Induction of endoplasmic reticulum stress-induced β-cell apoptosis and accumulation of polyubiquitinated proteins by human islet amyloid polypeptide

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Am J Physiol Endocrinol Metab 293: E1656–E1662, 2007. First published October 2, 2007; doi:10.1152/ajpendo.00318.2007.—The islet in type 2 diabetes is characterized by an ~60% β-cell deficit, increased β-cell apoptosis, and β-cell mass due to increased islet cell loss. We report that by 10 wk of age hIAPP mice develop diabetes with a deficit in β-cell mass due to increased β-cell apoptosis. The rIAPP transgenic mice counterparts do not develop diabetes or have decreased β-cell mass. Both rIAPP and hIAPP transgenic mice have increased expression of BiP, but only hIAPP transgenic mice have elevated ER stress markers (X-box-binding protein-1, nuclear localized CCAAT/enhancer binding-protein homologous protein, active caspase-12, and accumulation of ubiquitinated proteins). These findings indicate that the β-cell toxic effects of hIAPP depend on the propensity of IAPP to aggregate, but not on the consequence of protein overexpression.

Huang C-j, Haataja L, Gurlo T, Butler AE, Wu X, Soeller WC, Butler PC. Induction of endoplasmic reticulum stress-induced β-cell apoptosis and accumulation of polyubiquitinated proteins by human islet amyloid polypeptide. Am J Physiol Endocrinol Metab 293: E1656–E1662, 2007. First published October 2, 2007; doi:10.1152/ajpendo.00318.2007.—The islet in type 2 diabetes is characterized by an ~60% β-cell deficit, increased β-cell apoptosis, and β-cell mass due to increased islet cell loss. In contrast, we confine use of the term ER stress to describe the response to an increased burden of ER protein traffic (folding, posttranslational modification). This occurs most often because of an adaptive increase in the rate of protein synthesis, e.g., insulin in response to insulin resistance. In contrast, we confine use of the term ER stress to describe the circumstances when the ER fails to prevent aberrant protein folding and modification to the extent that apoptosis is initiated. In the context of amyloidogenic proteins, the failure of protein trafficking by the ER appears to result in formation of oligomers with the potential to cause membrane disruption. Addition of these oligomers extracellularly to cells causes plasma membrane disruption (16) and some features of ER stress (5) perhaps mediated through calcium influx (8).

β-Cells are particularly vulnerable to ER stress-induced apoptosis (11, 38). Overexpression of hIAPP induces β-cell apoptosis, characterized by activation of caspase-12 and increased expression and nuclear translocation of CCAAT/enhancer binding-protein homologous protein (CHOP) in a hIAPP transgenic rat model (15). On a note of caution, however, diabetes has developed unexpectedly in some β-cell-specific transgenic mouse models, e.g., mice expressing major histocompatibility (MHC) molecules on the insulin promoter (24). This calls into question whether development of diabetes in hIAPP expressing transgenic rodents really provides insight...
into a potential mechanism that might underlie the increased β-cell apoptosis in T2DM or is a nonspecific consequence of overexpression of IAPP per se.

The present studies were undertaken to address the following questions. First, do high expression rates of hIAPP in a transgenic mouse model reproduce the characteristics of increased β-cell apoptosis observed in the HIP rat (15)? Second, does induction of β-cell apoptosis by transgenic overexpression of hIAPP depend on its propensity to form toxic oligomers? To address these questions, we developed mice transgenic for rIAPP (r-TG) on the same promoter with the same transgenic expression levels of IAPP and on the same background as mice previously developed by us transgenically expressing hIAPP (h-TG) (17). In addition, we studied INS cells overexpressing hIAPP vs. rIAPP. We report increased β-cell apoptosis with characteristics of the ER stress pathway in h-TG but not r-TG mice.

The third objective of these studies was to take advantage of these different outcomes with comparable expression rates of hIAPP vs. rIAPP to examine some of the changes that have been attributed to the UPR and ER stress. Our goal was to establish which of these responses might be more reasonably considered as evidence of the UPR (i.e., successful adaptation to an increased ER synthetic burden) vs. those that might be more reasonably linked with induction of apoptosis, at least as induced by high expression rates of an amyloidogenic protein.

