Calcium-activated Calpain-2 Is a Mediator of Beta Cell Dysfunction and Apoptosis in Type 2 Diabetes*

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The islet in type 2 diabetes mellitus (T2DM) and the brain in neurodegenerative diseases share progressive cell dysfunction, increased apoptosis, and accumulation of locally expressed amyloidogenic proteins (islet amyloid polypeptide (IAPP) in T2DM). Excessive activation of the Ca2\(^{2+}\)-sensitive protease calpain-2 has been implicated as a mediator of oligomer-induced cell death and dysfunction in neurodegenerative diseases. To establish if human IAPP toxicity is mediated by a comparable mechanism, we overexpressed human IAPP in rat insulinoma cells and freshly isolated human islets. Pancreas was also obtained at autopsy from humans with T2DM and nondiabetic controls. We report that overexpression of human IAPP leads to the formation of toxic oligomers and increases beta cell apoptosis mediated by increased cytosolic Ca2\(^{2+}\) and hyperactivation of calpain-2. Cleavage of α-spectrin, a marker of calpain hyperactivation, is increased in beta cells in T2DM. We conclude that overactivation of Ca2\(^{2+}\)-calpain pathways contributes to beta cell dysfunction and apoptosis in T2DM.

Hyperglycemia in type 2 diabetes mellitus (T2DM) is due to impaired insulin secretion in the setting of relative insulin resistance (1). The islets of Langerhans in T2DM are characterized by a deficit in beta cells, increased beta cell apoptosis, and islet amyloid derived from islet amyloid polypeptide (IAPP), a 37-amino acid highly conserved peptide co-expressed and secreted with insulin by pancreatic beta cells (2, 3).

The pathology of the islet in T2DM and brain in neurodegenerative diseases such as Alzheimer disease share several parallels. In both, the loss of functional tissue is associated with deposition of a locally expressed protein with the potential to form amyloid fibrils (Alzheimer beta protein in Alzheimer disease and IAPP in T2DM) (2, 4). In both T2DM and Alzheimer disease, there has been a debate as to whether the amyloid deposits contribute to cell loss (the so-called amyloid hypothesis) or are secondary to the processes leading to cell loss. Evidence against a direct role of amyloid deposits on cell loss is the poor correlation between the extent of amyloid deposits and the severity of disease in both human and animal models (3, 5, 6). Moreover, preformed amyloid fibrils are not cytotoxic when applied to cells (7).

However, several lines of evidence are supportive of a role of cytotoxicity by amyloidogenic proteins. These include genetic predisposition in occasional families with mutations leading to increased amyloidogenicity of the amyloid protein (8) and reproduction of the disease phenotype in rodent models transgenic for the relevant human amyloidogenic protein (9). There is an increasing appreciation that the cytotoxic forms of amyloidogenic proteins are small nonfibrillar oligomers that may form in membranes and cause nonselective membrane permeability (7, 10, 11), the toxic oligomer hypothesis. Moreover, misfolding and aggregation of amyloidogenic proteins into toxic oligomers induce apoptosis through the mechanism of endoplasmic reticulum stress (ER stress) (12, 13).

The proximal molecular events that link formation of toxic oligomers and induction of ER stress are unknown. One plausible explanation is that local membrane instability caused by toxic oligomers permits unregulated Ca2\(^{2+}\) surges from the ER or other intracellular Ca2\(^{2+}\)-enriched compartments. Toxic IAPP oligomers appear to form and act intracellularly (14) within the secretory pathway (15). Moreover, they escape the secretory pathway, apparently by disrupting intracellular membranes (15). Therefore, disturbance of the usually discrete cellular compartmentalization of Ca2\(^{2+}\) is a logical candidate to link membrane-permeant toxic oligomers and induction of cellular dysfunction and, under more extreme circumstances, cell death. There is as much as a 10,000-fold concentration difference between cytoplasmic Ca2\(^{2+}\) (100 nm) and ER lumen Ca2\(^{2+}\) (0.5–1.0 mM) so that even modest disruption of the ER membrane integrity might activate aberrant cytoplasmic Ca2\(^{2+}\)-activated signaling pathways. Also, because a high ER Ca2\(^{2+}\) concentration is required for appropriate ER function, ER membrane disturbance might be expected to exacerbate the ER dysfunction that permitted protein misfolding initially.

In support of the postulate that unregulated Ca2\(^{2+}\) release from the ER to the cytoplasm might be a mediator of beta cell dysfunction and apoptosis in T2DM, it is well recognized that cytoplasmic Ca2\(^{2+}\) overload is a ubiquitous cause of cell death in...
neurons, cardiomyocytes, and insulin-producing beta cells (16, 17). Effectors or executors of calcium overload include protease calpains, kinases/phosphatases, calmodulin, and calcineurin (18). Sustained hyperactivation of calpain is provoked in many pathological processes, including ischemia, traumatic injury, and neurodegenerative disorders such as Alzheimer disease (17, 19, 20).

Calcium-dependent protease calpains belong to the cysteine protease family that has previously been implicated in the pathophysiology of several inflammatory disorders, including myocardial reperfusion injury, cerebral ischemia/reperfusion, circulatory shock, and T2DM (17). Insulin-producing beta cells express several calpains, including calpain-10, μ-calpain (or calpain-1), and m-calpain (or calpain-2). Polymorphisms in calpain-10 are associated with the risk of developing T2DM in some ethnic groups (21). The ubiquitous μ-calpains and m-calpains are activated by micromolar and millimolar levels of calcium, respectively. Calpains mediate a variety of physiological functions such as cytoskeleton remodeling, vesicle trafficking, and membrane fusion (22).

