null mice show hyperplastic but dysfunctional islets *(22–24)*. In contrast, IRS-2 knockouts show only mild growth retardation, but develop insulin resistance and β-cell hypoplasia leading to overt diabetes and early death *(23,25)*. Furthermore, IRS-2 knockouts show defects in the CNS that indicate a potential role for the substrate in neuronal function. For example, female IRS-2 knockouts are infertile, possess fewer ovarian follicles and have lower sex steroid, LH, and prolactin levels *(26)*. Pituitaries of female IRS-2 deficient mice are small and have a reduced number of gonadotrophs and the mutants show hypothalamic resistance to leptin. On the other hand, IRS-3 null mice develop normally and have normal glucose tolerance *(27)*. Although this substrate is normally abundantly expressed in adipose tissue, insulin signaling in adipocytes was found to be unaffected by the lack of IRS-3 *(27)*. IRS-4 deficient mice manifest mild growth defects and glucose intolerance and this is evident only in males because the IRS-4 gene is located on the X chromosome *(28)*. Knockout of the p85 regulatory subunit of PI 3-kinase in mice leads to increased insulin sensitivity and hypoglycemia *(29–31)*, while null mutants for Akt-2 (PKB-β) show insulin resistance in muscle and liver and an increased islet mass *(32)*. Interestingly, mutants for p70S6 kinase show reduced β-cell size, lower insulin secretion, and reduced pancreatic insulin content *(33)*. The phenotypes of the global knockouts of some of the major proteins in the insulin/IGF-I signaling pathway are shown in Table 1. A more detailed list can be found in other reviews *(34,35)*.

Tissue-Specific Transgenic and Knockout Models

The early death of the mice in global knockouts of the insulin and IGF-I receptors precludes a detailed examination of cellular and molecular alterations in the individual tissue(s) considered important for the effects of insulin and/or IGF-I. Therefore, to evaluate the role of each protein in specific tissues, several laboratories have adopted the approach of tissue-specific knockouts using the Cre/*loxP* technique *(9, 10,36,37)* or the expression of a dominant negative in specific tissues *(38–40)*. In most of these instances the mice are born normally and survive into adulthood and allow for a physiological, biochemical, and molecular examination of the target tissues important for glucose homeostasis. Traditionally, the tissues that were considered crucial targets of insulin action included the skeletal muscle, liver, and adipose. Some of these knockouts with some unexpected phenoypes are discussed below.

Mice with skeletal-muscle-specific insulin receptor knockout (MIRKO) display elevated fat mass, serum triglycerides, and free fatty acids—all features of the metabolic syndrome in humans *(41)*. Surprisingly, the MIRKO mice did not show glucose intolerance but had a reduced muscle glucose uptake in vitro *(41)* and during a euglycemic hyperinsulinemic clamp *(42)*. One interpretation of these data is that loss of functional insulin signaling in skeletal muscle promotes cross talk with other metabolic tissues such as the adipose. In contrast, muscle-specific disruption of the glucose transporter GLUT4 leads to glucose intolerance and insulin resistance due to a reduced glucose uptake in muscle *(43)*. Liver-specific deletion of the insulin receptor (LIRKO) leads to severely impaired glucose tolerance supporting a direct role for insulin in suppressing hepatic glucose production *(44)*. Adipose-tissue-selective insulin receptor knockout (FIRKO) mice have low fat mass and high serum leptin levels and the mutants are resistant to age-related, hypothalamic lesion-induced obesity and to obesity-related glucose intolerance *(45)*. Adipose-specific knockout of GLUT4 shows impaired insulin-stimulated glucose uptake in adipocytes, insulin resistance in muscle and liver, and, consequently, glucose intolerance and hyperinsulinemia *(46)*. The knockouts generated recently to examine the roles of specific proteins in the insulin/IGF-I signaling pathway in classic insulin target tissues are summarized in Table 2.

It has recently become clear that insulin signaling in non-classic target tissues, such as pancreatic islets and the CNS, also contribute importantly to glucose homeostasis, body weight regulation, and fertility by modulating glucose sensing and glucose metabolism, appetite, energy expenditure, and reproduction. The phenotypes of the two knockouts are discussed below.

