Endocrine Pancreas in Insulin Receptor–Deficient Mouse Pups
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Insulin receptor (IR)-deficient pups rapidly become hyperglycemic and hyperinsulinemic and die of diabetic ketoacidosis within a few days. Immunocytochemical analysis of the endocrine pancreas revealed that IR deficiency did not alter islet morphology or the number of β-, α-, δ-, and pancreatic polypeptide (PP) cells. The lack of IR did not result in major changes in the expression of islet hormone genes or of β-cell–specific marker genes encoding pancreas duodenum homeobox–containing transcription factor-1 (PDX-1), glucokinase (GCK), and GLUT2, as shown by reverse transcription–polymerase chain reaction analysis. The serum glucagon levels in IR-deficient and non diabetic littersmates were comparable. Finally, total insulin content in the pancreas of IR-deficient pups was gradually depleted, indicating sustained insulin secretion, not compensated for by increased insulin biosynthesis. These findings are discussed in light of recent results suggesting a role of IR in β-cell function. Diabetes 50 (Suppl. 1):S146–S149, 2001

Several studies using transgenic and knockout mice have established that the development and function of pancreatic β-cells are controlled by a number of genes encoding specific transcription factors such as pancreas duodenum homeobox–containing transcription factor-1 (PDX-1), hormones, growth factors such as IGF-I and IGF-II and their receptors, or proteins involved in glucose sensing such as glucokinase (GCK) and the glucose transporter GLUT2 (1,2).

The biological effects of insulin are mediated by the insulin receptor (IR), a heterotetrameric (α2β2) membrane tyrosine kinase. After insulin binding, the activated IR phosphorylates docking proteins such as insulin receptor substrate (IRS)-1 or IRS-2. These adaptor proteins subsequently recruit intracellular mediators, which in turn lead to the activation of various signaling pathways (3). Although muscle, liver, and fat tissue represent the major target tissues for the metabolic action of insulin, IR is thought to be present in most cells and its expression in the β-cell has also been documented (4). Despite a number of in vivo studies of humans or animal models, the question as to whether IR expression in β-cells has any functional significance has remained a matter of debate for several years. It has emerged from some recent work using purified β-cells or β-cell lines that insulin action through IR might play an important autocrine role in normal β-cell function. This conclusion is based on the observations that insulin can stimulate insulin gene transcription as well as insulin secretion in β-cells (5–7). It was further shown that the signaling pathways activated by IR that lead to the transcriptional upregulation of the insulin gene involve the IRS-2/phosphatidylinositol 3-kinase/p70 S6 kinase and the calcium/calmodulin-dependent protein (CaM) kinase cascades (5).

We and others have previously reported that targeted disruption of the IR gene (Insr) in the mouse resulted in diabetic ketoacidosis and neonatal lethality (8,9). IR deficiency did not preclude the development of a pancreas with both exocrine and endocrine components. IR-deficient pups were normal at birth, but rapidly became hyperglycemic and hyperinsulinemic. To gain further insight into the role of IR in β-cell growth and function, we analyzed in this work the endocrine pancreas in IR-deficient pups.

RESEARCH DESIGN AND METHODS

Animals and genotyping. The generation of mice with targeted disruption of the IR gene has been described previously (8). IR-deficient mice produced by intercrossing heterozygous null mutants could be identified by their diabetic phenotype. The nondiabetic littersmates were genotyped by polymerase chain reaction (PCR) performed on tail DNA using Taq DNA polymerase ( Gibco) and primers specific for the neomycin phosphotransferase gene (5′-GTTTCCTCCG CGTCAGGCCA-3′/5′-GTCCGATAGCGGTCCGCCA-3′). The following reaction cycles were used: 95°C for 5 min, 65°C for 1 min, and 72°C for 1 min, followed by 36 cycles of 95°C for 20 s, 65°C for 30 s, and 72°C for 1 min and finally 72°C for 7 min. A PCR product of 620 bp was detected in heterozygous mutant pups.

Immunocytochemistry and quantification of islet cells. The splenic part of the pancreas of newborn mice was fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Dewaxed sections (5 μm) were first blocked for endogenous peroxidase activity by incubation in a methanol/H2O2 mixture, then rehydrated and treated with proteinase K (1–4 μg/ml) at 37°C. After preincubation in goat serum, the sections were exposed to guinea pig anti-insulin (Sigma) or rabbit anti-glucagon (Dako), anti-somatostatin (Euro-diagnostica), or anti-human pancreatic polypeptide (PP) (provided by Dr. L. I. Larsson) antibodies. Detection was by biotin-labeled goat anti-guinea pig IgG (Jackson Immunoresearch Labs) or goat anti-rabbit IgG (Immunotech), followed by incubation with peroxidase-labeled streptavidin (Dako) and development in diaminobenzidine-H2O2. The sections were counterstained with hematoxylin. Islet cells were quantified by counting immunopositive cells on six sections separated by more than 50 μm, followed by measurement of the surface area of the sections using Biocom Visiolab 1000 software.

