Fibrillogenic Amylin Evokes Islet \( \beta \)-Cell Apoptosis through Linked Activation of a Caspase Cascade and JNK1*

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Fibrillogenic human amylin elicits pancreatic \( \beta \)-cell apoptosis that may contribute to development of type-2 diabetes. Here, we demonstrated that activation of a caspase cascade is necessary for induction of apoptosis by fibrillogenic amylin variants in two pancreatic \( \beta \)-cell lines. Human amylin, as well as truncated 8–37 human amylin, evoked sequential activation of caspases-8 and -3, and apoptosis, whereas non-\( \beta \)-sheet forming and non-fibrillogenic homologs, such as [25,28,29 triprolyl] human amylin, did not, implying that the \( \beta \)-sheet conformer is required for human amylin-induced caspase activation. Significant inhibition of apoptosis was evoked by a selective caspase-1 inhibitor, indicating that caspase-1 is also essential for activation of the caspase cascade. Furthermore, we showed that specific jnk1 antisense oligonucleotides, which suppress phosopho-JNK1 expression, effectively decreased human amylin-induced activation of c-Jun. Studies of the interplay between the caspase cascade and the JNK pathway showed that both apoptosis and caspase-3 activation were suppressed by treatment with a JNK inhibitor and by transfection of antisense jnk1 oligonucleotides or antisense c-jun, whereas a selective inhibitor of caspases-1 and -3 prevented apoptosis but not c-Jun activation. Thus, the JNK1 activation preceded activation of caspases-1 and -3. However, selective JNK inhibition had no effect on caspase-8 activation, and selective caspase-8 inhibition only partially suppressed apoptosis and c-Jun activation, indicating that caspase-8 may partially act upstream of the JNK pathway. Our studies demonstrate a functional interaction of a caspase cascade and JNK1. Fibrillogenic amylin can evoke a JNK1-mediated apoptotic pathway, which is partially dependent and partially independent of caspase-8, and in which caspase-3 acts as a common downstream effector.

Pancratic islet amyloid commonly occurs in T2DM\(^*\) (type-2 diabetes mellitus), where it may contribute to islet \( \beta \)-cell dys-

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\(^\ddagger\) The abbreviations used are: T2DM, type-2 diabetes mellitus; Ac, acetyl; AFC, 7-amino-4-trifluoromethyl coumarin; AP-1, activator protein 1; AS-c-jun, antisense c-jun; ASO-jnk1, jnk1 antisense oligonucleotide; ASO-jnk2, jnk2 antisense oligonucleotide; BCA, bicinchoninic acid; ECL, enhance chemiluminescence; FMK, fluoromethyl ketone; GFP, green fluorescent protein; JNK, c-Jun NH\(_2\)-terminal kinase; POD, per-oxidase; S-c-jun, sense c-jun; SO-jnk1, jnk1 sense oligonucleotide; SO-jnk2, jnk2 sense oligonucleotide; z, benzylxocarbonyl.
Caspase Activation Mediates Human Amylin-evoked Apoptosis

Fat intake and/or changes in lipid metabolism could be factors leading to development of islet amyloid (26). The association of progressively decreasing islet mass with increasing amyloid deposits could lead to progressive impairment of insulin secretion, declining glucose tolerance, and eventually the development of fasting hyperglycemia (12).

The underlying molecular mechanisms by which human amylin elicits β-cell death remain to be fully elucidated. There is evidence that the cytotoxic action of human amylin correlates with an increased cellular oxidative response and low density lipoprotein uptake (27). It has also been proposed that human amylin might kill β-cells through aggregation and formation of membrane ion channels that could in turn disturb intracellular calcium homeostasis (28). However, our own studies of amylin-evoked cell death in RINm5F β-cells showed that calcium homeostasis remained normal until late in the cytotoxic process (29). There is also evidence that it is small or intermediate-size precursors of the human amylin fibrils rather than large, mature amyloid deposits that cause membrane instability and subsequent islet β-cell death (30). Furthermore, there is now substantive evidence that apoptosis (programmed cell death) is the mode of death induced by human amylin in islet β-cells (7, 9–11, 29). Contact of small human amylin aggregates with the β-cell membrane is probably required for the initiation of apoptosis, indicating that human amylin might exert a direct cytotoxic effect through membrane interactions (7). We have previously studied the cytotoxicity of fibril-forming amylin in cultured β-cells, where we demonstrated that exogenously applied human amylin significantly inhibits RINm5F β-cell proliferation, inducing apoptosis associated with increased expression of known apoptosis-related genes p53 and p21WAF1 (10). Human amylin elicited growth arrest and apoptosis in RINm5F cells in a manner dependent upon time and cell passage, indicating a link between aggregate-evoked apoptosis and the cell cycle (10). The roles of c-Jun and of the JNK (c-Jun NH2-terminal kinase/stress-activated protein kinase) pathway in this apoptotic process were also investigated. Human amylin treatment caused increased expression and site-specific phosphorylation of c-Jun accompanied by increased activator protein 1 (AP-1) DNA binding and c-Jun transcriptional activity (9). In addition, human amylin evoked activation of Jun kinase, and apoptosis was suppressed by expression of an antisense c-jun (AS c-jun) oligonucleotide (8, 26, 31). Consequently, targeting the β-cell death process evoked by human amylin is increasingly recognized as a new potential therapeutic approach for suppression of β-cell failure in T2DM.

