Islet amyloid polypeptide (amylin)-deficient mice develop a more severe form of alloxan-induced diabetes

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Mulder, Hindrik, Samuel Gebre-Medhin, Christer Betsholtz, Frank Sundler, and Bo Ahren. Islet amyloid polypeptide (amylin)-deficient mice develop a more severe form of alloxan-induced diabetes. Am J Physiol Endocrinol Metab 278: E684–E691, 2000.—To examine whether islet amyloid polypeptide (IAPP), other than through amyloid formation, may be of importance in diabetes pathogenesis, IAPP-deficient mice (IAPP−/−) were challenged with alloxan (day 0). Diabetes in IAPP−/− mice was more severe at day 35, indicated by greater weight loss; glucose levels were higher in alloxan-treated IAPP−/− mice, whereas insulin levels were lower, indicating a greater impairment of islet function. Accordingly, glucose levels upon intravenous glucose challenges at days 7 and 35 were consistently higher in alloxan-treated IAPP−/− mice. At day 35, insulin mRNA expression, but not β-cell mass, was lower in untreated IAPP−/− mice. Yet, upon alloxan administration, β-cell mass and numbers of β-cell-containing islets were significantly more reduced in IAPP−/− mice. Furthermore, they displayed exaggerated β-cell dysfunction, because in their remaining β-cells, insulin mRNA expression was significantly more impaired and the localization of glucose transporter-2 was perturbed. Thus the lack of IAPP has allowed exaggerated β-cell cytotoxic actions of alloxan, suggesting that there may be beneficial features of IAPP actions in situations of β-cell damage.

gene knockout; β-cell mass; insulin messenger ribonucleic acid; glucose transporter-2; glucose tolerance

ISLET AMYLOID POLYPEPTIDE (IAPP, also designated amylin) is a normal constituent of pancreatic β-cells in nearly all species examined so far (21). Given this conserved expression, as well as the regulation of IAPP expression (19) and secretion (14) by glucose, a regulatory role for IAPP in glucose homeostasis is likely. However, despite considerable efforts to clarify this issue, which include showing that IAPP inhibits insulin release (1) and peripheral glucose utilization (8), a clear physiological role of the peptide has yet to be revealed. Nevertheless, recent findings in mice with a targeted disruption of the IAPP gene (9) favor the idea that IAPP under normal conditions indeed acts in islets, inhibiting insulin release, and possibly also in the periphery, restraining insulin-stimulated glucose utilization. Thus the physiological relevance of the previously found metabolic actions of IAPP has been reinforced, and it appears likely that IAPP is an insulin counterregulatory β-cell hormone.

Previously, the debate on the role of IAPP in the pathogenic events leading to non-insulin-dependent diabetes mellitus (NIDDM) has focused on the amyloid-forming capacity of the peptide (13). Whether the metabolic effects or other actions of IAPP could also play a role in the pathogenesis of diabetes is not known. Nonetheless, perturbed glucose-stimulated insulin secretion and insulin resistance are both hallmarks of NIDDM, and the dual insulin-antagonistic effects of IAPP (1, 8) could contribute to both these aspects of the disease. Interestingly, under diabetic conditions in rodents, IAPP is overexpressed in islets compared with insulin (20), an overexpression that is matched by an increased ratio of IAPP to insulin for peptide content in the pancreas (18) and secretion (11). It is therefore conceivable that the insulin-antagonizing metabolic effects of IAPP will be pronounced by such an overexpression. This could be harmful in individuals at risk of developing NIDDM and further contribute to the development of the disease. Alternatively, the possibility also remains that overexpression of IAPP could be a beneficial adaptation to islet perturbations in the development of diabetes. In this scenario, there are several putative mechanisms by which IAPP could serve to protect β-cells under hyperglycemic and/or diabetic conditions; these include enhancement of islet microcirculation (28) and limitation of prolonged β-cell depolarization (31). To explore these issues, it would be desirable to examine the onset and course of experimental diabetes when the effects of IAPP are eliminated. The feasibility, however, of such studies is hampered by the lack of the identity of an IAPP receptor(s) and, consequently, reliable IAPP antagonists. However, the recent generation of IAPP-deficient mice (IAPP−/−) (9) allowed us to investigate whether a lack of IAPP will affect the development of diabetes. To this end, we used the

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β-cell-specific cytotoxic agent alloxan, a high dose of which induces permanent diabetes in mice.