METHODS

Transgenic models. The rIAPP transgene used in this study is identical to the transgene previously referred to as RIPHAT (rat insulin promoter hIAPP transgenic) (7, 17) other than the substitution of the mouse IAPP coding region in place of the human sequence. The hIAPP coding fragment was removed from the RIPHAT transgene by partial digests with the restriction endonucleases ApaI and NcoI. The mouse IAPP cDNA (270 bp) was generated by PCR, sequenced, and ligated to the digested RIPHAT transgene construct. As with the mice transgenic for hIAPP, purified linear DNA was injected into pronuclei of fertilized oocytes obtained from superovulated FVB/N strain females. For Southern blots, genomic DNA was isolated from tail clips using QiaQuick Preps (Qiagen, Valencia, CA), digested with EcoRI and BamHI restriction enzymes, and subjected to agarose gel electrophoresis prior to membrane transfer. To conform with established guidelines of the MGD Nomenclature Committee, the two generated lines overexpressing the rIAPP transgene are referred to as FVB/N-Tg(Iapp)3Wcs/− and FVB/N-Tg(Iapp)6Wcs/−. Both these lines were self-crossed to generate their homozygous counterparts: FVB/N-Tg(Iapp)3Wcs/Tg(Iapp)3Wcs and FVB/N-Tg(Iapp)6Wcs/Tg(Iapp)6Wcs. For brevity, the latter homozygous line is referred to as r-TG in this report. These transgenic mice were compared with age-matched animals from the host FVB/N strain, which we refer to as nontransgenic controls, and to age-matched mice homozygous transgenic for hIAPP (h-TG), the generation of which has been previously reported (17).

The studies were approved by the UCLA Animal Use and Care Committee. Mice were kept on a 12:12-h light-dark cycle and fed standard rodent chow ad libitum (Purina Laboratory Rodent Diet no. 5001). For studies of fasted animals, chow was removed in the early evening and blood obtained for analysis between 8 and 9 AM the following day.

At 10 wk of age, blood samples for measurement of blood glucose were obtained by retroorbital venous sampling, as previously described (36). Blood glucose concentrations were measured by the glucose oxidase method using a One Touch II Glucometer (Lifescan, Milpitas, CA). Plasma insulin and IAPP concentrations were measured as previously described (3, 36). Following euthanasia at 10 wk of age, the complete pancreas was rapidly dissected from each mouse, and all fat and non-pancreas tissue was trimmed. The pancreas was weighed, fixed in formaldehyde, and then embedded in paraffin. Sections of pancreas were then taken through the fixed tissue in the plane of embedding so that a near-complete section of pancreas (head, body, and tail) through its maximum width was obtained with each section.

Immunohistochemistry. To evaluate β-cell mass and β-cell apoptosis, adjacent sections were immunostained for insulin as previously described (1–3, 25); double stained for insulin and the terminal

Fig. 1. Fasting glucose and insulin concentrations and body weight in non-transgenic controls (CTL, n = 6), homozygous mice transgenic for rodent islet amyloid polypeptide (rIAPP) (r-TG, n = 10) and homozygous mice transgenic for human (h)IAPP (h-TG, n = 7) at 10 wk of age.

Fig. 2. β-Cell mass and β-cell apoptosis in CTL (n = 6), r-TG (n = 10), and h-TG (n = 7) mice at 10 wk of age.
To determine the presence of ER stress, sections of pancreas were double-stained for insulin and CHOP and in subsequent sections for insulin and caspase-12. Primary antibodies anti-CHOP (mouse monoclonal and rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-caspase-12 (rat monoclonal Sigma, St. Louis, MO; rabbit polyclonal, Biovision, Mountain View, CA) or anti-ubiquitin (rabbit polyclonal, Dako, Carpinteria, CA) and guinea pig anti-insulin (Zymed, South San Francisco, CA) were diluted to 1:100. Donkey-derived secondary antibodies conjugated to Cy3 or FITC were diluted to 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA). All slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) with 4,6-diamidino-2-phenylindole (DAPI).

Morphometric analysis. The \(\beta\)-cell mass for each mouse was measured by first obtaining the fraction of the cross-sectional area of pancreatic tissue (exocrine and endocrine) positive for insulin staining and then multiplying this by the pancreatic weight. This was performed using an Olympus IX70 inverted microscope (Olympus America, Melville, NY) connected to a Hewlett-Packard computer loaded with Image Pro-Plus software version 4.5.1 (Media Cybernetics, Silver Spring, MD).