This study was designed to test the hypothesis that IAPP-mediated ER stress-induced apoptosis is mediated in part through increased cytosolic Ca\(^{2+}\) and activation of Ca\(^{2+}\)-dependent calpain. We report that overexpression of human IAPP in INS 832/13 cells and isolated human islets led to increased cytoplasmic Ca\(^{2+}\), activation of Ca\(^{2+}\)-sensitive calpain-2, and beta cell apoptosis. Inhibition of calpain by calpeptin attenuated the toxicity of human IAPP. Also, we detected calpain-cleaved fragments of the cytoskeleton protein α-spectrin, a surrogate indicator of Ca\(^{2+}\)-initiated and calpain-mediated cytotoxicity, in beta cells of humans with T2DM.

**EXPERIMENTAL PROCEDURES**

**Human and Rodent Prepro-IAPP Adenovirus**

Adenovirus generation and transduction were performed according to the procedure described by Huang et al. (13). Briefly, to generate human and rat prepro-IAPP adenovirus, KpnI and Xhol or EcoRI and EcoRV restriction sites, respectively, were introduced in the front of ATG and after the stop codon. A 290-bp human prepro-IAPP PCR fragment was digested with KpnI and Xhol, and a 300-bp rat prepro-IAPP PCR fragment was digested with EcoRI and EcoRV. The fragments were inserted into pENTR2B and subsequently into pAd/CMV/DEST adenovirus vector (Invitrogen). Recombinant adenoviruses expressing human and rat prepro-IAPP (hiAPP and rIAPP, respectively) were generated and purified according to the manufacturer’s instructions (Clontech).

**Cell Lines**

Rat insulinoma cell line INS 832/13 was kindly provided by Dr. C. Newgard (Durham, NC) (23). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM Hepes, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 50 μM 2-mercaptoethanol (Sigma) in a 37°C 5% CO\(_2\) tissue culture incubator. Rat insulinoma cells (RIN) that express a low level of endogenous IAPP were used to establish the dose-response relationship between the m.o.i. of adenovirus-expressing hIAPP or rIAPP (Fig. 1, A and B) and protein expression levels. We chose to use 400 m.o.i. for all experiments with INS 832/13 cells. The cells were transduced with adenoviruses expressing human or rodent IAPP and then 48 or 72 h later were washed with PBS and either fixed for immunocytochemistry or lysed by boiling in Laemmli sample buffer for immunoblotting. Protein concentrations were determined using the DC protein assay (Bio-Rad). Calpeptin and BAPTA-AM were from Calbiochem.

**Human Islets**

Isolated human islets were obtained from the Islet Cell Resource Consortium. The islet purity was 90–95% as assessed by dithizone staining. The donors, aged 35–55 years, were heart-beating cadaver organ donors, and none had a previous history of diabetes or metabolic disorders. Islet viability was assessed by the live/dead kit (Molecular Probes). Islets were cultured in a 6-well plate or 4-well chamber slide in RPMI 1640 medium (5.5 mM glucose) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Invitrogen).

To ensure that islets were functional and viable during the course of the experiment, we performed functional evaluation (glucose-stimulated insulin secretion) of isolated islets. After 72 h in culture, 40 islets were preincubated for 1 h in complete RPMI 1640 medium containing 4.0 mM glucose. The medium was replaced with fresh medium containing either 4.0 mM glucose or 16.7 mM glucose for 5 min, and aliquots were collected for insulin measurements. Islets were lysed for insulin content measurement. Insulin concentration was measured by a human insulin enzyme-linked immunosorbent assay kit (Linco Inc., St. Charles, MO).

**Western Blot Analysis**

Proteins (20–40 μg per lane) were separated on 4–12% Bis-Tris NuPAGE gels and blotted onto a polyvinylidene difluoride membrane (Pall, Ann Arbor, MI). Membranes were probed with rabbit antibodies against calpain-2, cleaved caspase-3, poly(ADP-ribose) polymerase, β-actin (Cell Signaling Technology, Beverly, MA), and cleaved spectrin (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were from Zymed Laboratories Inc. Proteins were visualized using enhanced chemiluminescence (Milipore), and protein expression levels were quantified using Labworks software (UVP, Upland, CA). The membranes were reused after stripping with Pierce stripping buffer for 20 min at room temperature (Pierce).

**Cytosolic Ca\(^{2+}\) Measurement Using Reporter System**

To measure the elevation of cytosolic Ca\(^{2+}\), we used the NFAT-SEAP reporter system (Clontech) based on the activation of calcineurin by the elevation of cytosolic Ca\(^{2+}\) (24). Calcineurin is a calmodulin-dependent phosphatase (PP2B) that de-phosphorylates and activates the transcriptional factor nuclear factor of activated T-cells (NFAT) (24). INS 832/13 cells were plated in 4-well chamber permnox slides (Nunc, Rochester, NY) at 80,000 cells per well and cultured for 16–24 h. Cells were transfected overnight with the NFAT-SEAP construct (1.0 μg of plasmid DNA per well) using Lipofectamine (Invitrogen) and
then transduced with adenoviruses expressing hIAPP versus rIAPP at 400 m.o.i. Medium was collected 6, 12, and 18 h after transduction, and the SEAP activity was measured according to the manufacturer’s instructions (Calbiochem). Similar procedure was used for isolated human islets.