Apoptosis is a genetically controlled process of cell suicide that is essential for the normal development and tissue homeostasis of multicellular organisms (32). However, abnormal cell death regulation can contribute to a variety of diseases, including autoimmunity, and cancer, as well as neurodegenerative disorders and diabetes (33–36). One key component of the apoptotic machinery is the caspase family, which comprises a group of intracellular aspartyl-specific cysteine proteases with at least 15 members (35, 37, 38). Caspase activation plays a central role in the execution of apoptosis and caspases have been identified as targets for therapeutic intervention, where apoptosis occurs inappropriately (37, 39). Activation of caspase-3 is essential for the induction of apoptosis in mouse β-cells transfected with human Fas (40). Caspase-3 is activated by the signaling pathways for CD95/Fas and tumor necrosis factor α and is the furthest upstream caspase in the CD95 apoptotic pathway (37, 39). The activation of the caspase pathway and the resultant cleavage of substrate molecules, mediate a critical function in the regulation and execution of cell death.

However, the detailed biological roles of each caspase, their interrelationships and the interactions between caspase(s) and the other cellular signal pathways, such as the JNK or p38 pathways, in the regulation of apoptosis are not well defined and are currently the subject of intensive investigation. There is data indicating that the JNK and p38 pathways may function downstream of caspase activation in apoptotic signaling, because the activation of JNK and p38 by many apoptotic stimuli can be blocked by caspase inhibitors (35, 41, 42). In addition, death receptor-mediated JNK/p38 activation requires caspase-8, because Fas antibody did not activate JNK and p38 in caspase-8-deficient cells (41, 43). However, studies also showed that overexpression of upstream components of the JNK and p38 pathway, such as apoptosis signal-regulating kinase 1, can induce caspase activation and apoptotic cell death, indicating that JNK and p38 could act upstream of caspases in apoptotic signaling (44).

It is not known which caspase(s) play a major role in human amylin-induced apoptosis of islet β-cells and how caspase(s) might cooperate with JNK to regulate this apoptotic process. In the current study, possible roles for caspases in human amylin-induced apoptosis of islet β-cells were investigated using two insulinoma β-cell lines, rat RINm5F and human CM cells. We found that human amylin induced time- and dose-dependent activation of initiator caspase-8, caspase-1, and downstream effector caspase-3 and that apoptosis could be suppressed by treatment with selective caspase inhibitors. Caspase activation was also examined in β-cells treated with different human amylin variants. The non-fibrillogenic agonist, [25,28,29triprolyl]human amylin, elicited neither apoptosis nor caspase activation, whereas by contrast, the truncated but fibrillogenic α-37human amylin evoked cytotoxicity similar to that of the wild-type protein. Further studies on the relationship between caspase activation and the JNK pathway demonstrated that human amylin might simultaneously induce multiple pathways that mediate apoptotic cell death. We showed here that JNK1 activation occurs upstream of caspase-3, because blocking of the JNK1 activation by a specific jnk1 antisense oligonucleotide inhibited caspase-3 activation. In addition, activation of the JNK pathway is dependent on the initiator caspase-8, because suppression of caspase-8 activity significantly, although incompletely, inhibited c-Jun activation. However, the JNK pathway could also be activated independent of caspase-8 activation. We expect that these studies will lead to a greater understanding of the molecular basis for the cause and progression of β-cell dysfunction in T2DM and may thus reveal new therapeutic targets for this common disease.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The rat insulinoma cell line RINm5F, was a gift of Dr. H. K. Oie (National Institutes of Health, Bethesda, MD) and the human insulinoma cell line CM, was kindly provided by Dr. P. Pozzilli (Department of Diabetes & Metabolism, St. Bartholomew’s Hospital, London). This cell line was established from cells isolated from ascitic fluid of a patient with a malignant insulinoma (45). RINm5F and CM cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen), 290 mg/ml l-glutamine, 100 IU/ml penicillin, 10 μg/ml streptomycin, and 2.5 mg/ml NaHCO3 at 37 °C in a humidified incubator with 5% CO2 as previously described (9, 10). All experiments were performed using RINm5F cells between passages 26 and 35 and CM cells between passages 6 and 28. For amylin treatment, stock solutions (500 μM) were prepared by dissolving synthetic human amylin (Lot 524836, Bachem, CA), rat amylin (Lot ZM275, Bachem, CA), or [25,28,29triprolyl]human amylin (L31214, Auspep) powder in water and incubating at room temperature for 10 min.
Cells were cultured to ~70% confluence, after which peptides were added to a final concentration of 10 μM. Cultures were then incubated for further periods of 0.5, 1, 2, 4, 8, 16, or 24 h, respectively. Untreated control cells were also cultured and processed in an otherwise equivalent manner.