Insulin and IGF-I Receptors in the Islets

Several investigators have attempted to demonstrate the presence of insulin receptors in the islet β-cells since the 1980s (reviewed in ref. *47*). Although IGF-I receptors have been shown to be present on both β- and α-cells *(48,49)*, the presence of insulin receptors in the β-cells has been more difficult to prove. This may be related, in part, to the continuous secretion of insulin by the β-cell and a potential downregulation of the insulin receptor and to the lack of availability of a suitable antibody for immunohistochemistry. To circumvent these technical drawbacks Rothenberg and colleagues performed single-cell PCR using DNA prepared from primary β-cells to conclusively show the presence of the receptor *(50)*. Furthermore, islet β-cells have been shown to express all four insulin receptor substrates (IRS), -1, -2, -3, and -4, and data from several other labs provide ample evidence for a functional insulin/IGF-I signaling pathway in the islets (reviewed in refs. *35,47*). An orphan receptor of the insulin receptor subfamily—the insulin receptor-related receptor (IRR)—has been reported to be expressed at higher levels than the insulin or IGF-I receptors *(51)*. However, mice deficient for IRR failed to manifest alterations in islet morphology or secretory function

Table 1
Global Knockouts

Protein	Phenotype	Reference
Insulin	Intrauterine growth retardation, Neonatal lethality, Ketoacidosis, Liver steatosis	Duvillie et al., 1997 *(11)*
IGF-I	Dwarfism, Variable survival	Liu et al., 1993 *(13)*, Powell-Braxton et al., 1993 *(14)*
Insulin receptor	Neonatal lethality, Ketoacidosis	Accili et al., 1996 *(16)*, Joshi et al., 1996 *(17)*
IGF-I receptor	Dwarfism Neonatal lethality	Liu et al., 1993 *(13)*
IRS-1	Post-natal growth retardation	Araki et al., 1994 *(20)*
	Insulin resistance	Tamemoto et al., 1994 *(21)*
	Islet hyperplasia	
	Insulin secretory defect	Kulkarni et al., 1999 *(22)*
		Aspinwall et al., 1999 *(24)*
		Kubota et al., 2000 *(23)*
IRS-2	Insulin resistance	Withers et al., 1998 *(25)*
	Diabetes	Kubota et al., 2000 *(23)*
	Islet hypoplasia	
IRS-3	Relatively normal	Liu et al., 1999 *(27)*
IRS-4	Mild glucose intolerance	Fantin et al., 2000 *(28)*
PI 3-kinase isoforms		
p85α	Increased insulin sensitivity, Hypoglycemia	Terauchi et al., 1999 *(30)* Mauvais-Jarvis et al., 2002 *(31)*
p85β	Increased insulin sensitivity, Hypoglycemia	Ueki et al., 2002 *(29)*
GLUT4	Growth retardation, Reduced adipose tissue, Cardiac hypertrophy, Normal glucose tolerance.	Katz et al., 1995 *(111)*
Akt1	Growth retardation, Increased apoptosis, Normal glucose tolerance.	Chen et al., 2001 *(112)*
Akt2	Insulin resistance in liver and muscle, Increased islet mass.	Cho et al., 2001 *(32)*
P70S6kinase (S6K1)	Hypoinsulinemia, Glucose intolerance, and Reduced beta-cell size.	Pende et al., 2000 *(33)*
ACRP30 (Adiponectin)	Insulin resistance	Maeda et al., 2002 *(113)*
	Increased neointimal formation	Kubota et al., 2002 *(114)*
	Increased beta-oxidation	Ma et al., 2002 *(115)*
Insulin receptor-related receptor	Normal phenotype	Kitamura et al., 2001 *(52)*

[a]A summary of global knockouts in the insulin/IGF-I signaling pathway. Some knockouts may not be included for lack of space.

(52). A more-detailed discussion of these and other transgenic studies is beyond the scope of the current review and can be found in recent publications *(7,34,47)*.