The frequency of \(\beta\)-cell apoptosis was computed by examination of the TUNEL-positive \(\beta\)-cells in the islets. The frequency of \(\beta\)-cell apoptosis was expressed as events per islet. To control for differing \(\beta\)-cell numbers in different islet cross sections, the frequency of these events was also computed as the number of positive cells per islet per insulin staining area in each pancreatic section. The proportion of \(\beta\)-cells (insulin stained) with nuclear CHOP was determined in 20 representative islets from each mouse. Likewise, the proportion of \(\beta\)-cells stained for perinuclear caspase-12 and ubiquitin were also evaluated.

Mouse islet isolation. After an overnight fast, mice were euthanized using isoflurane. Blood was collected as described above (23). The bile duct was clamped at the entrance to the duodenum and cannulated, and the pancreas was perfused with 2 ml of collagenase solution: HBSS (Invitrogen, Carlsbad, CA) supplemented with 25 mM HEPES (Invitrogen), 0.23 mg/ml liberase (Roche, Penzberg, Germany), and 0.1 mg/ml DNAse (Roche). Pancreas was removed, transferred into a glass vial containing 2 ml of ice-cold collagenase solution, and then dispersed by shaking for 30 s. Tissue digest was washed four times with ice-cold HBSS, and then islets were separated from exocrine debris by Histopaque gradient (Sigma). After centrifugation, the medium/Histopaque interface was collected and washed, and islets were handpicked under the microscope. Right after being picked, isolated islets were lysed for protein analysis.

Cell culture. Rat insulinoma cell line INS832/13 (13) was kindly provided by Dr. C. Newgard (Durham, NC). Cells were cultured in...
RPMM 1640 supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, penicillin-streptomycin (Invitrogen), and 50 μM 2-mercaptoethanol (Sigma) at 37°C with 5% CO2. Human and rodent prepro-IAPP-EGFP adenoviruses have been described previously (15). The cells were transduced with adenoviruses expressing human or rodent IAPP-EGFP or GFP at MOI = 100. Forty-two hours after transduction, cells were washed with PBS and lysed by boiling in Laemmli sample buffer. Protein concentrations were determined using the BCA protein assay (Bio-Rad, Hercules, CA).

Western blot analysis. Proteins (20 μg/lane) were separated on 4–12% Bis-Tris NuPAGE gels and blotted onto a PVDF membrane (Pall, Ann Arbor, MI). Membranes were probed with rabbit antibodies against BiP, β-actin (Cell Signaling Technologies, Beverly, MA), X-box-binding protein-1 (XBP-1; Santa Cruz Biotechnology), ubiquitin (Dako), hIAPP (25–37 aa; Peninsula Laboratories, San Carlos, CA), rat antibodies against caspase-12 (Sigma), mouse antibodies against CHOP (Santa Cruz Biotechnology) or guinea pig antibodies against insulin (Zymed) as primary antibodies. Horseradish peroxidase-conjugated secondary antibodies were from Zymed. Proteins were visualized using enhanced chemiluminescence (ECL, Millipore) and protein expression levels were quantified using Labworks software (UVP, Upland, CA). The membranes were reused after stripping with Pierce stripping buffer (Pierce, Rockford, IL).

Statistical analysis. Statistical comparisons were performed using the unpaired two-tailed Student’s t-test. Data in graphs are presented

Fig. 5. A: caspase-12 was not detected in wild-type (A–C) or r-TG mice (D–F) by immunohistochemistry. However, caspase-12 expression was present in a perinuclear pattern (arrow) in 42.0 ± 5.5% of β-cells of h-TG mice (G–I). Scale bar, 10 μM. B: representative immunoblot of total and cleaved caspase-12 of pooled islet lysates from 4–5 mice from each group.
as means ± SE. Findings were considered to be statistically significant at the $P < 0.05$ level.

RESULTS

Body weight, blood glucose, and insulin. By 10 wk of age, h-TG mice, but not r-TG mice, developed diabetes. Despite hyperglycemia, plasma insulin levels were decreased in h-TG vs. r-TG mice, implying defective insulin secretion. Consistent with diabetes-induced glycosuria, h-TG mice also had a modestly lower weight than r-TG or wild-type mice (Fig. 1).

β-Cell mass and apoptosis. At 10 wk of age β-cell mass was decreased by 48% ($P < 0.01$) in h-TG mice compared with wild-type mice (Fig. 2). The mechanism underlying this defect in β-cell mass was an approximately sixfold increase in β-cell apoptosis ($P < 0.05$) in h-TG mice vs. wild-type or r-TG mice. Consistent with our prior findings (17), we did not identify extracellular islet amyloid in h-TG mice at 10 wk of age. Islet amyloid was also not present in either r-TG mice or wild-type mice.