**Ratiometric Measurement of Cytosolic Ca**

INS 832/13 cells on coverslips were loaded with the fluorescent Ca** indicator Fura-2 by incubation in media containing 5 \( \mu M \) Fura-2/AM (Molecular Probes, Eugene, OR) for 60 min at 37 °C (25). Coverslips were then mounted in an experimental chamber (RC-25F; Warner Instrument Corp.) that was perfused with culture medium at 1.5 ml/min. The perfusion medium was heated using an in line heater (TC-344B; Warner Instrument Corp.), which maintained bath temperature at 37 °C. The chamber was placed on the stage of an inverted microscope (Zeiss TV 100; Carl Zeiss, Inc., Thornwood, NY) with attached digital imaging system (Attofluor; Atto Instruments, Rockville, MD) with electronically controlled excitation filter positions (software RatioVision). Cells were continuously perfused with media for 3–5 min before the start of the acquisition of data. Ratios of images (340 nm excitation/380 nm excitation, emission filter 520 nm) of 30–50 cells per field were obtained at 30-s intervals. A region of interest was defined over each cell, and the average ratio intensity over the region was converted to \([Ca^{2+}]_i\), using a calibration curve constructed with a series of calibrated buffered calcium solutions (calcium calibration buffer kit number 2; Molecular Probes). For each cell, \([Ca^{2+}]_i\), values and their times of acquisition were stored on computer disk.

**Calpain Activity Assay**

The fluorometric calpain activity assay is based on the detection of the cleavage of the calpain substrate Ac-LLY-amino-4-trifluoromethylcoumarin (calpain activity assay kit, Biovision, Sunnyvale, CA). Upon cleavage of this substrate in lysates, free amino-4-trifluoromethylcoumarin emits a yellow-green fluorescence (505 nm), which is detected by the CCD camera (UVP, Upland, CA). The detection of IAPP toxic oligomers in these pancreas samples was reported previously (15).

**ATPase inhibitor, 0.1 \( \mu M \) thapsigargin, was used as a positive control.**

**Human Pancreatic Tissue**

Institutional Review Board approval was obtained from both the Mayo Clinic (Institutional Review Board number 1516-03) and UCLA (number 06-04-021-01). We obtained human pancreatic tissue at autopsy from seven obese humans with T2DM and seven age- and BMI-matched nondiabetic controls (Table 1). In addition, aliquots of nine surgically removed human pancreatic tissue specimens (pancreatectomy for tumor but non-tumor-affected pancreas used for study) from T2DM (\( n = 3 \)) and control subjects (\( n = 6 \)) were studied with UCLA Institutional Review Board approval (Fig. 6C). The detection of IAPP toxic oligomers in these pancreas samples was reported previously (15).

**Immunocytochemistry**

For immunocytochemical analysis, cells were plated in 8-well chamber Permanox slides (Nunc, Rochester, NY) at 80,000 cells per well and cultured for 48 h. Cells were then transduced with adenoviruses expressing human or rodent IAPP at 400 m.o.i. Forty eight hours later, cells were gently washed with PBS and fixed with 4% paraformaldehyde (Sigma) for 20 min at room temperature. After washing, cells were permeabilized with 0.4% Triton X-100/Tris-buffered saline for 20 min at room temperature, blocked with 3% bovine serum albumin, 0.2% Triton X-100/Tris-buffered saline for 1 h at room temperature, and incubated overnight at 4 °C with anti-calpain antibody (catalog no. 208755, Calbiochem) diluted 1:100 with 0.2% Tween 20, 3% bovine serum albumin/Tris-buffered saline, followed by Cy3-anti-rabbit IgG (1:200, 1 h at room temperature; Jackson ImmunoResearch Laboratories, West Grove, PA).

To assess cell death, 48 or 72 h after transduction, culture medium was replaced with medium containing 50 \( \mu g \)/ml pro-
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FIGURE 1. Overexpression of hIAPP induces apoptosis in beta cells. A, there was an m.o.i.-dependent increase in IAPP expression and apoptosis (cleaved caspase-3 [Cl.Casp-3]) in rat insulinoma cells (RIN) transduced with hIAPP adenovirus at different m.o.i. for 48 h. B, in RIN cells, IAPP overexpression at 400 m.o.i. was comparable for human and rodent IAPP. Cntl, control. C, toxic IAPP oligomers were detected by cryo-immunogold labeling (A11 antibody) in INS 832/13 cells overexpressing hIAPP (panels a–c) to a greater extent than in cells overexpressing rIAPP (panel d) at the same m.o.i. AdV, adenovirus. D, hIAPP overexpression induces apoptosis in INS 832/13 cells to a much greater extent than rIAPP (400 m.o.i., 48 h; please note comparable protein overexpression shown in B). Data are the mean ± S.E. from four independent experiments; *, \( p < 0.05 \) compared with control; *, \( p < 0.05 \) compared with rIAPP overexpressing cells. ProCasp-3, pro-caspase; PARP, poly(ADP-ribose) polymerase.

Propidium iodide (PI) (Sigma). Cells were cultured at 37 °C for 30 min, then washed once with PBS, and fixed with 4% paraformaldehyde for 20 min at room temperature. Slides were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Random areas (six per chamber) were imaged first in blue (DAPI) and then in red (PI or Cy3) channel.

Immunofluorescent staining of human pancreatic sections was performed as described (13). A specific antibody against the calpain-cleaved α-spectrin (Santa Cruz Biotechnology) (26, 27) was used as a surrogate for calpain hyperactivation. Sections were double-stained with cleaved spectrin and insulin antibodies.