Limitations to the Study of Islet Function In Vivo

The islet is made up of four different cell types—the majority (approx 80%) being insulin-secreting β-cells, approx 15% of glucagon-secreting α-cells, and the remainder (approx 5%) being made up of δ-cells and PP cells that secrete somatostatin and pancreatic polypeptide, respectively. The close association of these four different cell types underscores a potential need for intimate paracrine/autocrine interactions for optimal functioning of the islet, in vivo, but precludes the careful examination and precise definition of the regulatory factors and mechanisms that modulate the secretory and synthetic functions of each type of cell. A common test to evaluate islet function, in vivo, in both humans and rodents

Table 2
Tissue-Specific Knockouts

Protein	Phenotype	Reference
IGF-I		
Liver	Normal growth and development, Muscle insulin sensitivity.	Yakar et al., 1999 *(116)* Liu et al., 2000 *(117)* Yakar et al., 2001 *(118)*
Insulin receptor		
Muscle	Increased adiposity, Dyslipidemia and Glucose intolerance.	Moller et al., 1996 *(119)*
	Normal glucose tolerance, Elevated triglyceride and FFA levels	Bruning et al., 1998 *(41)*
β-cell	Glucose intolerance, Loss of acute phase insulin secretion, Reduced β-cell mass.	Kulkarni et al., 1999 *(54)*
Liver	Severe glucose intolerance, Hepatic dysfunction	Michael et al., 2000 *(44)*
Brain	Increased food intake and obesity in female Impaired spermatogenesis and ovarian follicle maturation	Bruning et al., 2000 *(65)*
Adipose	Protect against obesity and glucose intolerance Heterogeneity in cell size of white adipose tissue	Bluher et al., 2002 *(45)*
Insulin receptor		
Muscle and Adipose	Impaired glucose tolerance Insulin resistance	Lauro et al., 1998 *(40)*
IGF-I receptor		
β-cell	Impaired glucose tolerance Reduced glucose-stimulated insulin secretion	Kulkarni et al., 2002 *(66)* Xuan et al., 2002 *(67)*
GLUT4		
Muscle	Glucose intolerance, Reduced glucose transport	Zisman et al., 2000 *(43)*
Adipose	Glucose intolerance, Insulin resistance in muscle and liver	Abel et al., 2001 *(46)*
Insulin receptor and IGF-I receptor		
Muscle	Insulin resistance, β-cell dysfunction and diabetes	Fernandez et al., 2001 *(120)*

[a]A summary of recently created tissue-specific knockouts of proteins important for maintenance of glucose homeostasis. Some knockouts may not be included for lack of space.

involves the administration of a secretagogue—for example, glucose or amino acid—by the oral, intravenous, or intraperitoneal routes (in rodents only), and blood sampling to assess the first (acute) and second phase insulin release and/or C-peptide levels as measures of secretion *(53,54)*. Although these tests provide some information on secretory function, the precise cellular mechanisms involved in individual islet cell function are difficult to dissect in both humans and rodents. This has led to experiments on isolated islets and examination of their functions in perifusion and static incubation systems and the use of clonal α- and β-cell lines *(55–57)*. Thus, studies describing individual islet cell functions require a complement of in vitro and in vivo tests for a better physiological understanding of their regulatory mechanisms.

In this context, the development of genetic engineering techniques over the last decade, to create gain-of-function or loss-of-function mutations, is an excellent strategy to examine the function of specific proteins in the different cell types in the islet. Furthermore, the ability to "turn off" a gene encoding for a particular protein in a time-dependent manner provides a tool to simulate the gradual dysfunction that is usually observed in chronic diseases *(10)*. Although adaptation to the creation of a genetic mutation during embryonic life is a natural consequence of this method, the information obtained in studies in several biological disciplines using these methods has been extremely useful to unravel potentially novel and unexpected functions of proteins. These observations are comparable to those made from humans with naturally occurring genetic mutations,