MATERIALS AND METHODS

Experimental animals, glucose and insulin determinations, and tissue processing. As described elsewhere (9), by use of targeted mutagenesis in embryonic stem cells, the mouse IAPP gene was disrupted by deletion of a major part of exon 3, which encodes the mature peptide (7). The deficient IAPP expression in IAPP+/− mice was confirmed by the lack of exon 3 in mouse tail DNA subjected to Southern hybridization and by the lack of IAPP-like immunoreactivity in pancreatic islets (9) (see Fig. 5, C and D).

The mice were nonsibling F3-descendant males from one chimeric mouse. Age-matched (11–17 wk) IAPP+/− and their wild type (IAPP+/+) controls, respectively (day 0; n = 10 in each group), were given tail vein injections of alloxan monohydrate (Sigma, St. Louis, MO; 70 mg/kg, dissolved in 0.1 M citrate, pH 4.5) or saline. All animals were weighed once weekly. Plasma glucose levels were also determined once weekly in a retroorbital blood sample by use of the glucose oxidase method. At days 7 and 35, an intravenous glucose tolerance test (IVGTT) was performed. d-Glucose (1 g/kg) was injected into the tail vein of anesthetized mice, additional drugs were given intraperitoneally (midazolam, 0.4 mg/mouse; Dormicum, Hoffman-La Roche, Basel, Switzerland) + a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm, J. ansenn, Beerse, Belgium), and retroorbital blood samples were collected. Plasma insulin was determined once weekly by RIA; the insulin content was compared with a two-tailed Student’s t-test. A probability level of P < 0.05 was considered statistically significant.

RESULTS

Body weight and basal plasma glucose and insulin levels. The alloxan-treated IAPP−/− mice lost significantly more weight than the age-matched wild type controls (from 34.2 ± 1.0 to 26 ± 1.3 g vs. from 32 ± 1.0 to 30.2 ± 1.2 g; P < 0.001 for t-values), indicating that they were metabolically more severely affected by alloxan. As shown in Fig. 1A, this was further corroborated by the basal plasma glucose levels after alloxan treatment, which were more elevated in the IAPP−/− mice than in IAPP+/+ mice. Basal plasma insulin levels determined at the same time points (Fig. 1B) were consistently lower in the IAPP−/− mice after alloxan treatment compared with the alloxan-treated wild type mice, despite the higher glucose levels in the IAPP−/− mice, thus indicating a more extensive impairment of β-cell function. It should also be noted that there was a trend toward lower basal plasma insulin levels in IAPP−/− mice before alloxan treatment (Fig. 1B). A similar finding was made in additional untreated mice that were analyzed, here, basal plasma insulin levels were 165 ± 8 pmol/l in IAPP−/− mice vs. 191 ± 10 pmol/l in IAPP+/+ mice (P = 0.060).

Intravenous glucose tolerance test. To further explore the nature of the β-cell impairment, IVGTTs at days 7 and 35 after alloxan injection were performed (Fig. 2).
At both time points, the control IAPP<sup>+/−</sup> mice eliminated glucose faster than the IAPP<sup>−/−</sup> mice. This finding is in agreement with our previous data (9). In contrast, the alloxan-treated IAPP<sup>+/−</sup> mice had lost their enhanced elimination of glucose, compared with the alloxan-treated IAPP<sup>−/−</sup> mice; at both time points after alloxan treatment, the basal glucose level immediately before glucose injection was higher in the IAPP<sup>+/−</sup> mice and was still higher at 2 h compared with the IAPP<sup>−/−</sup> mice. Thus, taken together, the basal levels of glucose and insulin and the results from the glucose challenges demonstrate that alloxan-treated IAPP<sup>+/−</sup> mice display a more extensive impairment of β-cell function and, consequently, exaggerated diabetes compared with their wild type controls.