**Image Analysis**

To assess cell death, propidium iodide-positive cells were counted in each image and related to the total numbers of cells in the image (DAPI-labeled nuclei). To quantify the expression of calpain 2, the area labeled with anti-calpain 2-Cy3 was measured using Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD) and related to the total number of cells in the image (DAPI-labeled nuclei). For cleaved spectrin analysis, 10–15 islets from each human pancreas section were imaged. Total beta cells and beta cells stained for cleaved spectrin were counted (Table 1).

**Cryo-immunogold Labeling and Electron Microscopy**

**Sample Preparation**—INS 832/13 cells were transduced with hIAPP or rIAPP for 30 h and then fixed with 4% paraformaldehyde + 0.1% glutaraldehyde for 6 h, scraped, spun, and embedded and sectioned as described previously (28).

**Staining Procedure**—Sections were incubated with antibody against toxic oligomers (A11 (29)) diluted in 20 mM Tris, 150 mM NaCl, 1% bovine serum albumin at 4 °C overnight, washed three times with the same buffer, and then incubated with secondary antibody for 45 min at room temperature. After washing, the sections were fixed with 0.8% glutaraldehyde, treated with 1% uranyl acetate in 1.3% methylcellulose, and air-dried. Samples were analyzed within 1–3 days after staining using a JEM 1200-EX transmission microscope (JEOL, Japan) equipped with a BioScan 600W digital camera (Gatan, Inc., Pleasanton, CA).

**Confocal Time Lapse Microscopy**

Time-lapse confocal microscopy was used as described previously (30). Briefly, INS 832/13 cells were transfected with plasmid DNA expressing NFAT-GFP for 12 h, and then the
same cells were transduced with Ad-hIAPP at 400 m.o.i. Confocal time-lapse microscopy was conducted to follow individual cells in the presence with 500 ng/ml of PI for 2 h.

Statistical Analysis

All values were presented as the mean ± S.E. Student’s t test was performed to compare the differences between the rIAPP versus hIAPP adenovirus expressing cells or islets. Values of p < 0.05 were considered to be statistically significant.

RESULTS

Overexpression of Human IAPP in INS 832/13 Cells Reduces Cell Viability and Increases Cell Apoptosis

We previously reported that the overexpression of hIAPP with a green fluorescent protein tag induced ER stress in INS 832/13 cells (13). Because the presence of the green fluorescent protein tag may interfere or distort the toxic effects of hIAPP, we created an adenovirus expressing hIAPP without any tag. The nonamyloidogenic rIAPP was used as a control because it contains three proline residues in the 20–29-amino acid region of IAPP rendering it soluble. The sequence of IAPP in the mouse and rat is identical, hence the term rodent. To establish a dose response for m.o.i. of adenoviral expression and toxicity, and to ensure comparable expression of experimental hIAPP and control rIAPP, RIN cells were transduced with an m.o.i. of 150–500 with a resulting progressive increase in apoptosis quantified by cleavage of caspase-3 48 h after transduction (Fig. 1A). For subsequent experiments, we used an m.o.i. of 400 for hIAPP or rIAPP as a control for a comparable burden of protein expression (Fig. 1B). hIAPP expression caused increased apoptosis compared with control or rIAPP (Fig. 1D). Furthermore, toxic IAPP oligomers were detected in hIAPP-transduced INS 832/13 cells but rarely in cells transduced with rIAPP (Fig. 1C).

Cytosolic Calcium Is Increased in INS 832/13 Cells Overexpressing Human IAPP

Having established that the new hIAPP adenovirus without a tag induced apoptosis in INS 832/13 cells, we sought to determine what caused apoptosis in INS 832/13 cells overexpressing hIAPP. Because hIAPP oligomers may compromise cellular membrane integrity (7, 31, 32), we designed several experiments to establish if overexpression of hIAPP induces an increase in cytosolic Ca2++. First, we established that the new hIAPP protein expression was detected 4–6 h after viral infection (data not shown), and the cytosolic Ca2++ was elevated 12 h (Fig. 2A, reporter assay) and 6 h (Fig. 2B, ratiometric assay) after adenoviral transduction. We observed an increased level of cytosolic Ca2++ in cells overexpressing hIAPP and a more modest increase in cells overexpressing rIAPP compared with nontransduced control cells (Fig. 2A). The minor effect of rIAPP was not detected by the ratiometric assay, which measured the cytosolic Ca2++ in less than a second, but was detectable by the reporter assay, which measured the accumulative effects of cytosolic Ca2++ elevation over 12 h. The increased Ca2++ levels in hIAPP-expressing cells may come from two sources as follows: adenoviral transfection itself can increase Ca2++ levels (33) or the increased number of peptides entering the ER can permit some passive leakage of Ca2++ through the ER translocon pore (34). We employed confocal time-lapse video micrography to show that hIAPP-induced abnormal elevation of cytosolic Ca2++ ions is followed by cell death (Fig. 2C, right panel). In Fig. 2C, left panel, one cell in the

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apoptosis was obtained using Ca$^{2+}$ chelator. When the intracellular calcium chelator BAPTA-AM was added 4 h after viral transduction, apoptosis (caspase-3 activation) was reduced at a dose of 0.25 μM (Fig. 2D). We do not see significant protective effect at higher concentrations of BAPTA-AM. We believe that at higher concentrations BAPTA-AM penetrates the ER lumen and depletes ER Ca$^{2+}$ leading to loss of function of ER chaperones, compromising protein folding and thus compromising its protective effects (35).