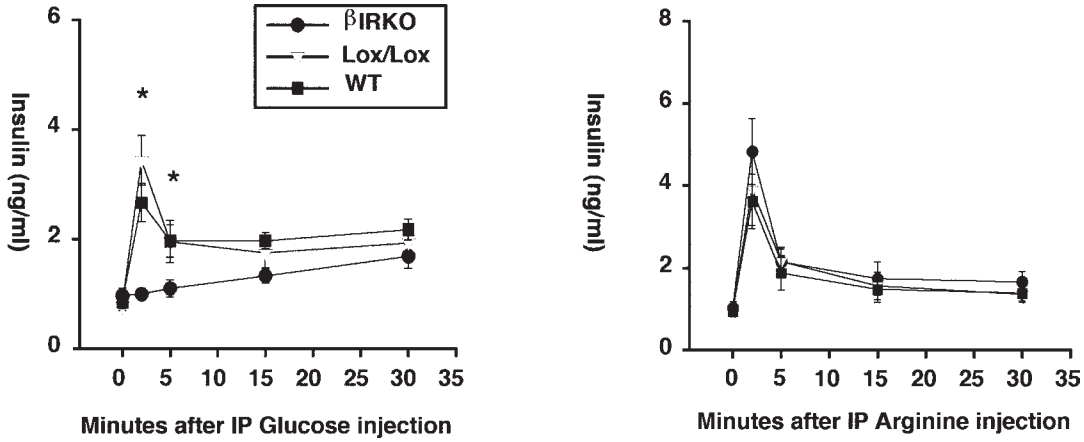

Fig. 1. Loss of acute phase insulin secretion in response to (**left**) glucose but not (**right**) arginine stimulation, in vivo, in mice deficient in β-cell specific insulin receptors (βIRKO). (Reproduced with permission from Kulkarni et al., *Cell* **96,** 329–339, 1999.)

who of necessity adapt to the mutation, but, nevertheless, provide important clues to understand the function of the proteins encoded by the gene(s).

Insulin Receptor Knockout in the Islet β-Cells

The availability of the Cre/*lox* technique has enhanced the ability of investigators to examine the role of different proteins in tissues that develop under the influence of a specific promoter. This strategy is especially useful in the islet, which is made up of four types of cells, each under the control of a different promoter. The detailed methodology for the Cre/*lox* technique is available from several recent reviews *(10,37)* and from other authors in this issue. Briefly, this requires the creation of two transgenic mice. First, *loxP* sites are inserted to flank a gene of interest to create a homozygous "floxed" mouse. The second requirement is a mouse expressing Cre recombinase in a tissue-specific manner. The tissue specificity of Cre expression is governed by the promoter used in the experiment. Breeding the "floxed" mouse with a mouse expressing Cre recombinase yields many genotypes including the knockout—a mouse homozygous for the *loxP* sites and expressing Cre on the chosen promoter.

Insulin expression is observed as early as embryonic d 9 when the rat insulin promoter is expressed in islet cells *(54, 58,59)*. Therefore, the rat insulin promoter is considered ideal for the tissue-specific expression of Cre recombinase in studies to evaluate the functions of proteins specific to the β-cells. The details relating to the creation of the β-cell specific insulin receptor knockout (βIRKO) mouse have been described elsewhere *(54)*. A characteristic feature in these knockouts, maintained on a mixed (C57Bl X 129Sv X DBA/2) genetic background, is a glucose-specific loss of acute phase insulin secretion that is secondary to altered glucose sensing (Fig. 1) *(54)*. The knockouts manifest progressive glucose intolerance with a reduction in islet size and reduced pancreatic and islet insulin content (Fig. 2).

