Compensatory Responses in Mice Carrying a Null Mutation for \( \text{Ins1} \) or \( \text{Ins2} \)

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Intrauterine growth retardation and postnatal acute diabetes result from insulin deficiency in double homozygous null mutants for \( \text{Ins1} \) and \( \text{Ins2} \) (Duvillée B, et al., Proc Natl Acad Sci U S A 94:5137–5140, 1997). The characterization of single homozygous null mutants for \( \text{Ins1} \) or \( \text{Ins2} \) is described here. Neither kind of mutant mice was diabetic. Immunocytochemical analysis of the islets showed normal distribution of the endocrine cells producing insulin, glucagon, somatostatin, or pancreatic polypeptide. Analysis of the expression of the functional insulin gene in \( \text{Ins1}^{+/−} \) or \( \text{Ins2}^{+/−} \) mice revealed a dramatic increase of \( \text{Ins1} \) transcripts in \( \text{Ins2}^{+/−} \) mutants. This compensatory response was quantitatively reflected by total pancreatic insulin content similar for both types of mutants and wild-type mice. Moreover, both mutants had normal plasma insulin levels and normal glucose tolerance tests. The determination of \( \beta \)-cell mass by morphometry indicated \( \beta \)-cell hyperplasia in the mutant mice. The \( \beta \)-cell mass in \( \text{Ins2}^{+/−} \) mice was increased almost threefold, which accounts for the increase of \( \text{Ins1} \) transcripts in \( \text{Ins2}^{+/−} \) mutants. This study thus contributes to evaluate the potential of increasing the \( \beta \)-cell mass to compensate for low insulin production. Diabetes 50 (Suppl. 1): S150–S153, 2001

Type 2 diabetes is characterized by both insulin resistance and \( \beta \)-cell failure. A number of studies suggest that insulin resistance does not result in overt diabetes as long as the \( \beta \)-cells are able to secrete increasing amounts of insulin (1). Major effort has been devoted to understanding the molecular mechanisms of transcriptional regulation of the insulin gene by glucose or other nutrients (2–4). The proliferation potential of the \( \beta \)-cells was clearly demonstrated in the adult hamster by wrapping the head of the pancreas with cellophane (5). A transient important increase in \( \beta \)-cell mass was also reported in rats during gestation (6). Moreover, it was shown in the rat that a 24-h glucose infusion resulted in significant increase of the \( \beta \)-cell mass (7,8).

In recent years, pronuclear injection and knockout transgenic approaches have been extensively applied to study the effects of manipulating the expression of genes involved in \( \beta \)-cell development and insulin gene expression, such as the transcription factor PDX-1, or proteins playing a crucial role in insulin secretion, such as glucokinase or GLUT2 (9,10). The role of the insulin receptor or the type 1 receptor for IGFs in \( \beta \)-cell growth and function was also investigated (11). The ability to increase \( \beta \)-cell mass to overcome insulin resistance was very convincingly demonstrated in knockout mice for insulin receptor substrate (IRS)-1 (12,13), an intracellular mediator of insulin signaling, whereas the development of overt diabetes in IRS-2-deficient mice was accompanied by a reduced \( \beta \)-cell mass (14).

We previously reported the phenotypic alterations resulting from total lack of insulin in double homozygous null mutant mice for the two nonallelic insulin genes (\( \text{Ins1} \) and \( \text{Ins2} \)) (15). The single \( \text{Ins1}^{+/−} \) or \( \text{Ins2}^{+/−} \) mutants were viable and fertile and did not present with major metabolic disorders. These mutants represent interesting tools with which to investigate the compensatory responses that may be operating in these mutant mice carrying only one functional insulin gene.