**Calcium-dependent Calpain Activity Is Increased in INS 832–13 Cells Overexpressing Human IAPP**

We further asked what was the consequence of increased cytosolic Ca$^{2+}$ induced by hIAPP. Among many cellular events initiated by increased cytosolic Ca$^{2+}$, calpain, a neutral Ca$^{2+}$-dependent protease, was a prime suspect. Previous study has shown that overexpression of Alzheimer amyloid precursor protein in cultured neurons induced activation of calpain (19). The expression of hIAPP in INS 823/13 cells induced increased calpain protease activity (Fig. 3A). This functional assay of calpain protease activity does not specify which calpain is activated, so a calpain-2-specific antibody was then used to address this question.

The antibody used to detect calpain-2 recognizes both the 80-kDa inactive and 58-kDa active forms of calpain-2 (catalog no. 208755, Calbiochem). Inactive calpain-2 is associated with the ER membrane at the cytosolic side (36), but following activation, calpain-2 is released from this site and rapidly degraded. Rapid degradation of activated calpain-2 is presumably to prevent this potent protease from degrading proteins distant from the intended local site of activation (37). Consistent with this, treatment with thapsigargin (ER Ca$^{2+}$-ATPase inhibitor) induced an almost complete loss of calpain-2 immunoreactivity on the ER surface (Fig. 3B, top right panel). Overexpression of hIAPP also caused a loss of calpain-2 immunoreactivity (Fig. 3B, lower right panel) compared with cells overexpressing rIAPP (Fig. 3B, lower left panel). To ensure the localization of calpain-2 on the ER of beta cells, we
performed ER fractionation and performed immunoblots using the same calpain-2-specific antibody. The result of enriched ER protein immunoblotting confirmed the observation from immunocytochemistry that treatment with thapsigargin led to the loss of inactive calpain in the ER-enriched fraction (data not shown). Immunoblotting showed that overexpression of hIAPP decreased total calpain content (Fig. 3C), presumably as a consequence of rapid degradation following Ca2+ activation (see above, Fig. 3B) (37). Furthermore, the observed hIAPP-induced elevated cytosolic Ca2+, increased calpain activity, and the decrease in total calpain protein content by immunoblot are consistent with Etoposide-induced cytosolic Ca2+ elevation, increased calpain activity, and decreased total calpain protein content (38).

**Inhibition of Calpain Activity by Calpeptin Reduces Toxicity of hIAPP in INS 832/13 Cells**

If calpain mediates hIAPP-induced apoptosis, then inhibition of calpain would be expected to reduce apoptosis. We chose the specific calpain inhibitor calpeptin, a fragment of the endogenous calpain inhibitor calpastatin, to test this hypothesis. A dose-response study was undertaken in INS 832/13 to identify a nontoxic dose (data not shown). Based on those findings, we chose to use calpeptin at 1.25 μM for 66 h. At that dose, caspase-3 cleavage and PI incorporation were decreased by 30 and 50%, respectively, in calpain-treated hIAPP-expressing cells compared with hIAPP-expressing cells without treatment (Fig. 4, A and B).

**Calcium Elevation and Calpain Activation Are Observed in Human Islets Overexpressing Human IAPP**

After establishing that overexpression of hIAPP induced increased cytosolic Ca2+, activation of calpain, and apoptosis in INS 832/13 cells, we extended these findings to isolated human islets. Expression of hIAPP increased cytosolic Ca2+ levels, monitored by the same Ca2+ reporter system (Fig. 5A). Activation of calpain by hIAPP was also confirmed in human islets assayed by calpain activity (Fig. 5B). Inhibition of calpain by calpeptin in human islets again led to attenuation of caspase-3 activation (Fig. 5C).

**Cleaved Spectrin Was Detected in Beta Cells in Humans with T2DM**

Having demonstrated that high expression levels of human IAPP induce increased cytosolic Ca2+ and hyperactivation of calpain, we posed the following question. Is there evidence of chronically increased cytosolic Ca2+ and hyperactivation of calpain in beta cells of humans with T2DM? Living beta cells from humans with T2DM are rarely available. Therefore, to address this, we analyzed the consequences of sustained elevated cytosolic Ca2+ and hyperactivation of calpain in sections of fixed paraffin-embedded human tissue available from T2DM.

The cytoskeletal protein α-spectrin (also called α-fodrin), when subjected to calpain cleavage, generates a specific α-spectrin fragment that can be detected as a measurement of calpain activity (39, 40). Using a specific antibody against the calpain-specific (26), cleaved fragment of α-spectrin, we detected increased cleaved fragments (Fig. 6, A and B) in beta cells from humans with T2DM (n = 7), compared with age- and BMI-matched nondiabetic subjects (n = 7). Exocrine cells also express α-spectrin, but the cleaved spectrin in T2DM was rarely detected in exocrine cells, ensuring that the detected calpain hyperactivation in beta cells was not an artifact of tissue collection or preservation. To substantiate this observation, we...
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examined the relationship between the frequency of cleaved spectrin and the frequency of intracellular toxic IAPP oligomers detected with a specific anti-toxic oligomer antibody (A11) in pancreatic beta cells of nine human pancreas samples obtained at surgery as reported previously (15). We observed a positive correlation \((r^2 = 0.8, p < 0.01)\) between the frequency of beta cells with cleaved spectrin and the oligomer-specific immunoreactivity (Fig. 6C). Using immunoblotting, we also detected cleaved spectrin in isolated human islets (Fig. 6D) and in INS 832/13 cells overexpressing hIAPP (Fig. 6E). In the same preparation, inhibition of calpain activation by calpeptin reduced the cleavage of spectrin induced by overexpressing hIAPP (Fig. 6D).