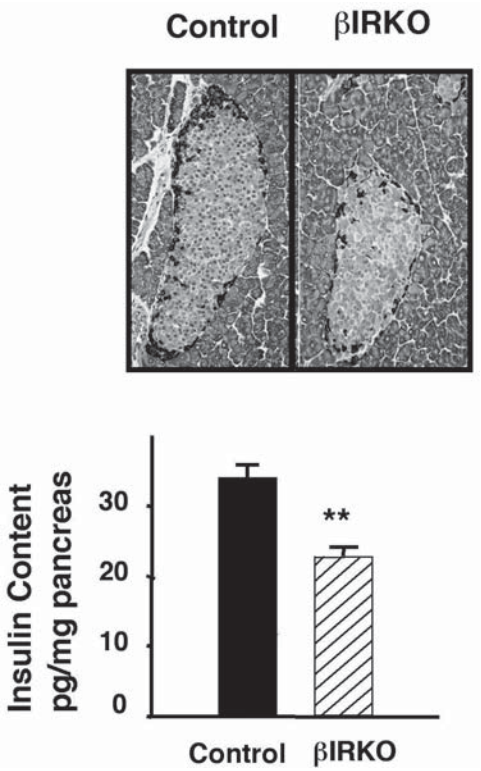

Fig. 2. Representative pancreas sections from control and β-cell insulin receptor knockout (βIRKO) mice showing (**top**) reduced islet size and (**bottom**) reduced pancreatic insulin content. (Reproduced with permission from Kulkarni et al., *Cell* **96,** 329–339, 1999.)

Following on from our original study, we have observed that some of the βIRKO mice become overtly diabetic as they age (7–8 mo) *(60)* and some become mildly obese *(60, 61)*. There are several factors that can account for this variation in the phenotype including the influence of the genetic background. Mice on a C57Bl6 background are known to

be susceptible to obesity *(62,63)*, and it is possible that some of the knockouts on the mixed background have a greater genetic contribution from the C57Bl6 strain and hence are more susceptible to weight gain. In this context experiments to evaluate the phenotype of the βIRKO mice on a pure B6 background will be interesting and are currently in progress in our laboratory. Alternatively, it is possible that the mild obesity observed in a fraction of the βIRKO mice is related to the CNS expression of the rat insulin promoter. Although the rat insulin promoter has been suggested to be expressed exclusively in the β-cells of the islet, a recent paper by Gannon et al. *(59)* indicates that the promoter is also expressed in the ventral cerebral cortical areas of the CNS. Using ROSA26 reporter mice to evaluate the expression of the rat insulin promoter2-Cre gene, the authors found that recombination occurred in approx 10% of the neurons in the region of the hypothalamus. However, it is not known whether the neurons that underwent recombination also express the neuropeptides that regulate appetite and body weight. In the light of these findings it will be important to investigate (a) the level of expression of the rat insulin promoter in the hypothalamus of the mouse brain as compared to its expression in the β-cells; (b) the precise location of the neurons that undergo recombination in different regions in the mouse brain and especially the potential co-expression of peptides involved in appetite control including leptin, melanocyte concentration hormone (MCH), neuropeptide Y (NPY), galanin, pro-opiomelanocortin (POMC), and so forth; and (c) the detailed ontogeny of the promoter during development and neonatal and adult life. These studies are currently in progress and the data from these experiments will be useful in the long term to examine the function of the promoter in normal physiology and as a reference for future transgenic studies. Although the rat insulin promoter continues to be an important tool for in vivo and in vitro investigation of the β-cells, it is desirable to obtain a Cre transgene that is driving expression exclusively in the β-cells using a suitable "fragment" of the pancreatic and duodenal homebox transcription factor gene (*pdx-1*) *(64)* or the neurogenin 3 promoters.

An observation relevant to this discussion is that mice with insulin receptor knockout in neuronal tissue (NIRKO mice) *(65)*, and presumably in hypothalamic neurons that regulate appetite, do not show alterations in islet function. NIRKO mice have circulating insulin levels in the normal range at least up to 6 mo of age and the females show a mild increase in insulin levels by 6–8 mo *(65)*. Furthermore, circulating C-peptide levels are in the normal range in the knockouts, suggesting a normal islet secretory function (R. N. Kulkarni, J. C. Bruning, unpublished observations). The mutants, however, manifest defects related to body weight regulation and reproduction (see discussion below). Thus, loss of functional insulin signaling in the brain, including the ventromedial hypothalamus, has little impact on gross islet morphology and secretory function in mice fed normal chow.