**RESEARCH DESIGN AND METHODS**

**Animals.** The generation of knockout mice for \( \text{Ins1} \) or \( \text{Ins2} \) was previously described (15,16). The experiments were performed using mutant and wild-type (wt) 129/Sv male mice usually at the age of 2–4 months. Insulin genes have been disrupted using two strategies: either by inserting a neomycin phosphotransferase (neo) gene cassette at the \( \text{Ins1} \) (\( \text{Ins1}^{+/−} \) mutants) or \( \text{Ins2} \) (\( \text{Ins2}^{+/−} \)–/– mutants) locus or by inserting the lacZ gene under control of the \( \text{Ins2} \) gene promoter together with the neo gene cassette (\( \text{Ins2}^{+/−} \)–/– mutants). Most of the experiments with \( \text{Ins2}^{−/−} \) mice were performed using the \( \text{Ins2}^{+/−} \)–/– mutants. \( \text{Ins2}^{−/−} \)–/– mutants were also included in some analyses and gave results comparable to those obtained with \( \text{Ins2}^{+/−} \)–/– mutants.

**Immunocytochemistry and morphometry.** Pancreases from \( \text{Ins1}^{−/−} \) or \( \text{Ins2}^{−/−} \) adult mice were fixed by immersion in 10% formaldehyde. Immunocytochemistry was performed on paraffin sections using rabbit polyclonal antibodies against C-peptide 1, C-peptide 2 (provided by O.D. Madsen), glucagon (Eurodiagnostica, Malmö, Sweden), somatostatin (Novocasta, Newcastle Upon Tyne, U.K.), or pancreatic polypeptide (PP) (Peninsula Labs, San Carlos, CA). Goat anti-rabbit serum coupled with peroxidase (Immunotech, Marseille, France) was used as the secondary antibody for antibodies against C-peptide. For glucagon, sections were incubated with swine anti-rabbit serum (Dako, Carpinteria, CA) and then with rabbit peroxidase anti-peroxidase (Dako). For antibodies against somatostatin and PP, goat anti-rabbit serum coupled with biotin (Immunotech) was used, followed by incubation with avidin-peroxidase. All reactions were revealed with diaminobenzidine (DAB). Sections were counterstained with hematoxylin and mounted.

Morphometry was performed with wt and mutant 129/Sv male mice at the age of 7–11 weeks. Pancreases were excised and weighed, and splenic parts were
fixed in Bouin’s solution and embedded in paraffin. Ten serial sections (7 μm) separated by an interval of 280 μm were immunostained for insulin. Sections were also immunostained with a guinea pig anti-insulin antibody (Dako) and then with rabbit anti–guinea pig IgG coupled with peroxidase (Dako). The antibody-peroxidase conjugates were revealed with DAB using a peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA). The areas of pancreatic sections and insulin-positive cells were measured using a microscope connected through a video camera to a computer using the software Biocom VisioLab 1 000 (Biocom, Les Ulis, France). The β-cell mass was calculated by multiplying the percentage of the areas of pancreas sections occupied by insulin-positive cells by the weight of the organ. Sections stained for insulin were counterstained with hematoxylin to determine the mean area of β-cells. The surface of groups of β-cells in islets, determined as above, was divided by the number of nuclei counted. Statistical significance was determined by analysis of variance using a StatView software package.

Reverse transcription–polymerase chain reaction analysis of Ins1/Ins2 transcripts. Total pancreatic RNA was prepared either from wt, Ins1−/−, or Ins2−/− mice or from the body of wt or Ins2−/− embryos at day E14.5 as described (15). Briefly, after reverse transcription (RT) of the insulin mRNAs, a unique primer pair was used for the polymerase chain reaction (PCR) amplification. The PCR products were digested with MstI and analyzed by Southern blotting using a single [32P]-labeled oligonucleotide probe, which revealed a fragment of 71 and 112 bp for Ins1 and Ins2, respectively (15). The primer set and the [32P]-labeled oligonucleotide probe used to analyze β-actin mRNA were previously specified (15).

Extraction of pancreatic proteins. Pancreatic extracts were prepared by homogenizing entire pancreas in acid/neutral mixture, and insulin was partially purified as described (17).