Insulin gene expression and storage. To examine the cellular events underlying the β-cell impairment, we evaluated insulin mRNA expression in islets, using quantitative in situ hybridization. In control mice, the mean OD of in situ hybridization labeling was significantly higher in IAPP<sup>+/+</sup> mice compared with IAPP<sup>−/−</sup> mice (Figs. 3 and 4). The lower expression of insulin in IAPP<sup>−/−</sup> mice suggests that insulin is synthesized at a lower rate in the absence of IAPP. To examine whether this reduced insulin mRNA expression correlates with reduced storage of the hormone, we determined the pancreatic insulin content in untreated IAPP<sup>+/−</sup> and IAPP<sup>−/−</sup> mice, age-matched with the alloxan-treated IAPP<sup>−/−</sup> and IAPP<sup>+/+</sup> mice and their controls in the

![Fig. 1. Basal glucose (A) and insulin levels (B) in control and alloxan-treated islet amyloid polypeptide (IAPP<sup>+/−</sup>) mice and their wild type controls (n = 10 in each group). Glucose and insulin levels at each time point were compared with a two-tailed t-test; *P < 0.05, ***P < 0.001.](http://ajpendo.physiology.org/)

![Fig. 2. Intravenous glucose tolerance test (1 g/kg) at days 7 (A) and 35 (B) in control and alloxan-treated IAPP<sup>+/−</sup> mice and their wild type controls (n = 10 in each group). In control mice, area under the curve (AUC) was calculated by the trapezoid model; in alloxan-treated mice such calculations were considered pointless because of the very high basal glucose levels. Control IAPP<sup>+/−</sup> mice eliminated glucose significantly faster at both time points; P < 0.001 and P = 0.004 at days 7 and 35, respectively.](http://ajpendo.physiology.org/)
study. Indeed, the pancreatic insulin content was lower in untreated IAPP\(^{-/-}\) mice than in their wild type controls (1.0 ± 0.1 vs. 1.4 ± 0.1 nmol/mg; \(P = 0.029\)).

After alloxan treatment, the mean OD of in situ hybridization labeling was significantly lower in the remaining \(\beta\)-cells in the IAPP\(^{+/+}\) and IAPP\(^{-/-}\) mice compared with their respective controls (Figs. 3 and 4). The insulin mRNA levels decreased to 43 and 55% of the untreated controls in the IAPP\(^{-/-}\) and IAPP\(^{+/+}\) mice, respectively, and the change was found to be significantly greater in IAPP\(^{-/-}\) mice. This indicates that \(\beta\)-cell function was more extensively perturbed by alloxan in IAPP\(^{-/-}\) mice than in their wild type controls. Moreover, the findings are in keeping with the more pronounced hyperglycemia/hypoinsulinemia and the more impaired glucose-stimulated insulin response in IAPP-deficient mice.

Immunocytochemistry. To further confirm the results of the analysis of insulin mRNA expression, we examined expression of islet-cell constituents at the protein level with immunocytochemistry. In control IAPP\(^{+/+}\) and IAPP\(^{-/-}\) mice, the number of islets and \(\beta\)-cells visibly appeared to be similar (Fig. 5, A, B, and D). As expected, IAPP-immunoreactive cells were found only in the wild type mice (Fig. 5, A and C). However, in the alloxan-treated IAPP\(^{-/-}\) mice, islets containing \(\beta\)-cells appeared to be scarcer and to harbor fewer such cells than in the alloxan-treated IAPP\(^{+/+}\) mice (Fig. 5, E and F).