In addition to examining the role of the insulin receptor, we and other investigators have used the Cre/*lox* approach to examine the role of other proteins in the β-cell. Using different floxed and Cre-expressing mice, we *(66)* and Accili's group *(67)* created the β-cell IGF-I receptor knockout. Unexpectedly, these mice develop β-cells and islets in a normal manner but show defects in glucose-stimulated insulin secretion secondary to reduced glucokinase and GLUT2 expression in the islets *(66,67)*. These results indicate that receptors other than the IGF-I receptor likely play a significant role in promoting the growth of β-cells and that IRS-2 has "IGF-I-independent" ligands that contribute to its potential β-cell growth effects. The findings from these Cre/*lox* studies also suggest that experiments using global knockouts should be treated with caution when extrapolating the findings to a specific tissue *(66–68)*. It is likely that disruption of proteins in the whole body have effects that impact on different tissues during early development that in turn lead to severe secondary effects in a tissue-specific manner.

Related Transgenic Models Created Using the Rat Insulin Promoter

Recently, several transgenic models have been developed using the rat insulin promoter to study β-cell function. Three of these involving the expression of growth factors —hepatocyte growth factor *(69)*, placental lactogen *(70)*, or the parathyroid-related protein *(71)*—have been separately targeted to the β-cell. Each of these models displays an increase in islet mass and insulin-mediated hypoglycemia. It will be useful to examine the potential cross-talk between the signaling pathways activated by the receptor tyrosine kinases for these growth factors and the insulin/IGF-I signaling pathway to dissect the mechanisms underlying the growth and apoptosis of islet β-cells. A schematic depicting the signaling pathways of potential growth factors in the islet β-cell is shown in Fig. 3. Another interesting model is the transgenic mouse over-expressing c-Myc in β-cells *(72)*. These mice develop early hyperplasia followed by apoptosis and overt diabetes indicating that c-Myc is involved in a complex interplay between proliferative and apoptotic pathways in the β-cells.

Other studies that have used the Cre/*lox* technique and the rat insulin promoter include the β-cell-specific disruption of the nuclear gene encoding mitochondrial transcription factor A (Tfam) *(73)*, disruption of the gene encoding for glucokinase *(74)*, deletion of foxa2 in the β-cell *(75)*, and *pdx-1* deletion in the β-cell *(76)*. Whether the expression of the rat insulin promoter in the CNS affects the phenotype in these different models is unclear.

Insulin Action in the CNS

In addition to the pancreatic islets, the CNS has been considered a nonclassic tissue for insulin action. However,