Western immunoblot analyses. Pancreatic extracts (100 μg) were run on 12.5% polyacrylamide gel in Tris-glycine buffer, pH 8.9, and electrophoresed onto nitrocellulose filters in the same buffer. The filters were soaked in 4% bovine serum albumin in 10 mmol/l phosphate buffer, pH 7.4, for 1 h at 40°C followed by photolinkage as described (15). The blots were washed twice at room temperature with phosphate-buffered saline containing 0.1% Tween 20 (PBST), washed once with PBST containing Blocking Reagent (Boehringer-Roche, Meylan, France), and incubated overnight at 4°C with guinea pig anti-insulin serum (Sigma, St. Louis, MO) in phosphate buffer. The blots were washed again with PBST and incubated with Blocking Reagent containing peroxidase-linked protein A (Amersham, Les Ulis, France) at room temperature. The filters were washed four times with PBST; incubated with enhanced chemiluminescence Western detection reagents (Amersham), and exposed to X-ray films.

Determination of insulin. Serum insulin levels or immunoreactive insulin content in pancreatic extracts were measured using a radioimmunoassay (RIA) kit (ICN Pharmaceuticals, Orsay, France).

Glucose tolerance tests. Glucose tolerance tests were performed in 4- to 6-month-old wt and Ins2−/− mice by intraperitoneal injection of 2 g/kg glucose (17) given after overnight fasting. Blood samples were collected from orbital sinus at the indicated times, and glucose levels were determined using a Glucometer II (Bayer Diagnostics, Puteaux, France).

RESULTS AND DISCUSSION

Endocrine pancreas in knockout mice for Ins1 or Ins2. Pancreatic sections were first stained with anti–C-peptide 1 or anti–C-peptide 2 antibodies specific for proinsulin 1 and proinsulin 2, respectively. As expected, positive staining was observed in Ins2−/− or Ins2−/− mice and for proinsulin 2 in Ins1−/− mice (Fig. 1). Immunocytochemical staining of the pancreatic sections was also performed using antibodies against glucagon, somatostatin, and PP (Fig. 1). The results indicate normal distribution of β-cells, α-cells, δ-cells, and PP cells in the islets in both types of mutants. Therefore, the overall islet morphology and the distribution of various endocrine cell types were not altered by targeted disruption of either Ins1 or Ins2.

Expression of the functional insulin gene in Ins1−/− or Ins2−/− mice. The expression of the active insulin gene copies in the homozygous null mutants for Ins1 or Ins2 was first analyzed by RT-PCR. As shown in Fig. 2A, Ins2 transcripts in Ins1−/− mice remained comparable to those of wt controls.

In contrast, a dramatic increase in Ins1 transcripts was observed in Ins2−/− mice, compensating for the absence of Ins2 transcripts, which represent the majority of total insulin transcripts in wt controls. To examine whether this compensation is occurring during fetal life, total RNA was extracted from the body of Ins2−/− embryos at day E14.5 and subjected to RT-PCR. The results presented in Fig. 2B show comparable amounts of Ins1 transcripts in Ins2−/− and wt controls, indicating that the compensation must occur during late gestation or postnatally. In this respect, it is interesting that transient glycosuria is often detected in Ins2−/− pups within the first week after birth. Finally, as expected, no insulin gene transcripts were detected in double homozygous null mutants for Ins1 and Ins2.

The expression of the functional insulin gene in Ins1−/− or Ins2−/− mice was also examined at the protein level. The results of Western blots are presented in Fig. 3. As expected, only insulin 2 was detected in Ins1−/− and insulin 1 in Ins2−/− mice. Plasma insulin levels in Ins1−/−, Ins2−/−, and wt mice were...
comparable (Table 1). The total immunoreactive insulin content in the pancreas from both kinds of mutants was determined by RIA using pancreatic extracts; the values were similar for 2- to 4-month-old animals in all groups (Table 1). Thus, the increase of \( \text{Ins}1 \) transcripts in \( \text{Ins}2 \)–/– mice described above leads to quantitative compensation at the protein level. In addition, \( \text{Ins}2 \)–/– mice showed glucose tolerance tests similar to those in wt animals (Fig. 4). To address the question of whether the compensatory increase of \( \text{Ins}1 \) transcripts in \( \text{Ins}2 \)–/– mice is accompanied by an increase in the \( \beta \)-cell mass, morphometric analysis of the pancreas in \( \text{Ins}1 \) or \( \text{Ins}2 \) knockout mice was performed.