We also examined islet expression of GLUT-2 in the mice, because it is considered to be responsible for the cellular uptake of alloxan into \(\beta\)-cells (23). Interestingly, whereas in both control IAPP\(^{+/+}\) and IAPP\(^{-/-}\) mice the expected localization of GLUT-2 immunoreactivity to the plasma membrane was seen (Fig. 6, A and B), most islets examined in the alloxan-treated IAPP\(^{-/-}\) mice displayed a diffuse GLUT-2 immunolabeling of the majority of the remaining islet cells (Fig. 6, C and D); such labeling, which suggests a cytoplasmic localization of the normally membrane-bound transporter, occurred only sporadically in the alloxan-treated IAPP\(^{+/+}\) mice.

Morphometry. To further evaluate the differential impact of alloxan in the IAPP\(^{+/+}\) and IAPP\(^{-/-}\) mice, we determined their numbers of \(\beta\)-cell-containing islets and \(\beta\)-cell mass. The lack of IAPP appeared not to have affected the number of such islets, because in sampled
pancreatic sections from both untreated groups, the number of islets containing insulin mRNA was similar (Fig. 7A). After alloxan treatment, the number of β-cell-containing islets was reduced in both groups of mice (Fig. 7A); the number of such islets, however, was significantly lower only in the IAPP<sup>−/−</sup> mice; a reduction to 43 vs. 63% of respective untreated control was seen, and this reduction was significantly greater in IAPP<sup>−/−</sup> mice. Accordingly, as reflected by area of β-cells labeled for insulin mRNA, β-cell mass was not significantly different in the control IAPP<sup>+/+</sup> and IAPP<sup>−/−</sup> mice (Figs. 3, A and B, and 7B). Alloxan treatment induced a significant reduction in β-cell mass in both strains of mice (Figs. 3, C and D, and 7B); this reduction to 31 vs. 38% of respective untreated control, however, was again significantly greater in IAPP<sup>−/−</sup> mice, as indicated by comparison of percent changes. These findings agree with our observations on insulin-immunoreactive cells (Fig. 5) and collectively demonstrate that diabetes in the mice was caused by reduced β-cell mass. Moreover, because both the reduction of β-cell mass and the signs of β-cell dysfunction are exaggerated in IAPP-deficient mice, islets lacking IAPP appear to be more vulnerable to alloxan.

**DISCUSSION**

There are now two phenomena that may link IAPP to the pathogenesis of diabetes. First, amyloid deposits are formed from the peptide in islets from subjects with
Amyloid formation could be causally linked to impairment of β-cell function, because in monkeys it precedes the development of diabetes (6) and, in vitro, human IAPP fibrils induce apoptosis in β-cells (16) and create pores in planar phospholipid bilayers (17). If amyloid formation, on the other hand, is a sequel to β-cell perturbation, it may interfere with nutrition of islet cells and their release of islet hormones and thereby worsen the condition of the disease. The possibility, however, remains that amyloid formation is an unavoidable event coupled to aging of β-cells; islet amyloid is also found in elderly nondiabetic individuals (13). Nevertheless, the findings of islet amyloid and hyperglycemia in transgenic mice with a targeted overexpression of human IAPP to β-cells again highlight the possibility that IAPP is indeed involved in the pathogenesis of NIDDM (30).

Second, because IAPP has previously been shown to restrain insulin release (1) and action (8), metabolic actions of the peptide may be harmful in diabetes. Hence, the overexpression of IAPP compared with insulin observed in experimental models of diabetes in rodents could further contribute to pathogenetic events in the disease (20). Therefore, it was anticipated that IAPP deficiency in mice would ameliorate alloxan-induced diabetes. To our surprise, we found that IAPP-deficient mice developed a more severe form of diabetes when challenged with alloxan; the basal glucose levels were consistently higher in alloxan-treated IAPP−/− mice, whereas basal insulin levels were lower. Also, the previously observed enhancement of glucose elimination under normal conditions in IAPP−/− mice (9) was reversed by alloxan treatment; although both groups of alloxan-treated mice eliminated glucose poorly, after the glucose challenges glucose levels were consistently higher in IAPP−/− mice. The morphological examination revealed that the underlying cause for this more severe diabetes phenotype is lower β-cell mass and insulin gene expression in IAPP-deficient mice after alloxan treatment. Moreover, statistical comparisons of percent changes in β-cell mass, number of β-cell-containing islets, and insulin mRNA levels indicated that these changes were significantly exaggerated in IAPP−/− mice, suggesting that islets lacking IAPP are more vulnerable to alloxan.