Reverse transcriptase–PCR analysis of pancreatic RNAs. Total pancreatic RNA from pools of four pancreata were prepared using SV Total RNA Iso-
expression of IGF-II in insulin itself (16). More recently, it was reported that over-
might stimulate cell proliferation to a greater extent than
mice become hyperglycemic. An attractive hypothesis is that
increased receptors that can efficiently bind IGF-I. One wonders
line derived from IGF-1R–deficient mice (16). It is also con-
lariferation has been clearly demonstrated using a fibroblast cell
IR activation by IGF-II could partly contribute to this effect.
Because this was mimicked in mice lacking IRS-2 (12,13).
An attractive hypothesis is that more efficient gl-
regulation of insulin gene transcription, the secreted insulin
mRNA with the various gene transcripts. PCR
products were run on agarose gels, transferred onto Hybond membranes,
and hybridized using 32P-labeled oligonucleotide probes. For insulin 1/
insulin 2, a unique primer pair was used for RT-PCR, and the product was
digested with MspI before Southern blot analysis using a single 32P-labeled
oligonucleotide probe, which revealed a fragment of 71 bp for insulin 1 and
of 112 bp for insulin 2. Quantification of PCR products was performed using
a Phosphorimagerequipped with ImageQuant software (Molecular Dynamics)
and was expressed in relation to internal control.
Measurement of pancreatic insulin content and serum glucagon levels. Pancreatic insulin was extracted using acid/ethanol and quantified by
radioimmunoassay (RIA) (ICN Pharmaceutical). Each extract was prepared
from a pool of five pancreata from newborn mice. Serum glucagon levels
were measured using a glucagon RIA kit (Linco Research).

RESULTS AND DISCUSSION
Islet morphology in IR-deficient mice. Pancreatic sections from IR-deficient and wild-type animals showed similar islet
morphology (Fig. 1A). In addition, quantitative analysis revealed that the numbers of the different islet cell types were also comparable (Fig. 1B). Thus, IR is not indispensable for islet formation and development of its various cell types.
IR and the type 1 receptor for IGFs (IGF-IR) are structurally
very similar and activate common signaling pathways (11).
Recent analysis of the endocrine pancreas in IGF-1R–deficient
mice revealed that the numbers of β- and α-cells were signifi-
cantly reduced (12,13). Thus, IGF-IR, in contrast to IR, plays a major role in the growth of the various endocrine cells. It was suggested that this growth-promoting function of IGF-1R could be achieved by IRS-2–mediated inhibition of apoptosis, because this was mimicked in mice lacking IRS-2 (12,13).
We previously reported that insulin deficiency in Ins1/Ins2
double knockout mice resulted in β-cell hyperplasia (10). Thus, the lack of insulin or of IR does not have the same con-
sequence on islet cell proliferation. In adult rats, short expo-
sure to high glucose can lead to increase in β-cell mass (14,15). However, hyperglycemia per se cannot be responsible for β-cell hyperplasia, since both insulin- and IR-deficient mice become hyperglycemic. An attractive hypothesis is that
lack of insulin could favor IGF-II action through IR, which
might stimulate cell proliferation to a greater extent than
insulin itself (16). More recently, it was reported that over-
expression of IGF-II in β-cells in transgenic mice resulted in
increased β-cell mass (17); it was proposed that more efficient IR activation by IGF-II could partially contribute to this effect.
That IGF-II action through IR can indeed stimulate cell pro-
liferation has been clearly demonstrated using a fibroblast cell
line derived from IGF-1R–deficient mice (16). It is also con-
ceivable that in the absence of insulin, the existence of empty
IRs might result in an increased number of IR–IGF-1R hybrid
receptors that can efficiently bind IGF-I. One wonders
whether these hybrid receptors, which cannot form in IR-defi-
cient mice, may have a greater mitogenic potential (18).
IR deficiency and gene expression in endocrine pancreas. Expression of genes encoding the four islet hormones was studied by RT-PCR. The results show that IR deficiency did not lead to major changes in the expression of these genes (Fig. 2A). Recent studies using purified β-cells or β-cell lines provided evidence that IR is involved in glucose regulation of insulin gene transcription, the secreted insulin acting on IR present on the β-cell (5). This mechanism of glu-
cose action can account for the finding that IR tyrosine phos-
phorylation was induced in β-cell lines stimulated with either
insulin or IGF-1 (19). Also, the effect of glucose on insulin gene transcription in β-cell lines was abolished in the presence
of insulin-signaling inhibitors that block phosphatidylinositol
3-kinase, CaM kinase, and p70 s6k (5). Furthermore, over-
expression of IR in β-cell lines resulted in increased cellular levels of insulin mRNAs (6). In the hyperglycemic IR-deficient
pups, one might have expected to find higher levels of insulin
transcripts than in normoglycemic wild-type pups. Because total insulin transcripts detected in IR-deficient and wild-
type pups were comparable, this suggests that transcriptional
activation of the insulin gene by high glucose might

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FIG. 1. Immunocytochemical analysis of pancreas. A: Pancreatic islets from IR-deficient and wild-type pancreata stained using antibodies against insulin, glucagon, somatostatin, and PP to detect β-, α-, δ-, and
PP cells, respectively. B: Quantification of β-, α-, δ-, and PP cells (from top to bottom) in pancreata from IR-deficient and wild-type mice. The numbers are expressed in relation to total area of the
counted tissue sections. Results are shown as means ± SE for four ani-
mals of each genotype. Bar = 40 µm.
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require the presence of IR on β-cells. To further explore this issue, we plan to achieve transgenic reconstitution of IR gene expression in β-cells in IR-deficient mice, which we also expect to be hyperglycemic, to see whether and to what extent the level of insulin transcripts can be increased.

