Dense Core Vesicle Proteins IA-2 and IA-2β
Metabolic Alterations in Double Knockout Mice
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IA-2 and IA-2β are members of the transmembrane protein tyrosine phosphatase (PTP) family located in dense core vesicles of neuroendocrine cells, including the β-cells of pancreatic islets. In the present study, by mating C57BL/6Nci IA-2β−/− mice with IA-2β−/− mice, we generated double knockout mice (IA-2β−/−/IA-2β−/−) to study the effect of the combined deletion of these two proteins on insulin secretion and blood glucose levels. The double knockout mice appeared healthy at birth and showed normal growth and development. Histological examination and immunostaining for insulin, glucagon, somatostatin, and pancreatic polypeptide revealed no difference between the double knockout and wild-type mice. Nonfasting blood glucose and insulin levels also were within the normal range. However, compared with the wild-type mice, the double knockout mice showed glucose intolerance and an absent first-phase insulin release curve. No evidence of insulin resistance was observed nor were there alterations in fasting blood glucose, insulin, or leptin levels in the double knockout mice maintained on a high-fat diet compared with the wild-type mice maintained on the same diet. In addition, to determine whether the combined deletion of IA-2 and IA-2β played any role in the development of diabetes in NOD mice, we generated double knockout mice on the NOD/LtJ background. The incidence of diabetes in these mice was not significantly different than that in the wild-type mice. Taken together, our experiments show that the dense core vesicle proteins IA-2 and IA-2β, alone or in combination, are involved in insulin secretion, but neither alone nor in combination are they required for the development of diabetes in NOD mice. Diabetes 54 (Suppl. 2):S46–S51, 2005

IA-2 is a major autoantigen in type 1 diabetes (1,2). Autoantibodies to IA-2 appear years before the development of clinical disease and are being widely used to identify individuals at high disease risk. Because most sera from diabetic patients that react with IA-2 also react with IA-2β, IA-2 rather than IA-2β has been used more frequently as the test antigen in clinical studies (3). IA-2 (also known as ICA512) and IA-2β (also known as phogrin) are members of the transmembrane protein tyrosine phosphatase (PTP) family (4–7). Because of mutations at critical sites in the protein tyrosine phosphatase core domain, both proteins are enzymatically inactive with standard substrates. Site-directed mutagenesis, however, can restore enzyme activity (8). Human IA-2 and IA-2β are located on two different chromosomes: 2q35 and 7q36, respectively (2). The intracellular domain of IA-2 and IA-2β are 74% identical, whereas their extracellular domains are only 26% identical. Electron microscopic studies revealed that IA-2 and IA-2β are located as transmembrane proteins in dense core vesicles (DCVs) and are expressed in many neuroendocrine cells (particularly brain, pituitary, pancreatic islets, and adrenals) throughout the body (4,6,9,10). The function of IA-2 and IA-2β, however, has remained unclear. Recently, by gene targeting, we succeeded in developing mouse lines deficient in IA-2 (IA-2β−/−/IA-2β−/−) or IA-2β (IA-2β−/−/IA-2β−/−) (11,12). Examination of these animals revealed normal blood glucose and insulin levels, but abnormal glucose tolerance tests and impaired insulin secretion.

The current experiments were initiated to generate double knockout (DKO) (IA-2−/−/IA-2β−/−) mice and to study the metabolic changes produced by the loss of both of these dense core vesicle proteins.

RESEARCH DESIGN AND METHODS

Generation of IA-2/IA-2β double knockout mice. Targeted disruption of the individual IA-2 and IA-2β genes was described previously (11,12). C57BL/6Nci eighth generation IA-2−/− mice were mated with C57BL/6Nci fourth generation IA-2β−/− mice and the double heterozygous outcomes were interbred to generate IA-2−/−/IA-2β−/− mice. To establish IA-2−/−/IA-2β−/−/NOD/LtJ congenic mice, NOD/LtJ tenth generation IA-2−/− mice were mated with NOD/LtJ fifth generation IA-2β−/− mice. NOD/LtJ congenic IA-2−/−/IA-2β−/− mice were intercrossed to generate IA-2−/−/IA-2β−/− and IA-2β−/−/IA-2β−/− mice. In the same way, IA-2−/−/IA-2β−/− and IA-2β−/−/IA-2β−/− mice on the NOD background were obtained by intercrossing of IA-2−/−/IA-2β−/−/NOD/LtJ congenic mice. Mice were bred and maintained under specific pathogen-free conditions. All procedures were approved by our Institutional Animal Care and Use Committee.