Because it is the lack of IAPP that separates IAPP−/− from IAPP+/+ mice at the genetic level, it is reasonable to assume that this lack is specifically responsible for the diabetes phenotype in IAPP-deficient mice that we describe here. Along these lines, it was recently shown that the targeted expression of calcitonin gene-related peptide (CGRP) to β-cells in nonobese diabetic mice prevents diabetes or decreases its incidence in such male and female mice, respectively (15). Because IAPP and CGRP exert similar effects in islets, possibly due to activation of the same receptors (27), it is not surprising that β-cell-targeted overexpression of CGRP results in a phenotype which, in effect, is an opposite of the exaggerated diabetes phenotype associated with IAPP deficiency in our genetic model. Moreover, in transgenic mouse overexpressing human IAPP in β-cells, insulin mRNA expression and storage are increased (5, 29); again, in this regard, this phenotype is the opposite of that which we describe here, in which insulin mRNA expression and storage already were lower in untreated IAPP-deficient mice compared with wild type control mice. Taken together with our data, the experiments with targeted expression of IAPP/CGRP to β-cells (5, 15, 29) argue that the phenotypes of these mice, as well as that of the IAPP−/− mice, are specific for the overexpression or lack of these peptides. Moreover, the previously described metabolic phenotype in male IAPP−/− mice, i.e., an exaggerated insulin response to glucose, was corrected by a randomly integrated β-cell-specific human IAPP transgene (9), again confirming that it is...
the lack of IAPP that is responsible for the phenotype observed.

Several possible mechanisms may underlie the aggravated diabetes in the IAPP-deficient mice. Khachatriyan et al. (15) reason that a local immunomodulatory action of CGRP prevents diabetes in their model. Local immunomodulation may involve control of islet circulation. Indeed, IAPP is known to act as a vasodilator (4) and has previously also been shown to increase the fractional blood flow through islets (28). Lack of such a blood flow increase in IAPP-deficient mice may impair islet regeneration after the alloxan insult. IAPP has also been shown to potently promote growth in cultured renal cells (10). Perhaps IAPP may act as a growth factor in islets, an effect that is lacking in IAPP-deficient mice and that could explain the persistence of impaired islet function. Also, it has previously been suggested that IAPP may limit prolonged depolarization of β-cells with ensuing elevation of intracellular Ca\(^{2+}\) levels, because IAPP hyperpolarizes the plasma membrane of patch-clamped β-cells (31). In IAPP-deficient mice, lack of such action, which we assume serves to protect β-cells from toxic effects of hyperglycemia, may aggravate β-cell damage in diabetes. In addition, the β-cell cytotoxic actions of alloxan may be associated with excessive cycling of Ca\(^{2+}\) through the mitochondrial membranes, which eventually are damaged (26). This leads to a decreased ability of mitochondria to retain Ca\(^{2+}\), their subsequent uncoupling, and impairment of ATP production, depriving the β-cell of energy. Therefore, if one role for IAPP is to reduce intracellular Ca\(^{2+}\) levels through membrane hyperpolarization (31), lack of this mechanism in the IAPP-deficient mice may potentiate the cytotoxic action of alloxan, presumably caused by a combination of cellular energy deprivation and constant elevation of intracellular Ca\(^{2+}\).