We further analyzed the expression of PDX-1, a key regulator of insulin gene transcription and the proposed mediator of the glucose responsiveness of insulin gene expression (20). In parallel with the equivalence of total insulin transcripts found in normal and mutant mice, PDX-1 gene expression was also unchanged in IR-deficient pups (Fig. 2B). PDX-1 has also been suggested to regulate the expression of other β-cell-specific genes, like those for the glucose-sensing proteins GCK and GLUT2 (1). RT-PCR revealed normal levels of GCK transcripts in hyperglycemic newborn mice lacking IR (Fig. 2B). This result is in agreement with an in vitro study showing no effect of glucose on GCK gene transcription in β-cells (21). In contrast, the RNA levels of GLUT2 were slightly increased in 3- to 4-day-old pups lacking IR (Fig. 2B), thereby confirming studies showing that hyperglycemia increases GLUT2 gene expression in β-cells (22).

In line with the similar levels of glucagon transcripts observed in the mutant and wild-type mice (Fig. 2A), serum glucagon levels in IR-deficient pups and their nondiabetic littermates were also comparable (Fig. 3). Thus, downregulation of glucagon secretion, which normally takes place under hyperinsulinemic and hyperglycemic conditions, was not seen in IR-deficient mice. This suggests a possible involvement of IR in the regulation of α-cell function. In the absence of counterregulatory insulin signaling, the normal serum glucagon levels must result in sustained glucagon action in the liver, leading to glycogen breakdown and increased ketogenesis.

**IR deficiency and hyperinsulinemia.** Because IR-deficient mice become both hyperglycemic and hyperinsulinemic, it is interesting to understand the mechanism leading to hyperinsulinemia in these mutants, i.e., lack of clearance versus increased insulin secretion from β-cells. It has been documented that insulin is eliminated from the circulation upon binding to IR in target tissues (23); therefore, it is conceivable

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**FIG. 2.** RT-PCR analysis of pancreatic gene expression. **A**: Analysis of transcripts for insulin 1/insulin 2, glucagon (Glu), somatostatin (Som), and PP. β-Actin (Act) mRNA was coamplified as control. Lower panel shows quantitative analysis. **B**: Analysis of the transcripts for PDX-1, GCK, and GLUT2. TBP mRNA was coamplified as control. Lower panel shows quantitative analysis. The quantitative data are presented as the ratio of the band intensities of the specific gene product in relation to internal controls. All RT-PCR reactions shown were performed using total RNA from IR-deficient and wild-type 3- to 4-day-old mice and the products were analyzed by Southern blotting. The ratio found with RNA from wild-type samples (stage P2; not shown) was normalized to 1. The sizes of the various PCR products correspond to 71 bp (insulin 1), 112 bp (insulin 2), 170 bp (glucagon), 165 bp (somatostatin), 150 bp (PP), 243 bp (β-actin), 275 bp (PDX-1), 162 bp (GCK), 150 bp (GLUT2), and 233 bp (TBP). Data are shown as means ± SE from triplicate assays.

**FIG. 3.** Serum glucagon levels. Glucagon was measured by RIA in serum pools from 2- to 4-day-old wild-type (n = 9) and IR-deficient (n = 8) pups. Serum from nondiabetic heterozygous IR knockout pups was pooled with that from wild-type mice. Data are given as means ± SE.

**FIG. 4.** Pancreatic insulin content. Insulin content was measured by RIA in pancreatic extracts prepared from wild-type and IR-deficient pups at 2 (P2) or 3–4 days (P3–4). Each extract was prepared from a pool of five pancreata. Data are given as means ± SE of four independent extracts for each genotype and age.
that reduced insulin clearance contributes to hyperinsulinemia in IR-deficient mice. However, and more importantly, insulin action through IR was also reported to play an active role in insulin secretion by β-cells (7). This issue was further investigated by generating mice with a β-cell–specific knockout of the IR gene (βIRKO). It was shown that insulin secretion in response to glucose was impaired in these mutants (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4).

We thus determined the total insulin content in pancreatic extracts from our hyperinsulinemic IR-deficient pups by RIA. Clearly, there exists a significant depletion of pancreatic insulin in mice lacking IR compared with wild-type controls (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4).

In addition, βIRKO mice become hyperinsulinemic with age. We thus determined the total insulin content in pancreatic extracts from our hyperinsulinemic IR-deficient pups by RIA. Clearly, there exists a significant depletion of pancreatic insulin in mice lacking IR compared with wild-type controls (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4). This depletion of insulin was greater at postnatal days 3–4, when the animals had been hyperglycemic for longer than 1 day (Fig. 4).

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