**DISCUSSION**

We tested the hypothesis that human IAPP-induced apoptosis is mediated through disturbance of intracellular Ca\textsuperscript{2+} homeostasis. We report the following: 1) that overexpression of hIAPP induced the formation of toxic IAPP oligomers and increased cytosolic Ca\textsuperscript{2+} concentration and hyperactivation of calpain-2 protease in insulin-secreting beta cells and/or isolated human islets; 2) that inhibition of calpain attenuated the toxicity of human IAPP in INS 832/13 cells and isolated human islets; and 3) that calpain-cleaved products of \(\alpha\)-spectrin were present in isolated human islets and INS 832/13 cells overexpressing hIAPP and were identified in pancreatic beta cells from humans with T2DM. These studies imply that chronically and aberrantly activated Ca\textsuperscript{2+}/calpain pathways contribute to the cytotoxicity of aggregated human IAPP. Moreover, these data suggest that dysfunction and increased beta cell apoptosis in T2DM are mediated, at least partially, through chronic overactive Ca\textsuperscript{2+}/calpain pathways. These findings support and extend the IAPP toxic oligomer hypothesis in T2DM.

Aberrantly increased cytoplasmic Ca\textsuperscript{2+} has been shown to mediate cellular dysfunction and cell death in neurodegenerative diseases, also characterized by abnormal intracellular aggregates of amyloidogenic proteins (17, 18, 41). We report that the overexpression of hIAPP in insulin-producing cells or islets leads to an early increase in cytoplasmic Ca\textsuperscript{2+}. This finding was obtained by using a gene reporter system, in which the Ca\textsuperscript{2+}-sensitive transcriptional factor nuclear factor of activated T-cells was linked to the secreted alkaline phosphatase to measure the accumulated effects of Ca\textsuperscript{2+} elevation. This finding was substantiated by the ratiometric measurement of intracellular Ca\textsuperscript{2+} using fura-2-AM (Fig. 2B), a standard protocol in the field (25).

The mechanism by which hIAPP aggregates cause inappropriately increased cytoplasmic Ca\textsuperscript{2+} is unknown but is most likely mediated by the property of hIAPP oligomers to induce
nonselective membrane leakage (7). In support of this, it has been shown that application of β-amyloid aggregates to cells caused increased cytoplasmic Ca\(^{2+}\) concentrations and subsequently reduced ER Ca\(^{2+}\) stores (42). It has been reported that exogenous hIAPP can activate transient receptor potential channels (43). Cytosolic Ca\(^{2+}\) elevation induced by hIAPP overexpression may arise as a result of hIAPP induced leakage into the cytosol from the secretory pathway or mitochondria because membrane-permeant IAPP toxic oligomers are identified at each of these sites (14, 15). Also, oligomeric β-amyloid-induced Ca\(^{2+}\) release can be blocked by the inhibition of the ER calcium release channels (42). Furthermore, increased cytoplasmic Ca\(^{2+}\) can be associated with the malfunction of both ER ryanodine and inositol 1,4,5-triphosphate receptors, which are substrates of activated calpains (44, 45). Because toxic hIAPP oligomers have been detected in the secretory pathway (15), they might also be secreted and act on the plasma membrane, a site that also leads to cytotoxicity (7, 10, 32, 46, 47) and elevated cytoplasmic calcium levels (18, 42).

An increase in cytosolic Ca\(^{2+}\) leads to activation of calpain, which is implicated in a number of pathological disorders, including brain ischemia, injury, and neurodegeneration (17). Inappropriately high calpain activation has been reported in the affected hippocampus in Alzheimer disease (16). Our data indicate that increased cytosolic Ca\(^{2+}\) concentrations preceded the hyperactivation of calpain. Furthermore, we showed that inhibition of calpain attenuated hIAPP-induced cytotoxicity, which is consistent with a recent report showing that the inhibition of calpain improves memory and synaptic transmission in a mouse model of Alzheimer disease with overexpression of human β-amyloid protein (48).

We also report that beta cells from humans with T2DM have cleaved α-spectrin, which is an indicator of a compromised cytoskeleton and cellular membrane. Scaffolding protein spectrin (αII) is an actin-binding protein and is expressed in most cells, including pancreatic endocrine and exocrine cells. Spectrin functions as a membrane stabilizer by forming trimers, tetramers, and higher polymers (49). αII spectrin has a μ-calpain cleavage site based on the secondary and tertiary conformational features surrounding the cleavage site, rather than the linear sequence (49). Activated calpain cleaves spectrin and generates 145- and 150-kDa fragments, recognized by a specific antibody (50). It has been reported that calpain-cleaved fragments of α-spectrin are a hallmark of hyperactivation of the calcium-calpain system (51), and injection of 6-hydroxydopamine into rodent brains resulted in calpain activation and the cleavage of α-spectrin. When treated with calpastatin, the cleavage of α-spectrin was greatly reduced (52).

Therefore, the presence of cleaved spectrin in beta cells from humans with T2DM, but rarely in age- and BMI-matched non-diabetic subjects, is consistent with chronic overactivation of Ca\(^{2+}\)-calpain pathways. Moreover, we report a positive correlation between the frequency of beta cells with cleaved spectrin (Fig. 6C) and the frequency of toxic oligomer-containing beta cells (15), consistent with the hypothesis that formation of intracellular toxic IAPP oligomers disrupt intracellular membrane fidelity with the adverse consequences of inappropriate activation of Ca\(^{2+}\)-sensitive pathways. These observations were further substantiated by the demonstration of the presence of cleaved spectrin in isolated human islets and INS 832/13 cells overexpressing hIAPP and reduction of spectrin cleavage by concurrent use of a calpain inhibitor (Fig. 6, D and E).