Because GLUT-2 is responsible for cellular uptake of both glucose and alloxan (23), increased GLUT-2 expression could form the basis for both the increased insulin response to glucose (9) and β-cell susceptibility to alloxan in IAPP\(^{-/-}\) mice. However, immunocytochemistry revealed no differential expression of GLUT-2 at the protein level in the untreated mice. Interestingly, a perturbed cellular expression of GLUT-2 was found in the alloxan-treated IAPP\(^{-/-}\) mice: although GLUT-2 normally (and in alloxan-treated IAPP\(^{+/+}\) mice) is localized to the plasma membrane (25), immunofluorescence in alloxan-treated IAPP\(^{-/-}\) mice indicated a cytoplasmic localization for GLUT-2. It has previously been reported that GLUT-2 is localized to the cytoplasm of β-cells in islets grafted to streptozotocin-diabetic rats (12). Moreover, it has previously been demonstrated that perturbed islet expression of GLUT-2 in experimental models of diabetes, e.g. Zucker diabetic fatty rats (24), accompanies progression of prediabetes to overt diabetes. Thus, in IAPP-deficient mice, the perturbed GLUT-2 expression may indicate β-cell failure, because it is likely that GLUT-2 in the cytoplasm will not function properly, hence impairing glucose responsiveness.

It has previously been shown that glucose hinders β-cell cytotoxicity conferred by alloxan (2), an effect conceivably due to a competition of glucose with alloxan for GLUT-2-mediated transport into β-cells (23). In addition, alloxan by itself is a β-cell secretagogue. This raises the possibility that the IAPP-deficient mice may also exhibit an exaggerated insulin response to alloxan, which would enhance glucose elimination. Under such circumstances, lower ambient glucose may result in reduced protection of β-cells to alloxan. Whether this holds true in the present case is not known, but a way to circumvent this would be to clamp plasma glucose during alloxan administration. Also, plasma glucose and insulin levels could be determined upon administration of alloxan.

The reason for lower insulin mRNA expression and storage in the IAPP-deficient mice, and whether they contribute to the exaggerated diabetes phenotype in IAPP\(^{-/-}\) mice, are unclear. It has been suggested that the increased insulin expression, storage, and release in transgenic mice overexpressing human IAPP may represent an adaptation to impaired insulin sensitivity (5, 29), because IAPP has previously been implicated in such impairment (8). Interestingly, female IAPP\(^{-/-}\) mice display a similar enhancement of glucose disposal to that in male IAPP\(^{-/-}\) mice (9) but lack the exaggerated glucose-stimulated insulin response; this suggests that the lack of IAPP in these mice confers an enhanced sensitivity to insulin. If this holds true, lower insulin mRNA expression and storage in the IAPP-deficient mice could have evolved in response to enhanced sensitivity to insulin. It is possible that such enhanced sensitivity may be of little importance in mice after alloxan treatment and that the lower insulin mRNA expression and storage in the IAPP-deficient mice will make them more susceptible to the β-cell cytotoxic actions of alloxan.

The increased susceptibility to alloxan in IAPP\(^{-/-}\) mice is also of interest in light of the emerging concept of β-cell rest as a means of hindering future diabetes development (3). This would infer that the increased susceptibility to alloxan in IAPP\(^{-/-}\) mice to some extent may be due to a lack of β-cell inhibition (1, 9) caused by the IAPP deficiency. This, however, needs to be examined in more detail.

In conclusion, we have found that IAPP-deficient mice display a more severe diabetes phenotype than wild type mice upon a challenge with alloxan, due to a greater impairment of β-cell function. This impairment is explained by lower β-cell mass and insulin expression in the IAPP-deficient mice after alloxan treatment compared with the alloxan-treated wild type mice. Because these perturbations were significantly greater in the IAPP-deficient mice, lack of IAPP from islets may confer an increased susceptibility to the diabetogenic actions of alloxan. In addition, lower insulin mRNA expression and storage in IAPP-deficient mice may be a contributing factor in the development of diabetes in such mice. The mechanism(s) underlying the observed phenomenon conceivably involves local actions of IAPP.
Unraveling of these is likely to shed new light on the pathogenesis of diabetes.

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