Western blot analysis. Antibodies to mouse IA-2 and IA-2β were prepared as described previously (11,12). Islets from IA-2−/−/IA-2β−/− or IA-2β−/−/IA-2β−/− mice were isolated using Percoll density gradient separation and hand-picked under a stereomicroscope. Equivalent size and numbers of isolated islets were sonicated. Proteins separated by SDS-PAGE were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Blots were detected by SuperSignal (Pierce, Rockford, IL).

Histology. Pancreata were collected in 10% neutral-buffer formalin and processed for paraffin embedding. Sections were stained with hematoxylin and eosin. Sections of paraffin-embedded pancreas were incubated with antibodies to insulin, glucagon, somatostatin, and pancreatic polypeptide (DAKO, Carpinteria, CA) followed by biotin-conjugated secondary antibody and streptavidin-peroxidase.

Blood tests. Nonfasting and fasting blood glucose were measured with a One Touch II blood glucose monitor (LifeScan, Milpitas, CA). Serum insulin levels were determined by ultrasensitive insulin enzyme immunoassay kits using mouse insulin standards (Alpco Diagnostics, Woburn, NH). Mice (14 months of age) were fasted overnight for glucose tolerance and insulin secretion tests. Animals were injected intraperitoneally with 2 g/kg body wt of glucose (glucose tolerance tests) or 3 g/kg body wt of glucose or 1 g/kg body wt of L-arginine (insulin secretion). For glucose tolerance tests, blood was drawn at
RESULTS

IA-2−/−/IA-2B−/− mice were generated by mating IA-2+/− with IA-2B+/− mice and the effect of the double knockout on insulin secretion and glucose metabolism was examined. The genotypes were determined by tail DNA PCR and confirmed by Western blot using antibodies specific for IA-2 and IA-2β. As seen in Fig. 1A, strong IA-2 and IA-2β bands were found in islet cell lysates of IA-2+/+/IA-2B+/+ mice, but were absent in the islet cell lysates of IA-2−/−/IA-2B−/− mice. Immunohistochemical analysis (Fig. 1B) using IA-2- and IA-2β-specific antibodies similarly showed the expression of IA-2 and IA-2β proteins in the islets of IA-2+/+/IA-2B+/+ mice, but not in the islets of IA-2−/−/IA-2B−/− mice. Immunohistochemical studies (Fig. 1C) failed to reveal any gross abnormality in the appearance of the IA-2−/−/IA-2B−/− pancreatic islets (Fig. 1C-a) or the staining pattern for insulin (Fig. 1C-b), glucagon (Fig. 1C-c), somatostatin (Fig. 1C-d), or pancreatic polypeptide (Fig. 1C-e) compared with IA-2+/+/IA-2B+/+ islets (not shown).

The effect of the DKO on body weight and nonfasting blood glucose and insulin levels is seen in Fig. 2. No significant difference was found in the IA-2−/−/IA-2B−/− male and female mice compared with the IA-2+/+/IA-2B+/+ male and female mice. In contrast, glucose tolerance tests were significantly elevated in IA-2−/−/IA-2B−/− mice compared with the IA-2+/+/IA-2B+/+ and IA-2+/−/IA-2B−/− mice (Fig. 3A). First-phase insulin secretion, after glucose stimulation, was absent in the IA-2−/−/IA-2B−/− mice compared with the robust response in the IA-2+/+/IA-2B+/+ mice (Fig. 3B). The remote possibility that blood glucose did not increase as rapidly or as much in DKO as in the wild-type mice was tested by measuring blood glucose at 5 min after injection (IA-2+/+/IA-2B+/+, 174 ± 15 mg/dl; IA-2−/−/IA-2B−/−, 167 ± 9 mg/dl). No significant difference was found.