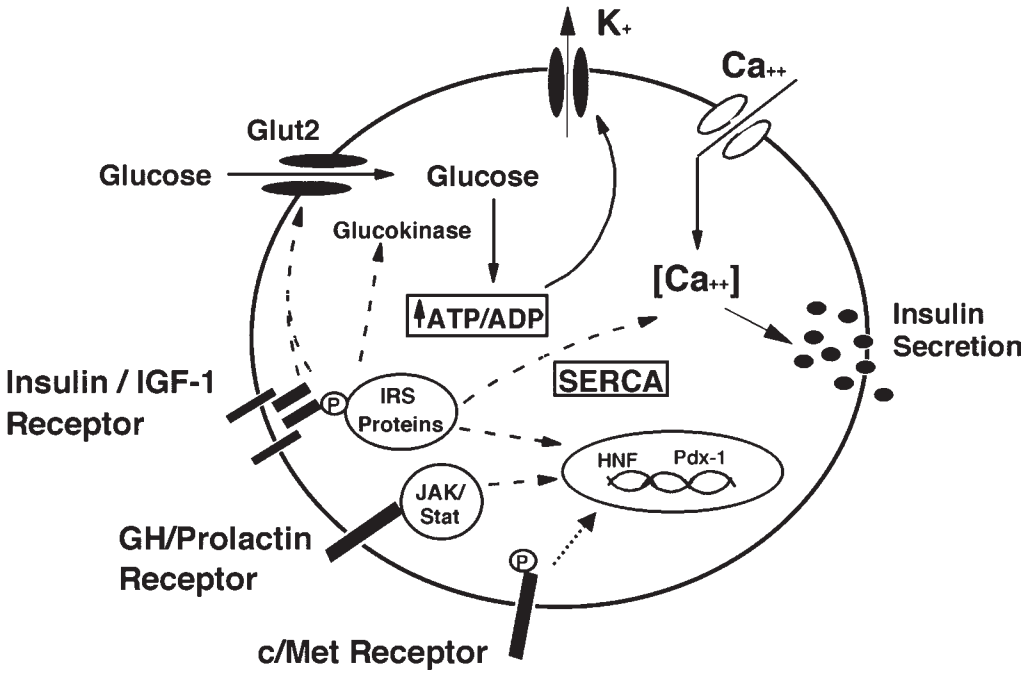

Fig. 3. A schematic showing the insulin/IGF-I and growth factor signaling pathways in the β-cell. The dotted lines indicate pathways with potential significance in downstream effects including β-cell transcription and insulin synthesis and secretion.

insulin receptors are widely expressed in the CNS, especially in the hypothalamus, pituitary, and the olfactory bulb *(77–79)*. In fact, insulin action in the brain has been associated with regulation of food intake, neuronal growth and differentiation, neurotransmitter release, and synaptic plasticity *(80–85)*. Consequently, defective insulin signaling in the CNS has been linked to altered food intake and body weight regulation, abnormal hepatic glucose production, and neurodegenerative disorders such as Parkinson's disease *(65,86,87)*. Several studies link altered insulin action to the development of Alzheimer's disease *(88–90)*. Analysis of brains from patients with Alzheimer's disease showed altered insulin receptor densities in the different cortical areas compared with the control group *(91)*. Treatment of hyperphagic obese Zucker rats with diazoxide resulted in lower calorie intake and reduced weight gain, and this was associated with enhanced brain capillary insulin binding *(92)*. Decreasing hypothalamic insulin receptors by intracerebroventricular injection of antisense oligonucleotides caused hyperphagia and insulin resistance accompanied by elevated neuropeptide Y and agouti-related peptide expression in the arcuate nucleus *(86)*. Insulin has been shown to rescue R28 cells—a rat retinal neural cell line—from apoptosis by a PI 3-kinase and Akt-mediated mechanism *(93)*. Upregulation of expression of insulin receptors in some parts of the hippocampus after training in mice suggests that insulin signaling is related to learning and memory *(89)*. Taken together these studies indicate the important role of insulin in diverse functions mediated by the CNS.

Limitations to the Study of CNS Function

Designing a suitable experiment to study CNS function has been difficult and challenging. This is largely due to the complex organization of neuronal tissue in different areas of the brain, the extensive networks that link different regions and subregions of the brain, and efferents that can rapidly influence the functions of other organ systems via the peripheral nerves. Studies in rodents have used the intracerebroventricular injection method to examine the effects of stimuli on appetite and body weight regulation. This technique involves the physical insertion of a suitable catheter for injection of test solutions and is necessarily associated with some destruction of neuronal tissue in the area of insertion. An additional drawback is the difficulty to accurately control and define the diffusion of the test substances around the site of entry. These methods are generally complemented by in vitro experiments to gain some insight into signaling pathways, neuronal growth and differentiation.

Insulin Receptor Knockout in the CNS

The use of genetic approaches and especially the Cre/*lox* technique circumvents some of the difficulties discussed above. To investigate the role of insulin signaling in vivo, Bruning et al. generated neuron-specific insulin receptor knockout (NIRKO) mice *(65)*. Insulin-receptor floxed mice were crossed with mice expressing Cre recombinase on the rat nestin promoter and enhancer *(94,95)*. Nestin is an inter-

mediate filament protein that is expressed in neuronal and glial precursor cells *(94)*.