There are other lines of evidence to support Ca\(^{2+}\)/calpain activation findings. Overexpression of calmodulin in mice results in insulin secretion defects, loss of beta cells, and diabetes (53, 54). Also, abnormal elevation of cytosolic Ca\(^{2+}\) induces ER stress and beta cell apoptosis by palmitate (55). Finally, ER stress is associated with Ca\(^{2+}\) efflux from the ER (56), and the ER stress marker, nuclear CHOP, was detected in beta cells from humans with T2DM but only rarely in beta cells from obese non-diabetic subjects (13).

In conclusion, we report that the overexpression of human islet amyloid protein hIAPP leads to the formation of toxic oligomers, an elevation of cytosolic Ca\(^{2+}\), and hyperactivation of the Ca\(^{2+}\)-dependent protease calpain. Furthermore, inhibition of calpain activity attenuates the beta cell apoptosis associated with chronic hyperactivation of Ca\(^{2+}\)/calpain. Identification of calpain-cleaved fragments of α-spectrin in beta cells from humans with T2DM but not in control subjects indicates that the Ca\(^{2+}\)-dependent protease calpain may play a key role in the pathophysiology of T2DM. These findings suggest that sustained hyperactivity of the Ca\(^{2+}\)/calpain signaling pathway may be the molecular basis for oligomeric protein conformational disorders such as T2DM and Alzheimer disease (16, 48).