In contrast to the absence of first-phase insulin secretion after glucose stimulation in the IA-2−/−/IA-2B−/− mice, there was no statistically significant difference in insulin secretion after arginine stimulation in the IA-2−/−/IA-2B−/− mice compared with the IA-2+/+/IA-2B+/+ mice (Fig. 4A). Although not a consistent finding, in some experiments, the fasting insulin levels were higher in the IA-2−/−/IA-2B−/− mice than in the IA-2+/+/IA-2B−/− mice (Fig. 3B and legend to Fig. 4A). To see if the deletion of both IA-2 and IA-2β might lead to insulin resistance, mice were injected with insulin and blood glucose levels were followed over the next 60 min. As seen in Fig. 4B, the decrease in glucose occurred at approximately the same rate in the IA-2−/−/IA-2B−/− mice as in the IA-2+/+/IA-2B−/− mice, ruling out insulin resistance.

To determine whether a high-fat diet would enhance the
glucose or insulin abnormalities resulting from the deletion of IA-2 and IA-2\(\beta\), 5-week-old mice were placed on a high-fat diet. On normal food, the body weights of the IA-2\(+/-\)/IA-2\(+/-\) and IA-2\(+/-\)/IA-2\(-/-\) mice were indistinguishable (Fig. 2A and B). In contrast, at the end of 15 weeks on a high-fat diet, the male and female IA-2\(+/-\)/IA-2\(+/-\) and IA-2\(+/-\)/IA-2\(-/-\) mice were indistinguishable (Fig. 2A and B). In contrast, at the end of 15 weeks on a high-fat diet, the male and female IA-2\(+/-\)/IA-2\(+/-\).

FIG. 2. Body weight, glucose, and insulin levels of IA-2\(+/-\)/IA-2\(+/-\) (■) and IA-2\(-/-\)/IA-2\(-/-\) (○) mice (n = 7–13 mice per group). Body weight at 12 weeks (A) and 20 weeks (B) of age is shown. Nonfasting blood glucose (C) and insulin (D) levels at 4 months of age are also shown.

FIG. 3. A: Glucose tolerance test. After overnight fasting, 4-month-old male and female IA-2\(+/-\)/IA-2\(+/-\) (●), IA-2\(+/-\)/IA-2\(-/-\) (▲), and IA-2\(-/-\)/IA-2\(-/-\) (○) mice were injected intraperitoneally with D-glucose (2 g/kg body wt) and blood glucose levels were measured at different times thereafter (n = 6–12 mice per group). Results are expressed as means ± SE. ¶P < 0.01, IA-2\(-/-\)/IA-2\(-/-\) vs. IA-2\(+/-\)/IA-2\(+/-\). *P < 0.05, **P < 0.01, IA-2\(-/-\)/IA-2\(-/-\) vs. IA-2\(+/-\)/IA-2\(-/-\).

B: Insulin secretion test. After overnight fasting, insulin secretion in response to intraperitoneal (IP) D-glucose (3 g/kg body wt) in 4-month-old IA-2\(+/-\)/IA-2\(+/-\) (●) and IA-2\(-/-\)/IA-2\(-/-\) (○) mice was measured over 20 min (n = 8–15 mice per group). Values are the mean ± SE. *P < 0.05, **P < 0.01.
2B⁺/⁺ mice gained ~29% more weight than the IA-2⁻/⁻/IA-2β⁻/⁻ mice (Fig. 5A and B) even though food intake did not seem to be significantly different between the two groups (Fig. 5C). Fasting blood glucose, insulin, and leptin levels also were not significantly different between the IA-2⁺/⁺/IA-2β⁺/⁺ and IA-2⁻/⁻/IA-2β⁻/⁻ mice (Fig. 5D, E, and F), although there were somewhat lower leptin levels in the IA-2⁻/⁻/IA-2β⁻/⁻ mice compared with the IA-2⁺/⁺/IA-2β⁺/⁺ mice.