The inactivation of insulin receptor in brain had no detectable impact on brain development and neuronal survival *(65)*. Female NIRKO mice showed increased food intake, and both male and female knockouts displayed diet-induced obesity and insulin resistance, increased body fat and elevated circulating leptin levels. The females showed a mild elevation in insulin levels at 6–8 mo of age. NIRKO mice showed impaired spermatogenesis and poor maturation of ovarian follicles, accompanied by low plasma luteinizing hormone (LH) levels. These data indicate an important link between insulin action in the brain and reproductive function.

Although some studies report the presence of nestin-positive cells in the islet *(96)*, others indicate that nestin is neither expressed in developing progenitor cells nor in differentiated pancreatic cells *(97)*. The lack of an altered islet function in the NIRKO mice (see discussion above) provides further evidence that expression of the nestin promoter and enhancer is unlikely to have functional consequences in the endocrine pancreas. The use of promoters that are more specific for different regions of the brain will be extremely useful to dissect the potential role of insulin/IGF-I in mediating the complex functions of the CNS.

Other studies using the nestin promoter include investigations to examine the role of the glucocorticoid receptor gene in the CNS *(95)*.

Role of Genetic Background

Considerable evidence exists with regard to the modification of monogenic disorders by the genetic background of inbred laboratory animals *(98–101)*. This may, in part, be due to the presence of modifier loci that may exaggerate or rescue the severity of a phenotype *(102)*. The identification of such modifier loci is a tedious process but provides insight into the pathophysiology of the disease *(102)*. Since a majority of transgenic and knockout experiments are currently performed in mice on a mixed background, the strain of mice is an important factor for consideration *(103,104)*. The investigation of genetically engineered mice on mixed or on a pure genetic background each has advantages. First, examination of phenotypes in mice on a mixed background may aid in the localization and identification of potential modifier loci that underlie the observed alterations. These findings gain relevance because most human populations are on a mixed genetic background and type 2 diabetes is a polygenic disease with a potential for genetic modification *(1,105)*. Second, mice on a mixed background reflect interactions between the background strains used in the creation, and the effect of the genetic manipulation itself and therefore have a greater chance of manifesting a "phenotype." A confounding factor is the effect of repeated intercrossing of mice already on a mixed genetic background. This would lead to subtle phenotypic changes over time due to a systematic influence of dominant or recessive traits of one or the other genetic strains. A rule of thumb used by some investigators is to start with examination of mice on a mixed background to avoid "missing" a phenotype and then to follow simple breeding strategies suggested to test flanking gene effects and minimize the confounding effects of background strains *(106,107)*. Although studying mice on a pure background has obvious advantages, a critical factor is the choice of the strain to backcross. For example, mice on the C57Bl6 background are more prone to obesity and glucose intolerance *(62,63,108)* while the 129Sv strain is relatively resistant to obesity. Thus, an obvious phenotype on the C57Bl6 may be completely missed if the 129Sv mouse strain is chosen for purification. Unfortunately, pure strain effects are difficult to predict and may require trial and error approaches with considerable investment of time, personnel, and resources. A related factor to consider when evaluating the "phenotype" is whether the efficiency of the conditional system itself is affected by the background strain. For example, the tetracycline-controlled transcriptional silencer system (tTS) has been shown to be different between different mouse strains *(109)*. It is possible, in theory, that the efficiency of the Cre/*lox* system is influenced by a specific mouse strain, although no detailed investigations are currently available in this regard.

Future Perspectives

Although there are some caveats to Cre/*lox* technology, the use of this technique has revolutionized the understanding of the pathophysiology of type 2 diabetes, obesity, and other diseases *(110)*. The technique is well established and should be considered a first choice for conditional gene deletion experiments. Recently, short inhibitor RNA sequences have been used to effectively silence genes and this technique appears to hold promise for use in single embryonic cells *(121,122)*. The availability and further development of these transgenic techniques complements the rapid advances in genome sequencing, gene array technology, and the ability to assess physiological alterations in murine models for the dissection of processes that underlie normal physiological mechanisms, and by extension, to a better understanding of the pathophysiological abnormalities of many disease states.

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