Expression of hIAPP vs. rIAPP in transgenic mice. At 10 wk of age, expression of IAPP/insulin was comparably increased (~3-fold) in h-TG and r-TG mice compared with wild-type controls (Fig. 3A). Since the antibody used for detection of IAPP binds to human and rodent IAPP, the expression is the sum of endogenously expressed IAPP as well as transgenically expressed IAPP.

UPR and ER stress. Transgenic or viral expression of either hIAPP or rIAPP resulted in increased expression of the ER chaperone protein BiP (GRP78; Fig. 3, B and C), consistent with induction of the UPR. Expression levels of CHOP and active XBP-1 were increased in isolated mouse islets and INS cells overexpressing hIAPP compared with rIAPP controls. Expression of activating transcription factor (ATF)-4 was increased in hIAPP- compared with rIAPP-expressing INS cells, although we were unable to detect ATF-4 in islet lysates from either transgenic mouse model or in wild-type mouse controls. We (15) previously reported that INS cells overexpressing hIAPP have increased apoptosis (TUNEL) compared with cells expressing a comparable level of rIAPP.

The accumulation of polyubiquitinated proteins is a characteristic of ER stress in unfolded-protein diseases (12, 29), and in the present studies this was identified by immunohistochemistry in h-TG but not r-TG mice (Fig. 4A). Western blotting also revealed increased polyubiquitinated proteins in h-TG vs. r-TG isolated mouse islets, with a modest increase in isolated r-TG islets compared with nontransgenic controls (Fig. 4B). Caspase-12 was detected in 42.0 ± 5.5% ($n = 6$) of β-cells of 10-wk-old h-TG but not detected in β-cells of r-TG or wild-type mice (Fig. 5A). Western blot analysis of isolated islets detected a marked increase in cleavage of caspase-12 in h-TG and a much more modest increase in r-TG mice vs. nontransgenic controls (Fig. 5B). It is not known whether the modest increase in polyubiquitinated proteins and activated caspase-12 detected by Western blotting in isolated islets from r-TG mice but not observed by immunohistochemistry of corresponding pancreas is because of greater sensitivity of Western blotting or due to the stress of islet isolation imposed on r-TG expression.

CHOP expression was not detected in 10-wk-old wild-type mice or r-TG (Figs. 3B and 6). In contrast, nuclear CHOP was

![Fig. 6. Nuclear CCAAT/enhancer binding-protein homologous protein (CHOP) was detected in ~1% of β-cells of h-TG (G–I) but not detected in islets from r-TG (D–E) or wild-type (WT; A–C) mice by immunohistochemistry. These results were consistent with Western blot experiments (see Fig. 3B). Scale bar, 10 μM.](http://ajpendo.physiology.org/)

**Fig. 6.** Nuclear CCAAT/enhancer binding-protein homologous protein (CHOP) was detected in ~1% of β-cells of h-TG (G–I) but not detected in islets from r-TG (D–E) or wild-type (WT; A–C) mice by immunohistochemistry. These results were consistent with Western blot experiments (see Fig. 3B). Scale bar, 10 μM.
detected in 0.8 ± 0.1% of β-cells of 10-wk-old h-TG mice (n = 3, Fig. 6, H and I). This finding was confirmed by immunoblotting (Fig. 3B).

DISCUSSION

We report that comparable overexpression of human but not rodent IAPP induces diabetes and increased β-cell apoptosis in homozygous transgenic mice. One potential action of overexpressed IAPP on β-cells to cause diabetes is the paracrine inhibition of insulin secretion through specific IAPP receptors (37). Consistent with this, the increased expression of soluble rodent hIAPP in the r-TG mice resulted in an increased fed but not fasted glucose after weaning (data not shown). However, the increased fed plasma glucose concentration resolved by 10 wk of age implying that some compensatory factor overcame the IAPP-induced inhibition of glucose-induced insulin secretion.

These two murine models provide an opportunity to compare β-cell regulatory factors with similar expression rates of hIAPP and rIAPP but with opposite outcomes. We evaluated some of the characteristics previously attributed to the UPR and ER stress. By this means we sought to distinguish responses that might be more appropriate to the UPR (when defined as a successful adaptation to an increased ER load) vs. ER stress-induced apoptosis, at least in the context of IAPP overexpression.