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REFERENCES
1. Weyer, C., Bogardus, C., Mott, D. M., and Pratley, R. E. (1999) J. Clin. Invest. 104, 787–794
2. Haataja, L., Gurlo, T., Huang, C. J., and Butler, P. C. (2008) Endocr. Rev. 29, 303–316
3. Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003) Diabetes 52, 102–110
4. Hardy, J., and Selkoe, D. J. (2002) Science 297, 353–356
5. Liu, L. F., Kuo, Y. M., Roher, A. E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J. H., Rydel, R. E., and Rogers, J. (1999) Am. J. Pathol. 155, 853–862
6. Janson, J., Soeller, W. C., Roche, P. C., Nelson, R. T., Torchio, A. J., Kretzler, D. K., and Butler, P. C. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7283–7288
7. Janson, J., Ashley, R. H., Harrison, D., McIntyre, S., and Butler, P. C. (1999) Diabetes 48, 491–498
8. Chartier-Harlin, M. C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J., et al. (1991) Nature 353, 844–846
9. Matvejenko, A. V., and Butler, P. C. (2006) Ilar J. 47, 225–233
10. Sparr, E., Engel, M. F., Sakharov, D. V., Sprong, M., Jacobs, J., de Kruijff, B., Höppner, J. W., and Killian, J. A. (2004) FEBS Lett. 577, 117–120
11. Jayasinghe, S. A., and Langen, R. (2007) Biochim. Biophys. Acta 1768, 2002–2009
12. Huang, C. J., Haataja, L., Gurlo, T., Butler, A. E., Wu, X., Soeller, W. C., and Butler, P. C. (2007) Am. J. Physiol. Endocrinol. Metab. 293, E1656–E1662
13. Huang, C. J., Lin, C. Y., Haataja, L., Gurlo, T., Butler, A. E., Rizza, R. A., and Butler, P. C. (2007) Diabetes 56, 2016–2027
14. Lin, C. Y., Gurlo, T., Kayed, R., Butler, A. E., Haataja, L., Glabe, C. G., and
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Butler, P. C. (2007) Diabetes 56, 1324–1332
15. Gurlo, T., Ryazantsev, S., Huang, C. J., Yeh, M. W., Reber, H. A., Hines, O. J., O’Brien, T., Glabe, C. G., and Butler, P. C. (2010) Am. J. Pathol.
16. Saito, K., Elce, J. S., Hamos, J. E., and Nixon, R. A. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2628–2632
17. Bevers, M. B., and Neumar, R. W. (2008) J. Cereb. Blood Flow Metab. 28, 655–673
18. Demuro, A., Mina, E., Kayed, R., Milton, S. C., Parker, I., and Glabe, C. G. (2005) J. Biol. Chem. 280, 17294–17300
19. Kuwako, K., Nishimura, I., Uetsuki, T., Saido, T. C., and Yoshikawa, K. (2004) J. Virol.
20. Norberg, E., Gogvadze, V., Ott, M., Horn, M., Uhlen, P., Orrenius, S., and Zhivotovsky, B. (2008) Cell Death Differ. 15, 1857–1864
21. Song, Y., You, N. C., Hsu, Y. H., Sul, J., Wang, L., Tinker, L., Eaton, C. B., and Liu, S. (2007) Hum. Mol. Genet 16, 2960–2971
22. Wu, H. Y., and Lynch, D. R. (2006) Mol. Neurobiol. 33, 215–236
23. Hohmeier, H. E., Mulder, H., Chen, G., Henkel-Rieger, R., Prentki, M., and Newgard, C. B. (2000) Diabetes 49, 424–430
24. Rao, A., Luo, C., and Hogan, P. G. (1997) Annu. Rev. Immunol. 15, 707–747
25. Young, S. H., Ennes, H. S., McRoberts, J. A., Chaban, V. V., Dea, S. K., and Mayer, E. A. (1999) Am. J. Physiol. 276, G1204–G1212
26. Cowan, C. M., Fan, M. M., Fan, J., Shehadeh, J., Zhang, L. Y., Graham, R. K., Hayden, M. R., and Raymond, L. A. (2008) J. Neurosci. 28, 12725–12735
27. Dutta, S., Chiu, Y. C., Probert, A. W., and Wang, K. K. (2002) Biol. Chem. 383, 785–791
28. Ryazantsev, S., Yu, W. H., Zhao, H. Z., Neufeld, E. F., and Ohmi, K. (2007) Mol. Genet. Metab. 90, 393–401
29. Kayed, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W., and Glabe, C. G. (2003) Science 300, 486–489
30. Saisho, Y., Manesso, E., Gurlo, T., Huang, C. J., Toffolo, G. M., Cobelli, C., and Butler, P. C. (2009) Am. J. Physiol. Endocrinol. Metab. 296, E89–E96
31. Khemtmourian, L., Killian, J. A., Höppenner, J. W., and Engel, M. F. (2008) Exp. Diabetes Res. 2008, 421287
32. Engel, M. F., Khemtmourian, L., Kleijer, C. C., Meeldijk, H. J., Jacobs, I., Verkleij, A. F., de Kruijff, B., Killian, J. A., and Höppenner, J. W. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 6033–6038
33. Michelaleti, F., Liprandi, F., Chemello, M. E., Ciarlet, M., and Ruiz, M. C. (1995) J. Virol. 69, 3838–3847
34. Van Coppenolle, F., Vanden Abeele, F., Slomianny, C., Flourakis, M., Hesketh, J., Dewailly, E., and Prevorskaya, N. (2004) J. Cell Sci. 117, 4135–4142
35. Paschen, W., Hotop, S., and Aufenberg, C. (2003) Cell Calcium 33, 83–89
36. Hood, J. L., Brooks, W. H., and Roszman, T. L. (2004) J. Biol. Chem. 279, 43126–43135
37. Johnson, G. V., and Guttmann, R. P. (1997) BioEssays 19, 1011–1018
38. Piwocka, K., Vejda, S., Cotter, T. G., O’Sullivan, G. C., and McKenna, S. L. (2006) Blood 107, 4003–4010
39. Pettigrew, L. C., Holtz, M. L., Craddock, S. D., Minger, S. L., Hall, N., and Geddes, J. W. (1996) J. Cereb. Blood Flow Metab. 16, 1189–1202
40. Takano, J., Tomioka, M., Tsubuki, S., Higuchi, M., Iwata, N., Itohara, S., Maki, M., and Saido, T. C. (2005) J. Biol. Chem. 280, 16175–16184
41. Giunti, R., Gamberucci, A., Fulceri, R., Bânhegy, G., and Benedetti, A. (2007) Arch. Biochem. Biophys. 462, 115–121
42. Ferreiro, E., Oliveira, C. R., and Pereira, C. M. (2008) Neurobiol. Dis. 30, 331–342
43. Casas, S., Novials, A., Reimann, F., Gomis, R., and Gribble, F. M. (2008) Diabetologia 51, 2252–2262
44. Rardon, D. P., Cefali, D. C., Mitchell, R. D., Seiler, S. M., Hathaway, D. R., and Jones, L. R. (1990) Circ. Res. 67, 84–96
45. Nagata, E., Tanaka, K., Gomi, S., Mibara, B., Shirai, T., Nogawa, S., Nozaki, H., Mikoshiba, K., and Fukushima, Y. (1994) Neuroscience 61, 983–990
46. Mirzabekov, T. A., Lin, M. C., and Kagan, B. L. (1996) J. Biol. Chem. 271, 1988–1992
47. Pereira, C., Ferreiro, E., Cardoso, S. M., and de Oliveira, C. R. (2004) J. Mol. Neurosci. 23, 97–104
48. Trinchese, F., Fa’, M., Liu, S., Zhang, H., Hidalgo, A., Schmidt, S. D., Yamaguchi, H., Yoshii, N., Mathews, P. M., Nixon, R. A., and Arancio, O. (2008) J. Clin. Invest. 118, 2796–2807
49. Stabach, P. R., Cianci, C. D., Glantz, S. B., Zhang, Z., and Morrow, J. S. (1997) Biochemistry 36, 57–65
50. Rajgopal, Y., and Vemuri, M. C. (2002) Neurosci. Lett. 321, 187–191
51. Siman, R., Zhang, C., Roberts, V. L., Pitts-Kiefer, A., and Neumar, R. W. (2005) J. Cereb. Blood Flow Metab. 25, 1433–1444
52. Grant, R. J., Sellings, L. H., Crocker, S. J., Melloni, E., Park, D. S., and Clarke, P. B. (2009) Neuroscience 158, 558–569
53. Epstein, P. N., Ribar, T. J., Decker, G. L., Yaney, G., and Means, A. R. (1992) Endocrinology 130, 1387–1393
54. Gómez Dunn, C. L., Atwater, I., Epstein, P. N., and Gagliardino, J. J. (1994) Virochows Arch. 425, 73–77
55. Gwiazda, K. S., Yang, T. L., Lin, Y., and Johnson, J. D. (2009) Am. J. Physiol. Endocrinol. Metab. 296, E690–E701
56. Resende, R., Ferreiro, E., Pereira, C., and Oliveira, C. R. (2008) J. Neurosci. Res. 86, 2091–2099