**\( \beta \)-Cell mass in \( \text{Ins}1 \)–/– or \( \text{Ins}2 \)–/– mice.** Pancreatic sections were stained with anti-insulin antibody to determine the

![FIG. 2. RT-PCR analysis of insulin gene expression. A: Amplification of transcripts for insulin 1/insulin 2 using total pancreatic RNA from wt, \( \text{Ins}1 \)–/–, \( \text{Ins}2 \)–/–, and double homozygous null mutant mice. B: Amplification of transcripts for insulin 1/insulin 2 using total RNA from the bodies of wt and \( \text{Ins}2 \)–/– embryos at day E14.5. All RT-PCR products were analyzed by Southern blotting, and the autoradiograms are presented. \( \beta \)-Actin mRNA was coamplified as an internal control. pBR322/Hpa II was used as a size marker.](image)

![FIG. 3. Western immunoblot analysis of pancreatic protein extracts from wt, \( \text{Ins}1 \)–/–, and \( \text{Ins}2 \)–/– mice. The primary anti-insulin antibody used detected both insulin 1 and insulin 2 in wt controls.](image)

![FIG. 4. Intraperitoneal glucose tolerance tests. The mean values obtained for five wt and six \( \text{Ins}2 \)–/– mice are presented.](image)

### TABLE 1

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<tr>
<th>Insulinemia (pg/ml)</th>
<th>Pancreatic insulin content (ng/mg pancreas)</th>
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<tbody>
<tr>
<td>wt</td>
<td>374.42 ± 76.16</td>
</tr>
<tr>
<td>( \text{Ins}1 )–/–</td>
<td>374.87 ± 55.26</td>
</tr>
<tr>
<td>( \text{Ins}2 )–/–</td>
<td>370.68 ± 53.39</td>
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Data are means ± SE. For insulinemia, \( n = 5, 6, \) and 11, for wt, \( \text{Ins}1 \)–/–, and \( \text{Ins}2 \)–/–, respectively. For pancreatic insulin content, \( n = 6, 6 \) and 8, for wt, \( \text{Ins}1 \)–/–, and \( \text{Ins}2 \)–/–, respectively. Serum insulin levels were determined after an overnight fast.
that compensation by increasing the β-cell mass in these mutants takes place, at least partly, within the first few days when these pups are probably hypoinsulinemic. It therefore appears that the lack of insulin or low insulin production could lead to β-cell hyperplasia. Concerning the possible mechanisms leading to increased β-cell mass in Ins1+/− or Ins2−/− mutants, it should be recalled that the insulin receptor (IR) can also bind IGF-II (19). In conditions where the competition with insulin is absent or low, IGF-II action through the IR might be favored, which could elicit more efficient mitogenic effects (20). In line with this hypothesis, β-cell hyperplasia was not detected in homozygous IR-deficient newborn mice (21,22). Further analysis of β-cell neogenesis, replication/proliferation, and apoptosis will shed some light on the molecular mechanisms responsible for increased β-cell mass in Ins1 or Ins2 knockout mutants. It would also be interesting to examine the time course of the appearance of β-cell hyperplasia in Ins2−/− mutants to see whether the β-cell mass continues to increase throughout adult life.

The importance of increasing the β-cell compartment to overcome insulin resistance by secreting higher amounts of insulin was clearly demonstrated from the analysis of knock-out mice for IRS-1 or IRS-2 (12–14). The study of Ins1 or Ins2 knockout mice now shows that increases in the β-cell compartment can also take place to overcome low insulin production by β-cells.

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REFERENCES