To determine if the deletion of both IA-2 and IA-2β would influence the development of diabetes in NOD mice, the most widely studied animal model for human type 1 diabetes (13), the incidence of diabetes in NOD IA-2⁻/⁻/IA-2β⁻/⁻ mice was compared with NOD IA-2⁺/⁺/IA-2β⁺/⁺ mice. The animals were genotyped and absence of IA-2 and IA-2β protein expression was confirmed by Western blot analysis using brain tissue (Fig. 6, inset). Approximately 45% of IA-2⁺/⁺/IA-2β⁺/⁺, 27% of IA-2⁻/⁻/IA-2β⁺/⁺, and 29% of IA-2⁻/⁻/IA-2β⁻/⁻ female NOD mice spontaneously developed diabetes by 40 weeks of age (Fig. 6). Although the percentage of IA-2⁺/⁺/IA-2β⁺/⁺ mice that developed diabetes was somewhat higher than that of the IA-2⁻/⁻/IA-2β⁺/⁺ or the IA-2⁻/⁻/IA-2β⁻/⁻ mice, the differences were not statistically significant, arguing that IA-2 and IA-2β alone (12,14) or in combination are not required for the development of diabetes in NOD mice.

**DISCUSSION**

Previously, we showed that the blood glucose and insulin levels of single knockout mice remained within the normal range (11,12). The current study shows that DKO mice similarly maintained their blood glucose and insulin levels within the normal range. Glucose tolerance tests, however, appeared to be more severely impaired in the DKO mice than in the single IA-2β (Fig. 3) or IA-2 knockout mice (11). This suggests that there might be a mild to moderate additive inhibitory effect on the capacity of DCVs to secrete insulin by knocking out both proteins. In fact, the first-phase glucose-induced insulin release curves were absent or more depressed than observed previously in single knockout mice (11,12). In contrast to the depressed glucose-dependent insulin release in DKO mice, arginine-dependent insulin release, which is thought to act, at least in part, by a mechanism independent of that used by glucose (15,16), was preserved, suggesting that the arginine pathway is not as heavily dependent on the presence of IA-2 or IA-2β. As anticipated, mice in which...
both DCV proteins were knocked out showed no evidence of insulin resistance, supporting the contention that the glucose intolerance was due to impaired insulin secretion and not impaired glucose disposal. The possibility that weight gain might alter the metabolic profile of DKO mice was examined by putting the mice on a high-fat diet. Somewhat to our surprise, the DKO mice gained considerably less weight than the wild-type mice, but the glucose, insulin, and leptin levels of the two groups were not significantly different. The reason for the failure to gain weight is not clear, but the knockout of IA-2 and IA-2β might affect the secretion of growth-regulating factors in the DCVs of other neuroendocrine cells.

A number of possibilities exist as to how deletion of IA-2 and/or IA-2β might suppress glucose-induced insulin release (17–20). These range from effects on the biogenesis of DCVs, to the stability of DCVs, to trafficking, docking, priming, fusion, and exocytosis. IA-2/IA-2β may exert a
the NOD DKO experiments add support to the argument that type 1 diabetes have autoantibodies to IA-2 or IA-2β. On the human level it is well known that not all individuals have both IA-2 and IA-2β, diabetes develops spontaneously in NOD mice in which cyclophosphamide. The present experiments show that treatment of diabetes in NOD mice (12,14). In those experiments, diabetes was induced by treatment with cyclophosphamide. The present experiments show that diabetes develops spontaneously in NOD mice in which both IA-2 and IA-2β have been knocked out. Because at the human level it is well known that not all individuals with type 1 diabetes have autoantibodies to IA-2 or IA-2β, the NOD DKO experiments add support to the argument that neither of these proteins alone or in combination is required for the development of diabetes. Instead, the autoimmune response to IA-2 and IA-2β may be a consequence of and perhaps a contributor to the disease.

In conclusion, the single and double knockout mice have provided us with important information about the role of the DCV proteins IA-2 and IA-2β in insulin secretion and glucose intolerance. The NOD knockout mice have shown us that neither of these proteins is required for the development of diabetes. Still unanswered, however, is what makes these two proteins, and not the myriad of other β-cell proteins, autoantigens in type 1 diabetes.

REFERENCES