Overexpression of either hIAPP or rIAPP provoked a comparable increased expression of the ER chaperone protein BiP, implying that this was in response to an increased ER load. As such, we would classify the adaptive increase in BiP as primarily related to the UPR, but in itself this adaptive increase was insufficient to prevent the toxic effects of overexpression of hIAPP in all β-cells. In contrast, induction and nuclear translocation of CHOP was more characteristic of hIAPP than rIAPP expression. We detected nuclear translocation of CHOP in pancreatic sections of h-TG but not r-TG mice with a comparable frequency (~1%) to that of β-cell apoptosis. Because hIAPP induced ER stress-mediated apoptosis is at least partially abrogated by CHOP inhibition (15), CHOP appears to play a contributory role in signaling hIAPP-induced ER stress.

If CHOP expression and nuclear translocation is involved in signaling hIAPP-induced apoptosis, do CHOP-positive nuclei coincide with TUNEL-positive nuclei in h-TG mice? We were unable to convincingly demonstrate concordant staining of β-cells in h-TG mice for CHOP and TUNEL. We postulate that this is because nuclear CHOP translocation initiates apoptosis, whereas execution of apoptosis (as detected by TUNEL staining to indicate activation of endonucleases) occurs later. Also, it is reasonable to suspect that the latter leads to degradation of CHOP. This is consistent with studies in INS cells in which we reported that nuclear CHOP preceded execution of apoptosis as detected by TUNEL (15). We occasionally observed concordant nuclear CHOP and TUNEL in hIAPP-expressing INS cells. However, the frequency of the concurrent staining was low. It is not possible to perform a comparable time course study in pancreatic tissue, and, given the low frequency (~1%) of nuclear CHOP-positive and TUNEL-positive β-cells in h-TG mice compared with INS cells expressing hIAPP (~5%), the chances of identifying coincident staining in tissue is much decreased. Clearly, future studies to establish the effects of CHOP knockdown in vivo in h-TG mice will provide more definitive insights into the role of CHOP as an intermediary in hIAPP-induced ER stress.

We also detected active XBP-1 in both transgenic mice and INS cells expressing hIAPP vs. rIAPP. These data suggest that active XBP-1 in the context of hIAPP overexpression is associated with ER stress rather than just the UPR as defined here. Active XBP-1 is known to upregulate the ER-associated degradation (ERAD) system, presumably to clear irreversibly misfolded proteins or protein aggregates (38). It is therefore of interest that accumulation of polyubiquitinated proteins was observed in h-TG mice to a much greater extent than in r-TG mice in these studies.

The nature of the polyubiquitinated proteins in β-cells of h-TG mice is as yet undefined. We previously reported that during the period of maximal β-cell loss in h-TG mice there are abnormal nonfibrillar intracellular IAPP aggregates confined to β-cells and associated with locally disrupted membranes (17, 31). We hypothesized that these aggregates form intracellularly and might represent the toxic form of IAPP (31). Since then, a better appreciation of the nature of the cytotoxic forms of amyloidogenic proteins has begun to emerge (9, 16, 18, 20, 21, 23, 26, 40). The most toxic form of amyloidogenic proteins are nonfibrillar oligomers that interact with membranes to cause nonselective ion channel membrane leakage (9, 10, 20, 21, 26, 28) and might be expected to disrupt membranes within the secretory pathway such as ER. It is also possible that they are secreted, and that they cause cytotoxicity by disrupting the plasma membrane and/or activating cell surface death receptors (8, 16, 35, 39, 40).

Consistent with previously reported findings in the HIP rat, we report that expression and activation of caspase-12 is increased in h-TG vs. r-TG mice. Although activation of caspase-12 is associated with ER stress in rodents (30), it does not appear to be an important mediator of the resulting apoptosis (33).

In summary, h-TG mice develop diabetes characterized by loss of β-cell mass and increased β-cell apoptosis, due at least in part to β-cell ER stress and accumulation of polyubiquitinated proteins. In contrast, mice that express a comparable burden of soluble rIAPP do not develop diabetes, lose β-cell mass, or have ER stress-induced β-cell apoptosis or an accumulation of polyubiquitinated proteins. These findings ensure that the β-cell toxic actions of overexpression of hIAPP are not simply a nonspecific consequence of the increased synthetic and folding burden placed upon the ER. They also give further impetus to the hypothesis that membrane-disrupting oligomers of amyloidogenic proteins initiate apoptosis, at least in part, by inducing ER stress and disrupting the proteasome and are consistent with recent studies in humans with T2DM (15, 22).

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GRANTS

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