For JNK inhibition treatment, we employed the JNK inhibitor 1, (L)-Form (Calbiochem), which contains the minimal 20-amino acid inhibitory domain of islet brain protein (critical for interaction with JNK) linked to the 10-amino acid HIV-TAT$_{22-33}$ sequence as a carrier peptide and containing two proline residues as a spacer (46). JNK inhibitor 1 or JNK inhibitor 1-negative control peptide were applied to RINm5F or CM cell cultures, at final concentrations of 1 μM, 1 h before the addition of human amylin. Caspase activities were measured after 16 h treatment and apoptosis after 24 h treatment.

**Analysis of Caspase Activity**—RINm5F and CM cells were cultured in 24-well plates and exposed to synthetic wild-type human amylin or rat amylin, α,β,γ,δ-human amylin or [25,28,29]triprolyl human amylin for specified time periods as described above. For treatment with caspase inhibitors, the specific peptide–FMK inhibitors (Bachem, Switzerland) were dissolved in Me$_2$SO and added directly to the cultured cells (1 μg/ml), 30 min prior to amylin treatment. Control cells received the same volume of Me$_2$SO alone (final Me$_2$SO concentration of 0.1% v/v), which had no effect on cell proliferation and viability. Cells were rinsed with PBS, and the cell lysate was prepared by adding 100 μl of lysis buffer (10 mM HEPES, pH 7.4, 1% EDTA, 0.1% CHAPS, 0.1% DTT) to each sample and freshly added 5 mM dithioretilol and 1× Protease Inhibitor Mixture, Roche Applied Science. The plate was rocked for 20 min on ice to facilitate lysis, followed by three to four freeze-thaw cycles with suspension and transfer between dry ice and room temperature. The lysed cells were centrifuged at 4 °C for 30 min at 13,000 × g, and the supernatant was collected for caspase activity assay in a 96-well plate. The protein concentration was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). Caspase activity was quantified by measuring liberation of 7-amino-4-trifluoromethyl coumarin (AFM) from the specific caspase AFC substrates (Bio-Rad) according to the manufacturer’s instructions. 100 μg of each cell extract was incubated in reaction buffer (20 mM HEPES, pH 7.2, 2 mM EDTA, 1% CHAPS with freshly added 10 mM DTT) containing 40 ng/μl fluorogenic AFC substrate in the presence or absence of caspase inhibitor in a black 96-well plate at 37 °C for 3–4 h. The AFC released was measured on a fluorescence plate reader (Spectra Max 340, Molecular Devices) at 540-nm emission.

**Immunocytochemistry**—Human CM cells were cultured on 24-well plates and exposed to wild type human or rat amylin, α,β,γ,δ-human amylin, or [25,28,29]triprolyl human amylin, for 16 h as described above. Cells were then fixed with 4% paraformaldehyde in 0.1 x phosphate buffer (pH 7.4) for 15 min and subjected to immunocytochemical staining as previously described (9). Fixed cells were incubated overnight at 4 °C with rabbit anti-cleaved-caspase-3 antibody (Cell Signaling, 1:250 dilution, 200 μl/well). Cells were washed three times for 10 min with PBS and incubated with biotinylated goat anti-rabbit IgG (1:250, Sigma) for 4 h at room temperature, followed by further PBS washes and incubation with extrAvidin-peroxidase (1:250, Sigma) for 2 h. The reaction was visualized by the addition of 0.01% diaminobenzidine and hydrogen peroxide. Immunostained cells were viewed and photographs taken using inverted-phase microscopy (Nikon).

**Cell Transfection**—Sense and antisense-c-jun constructs were prepared and transfected into CM cells using FuGENE 6 (Roche Applied Science) as previously described (9). First, cells were plated on 24-well plates at a density of 2 × 10$^5$ cells per well, 24 h before transfection. A total of 0.6 μg of plasmid DNA was mixed with 3 μl of FuGENE 6 transfection reagent in OPTI-MEM 1-reduced serum medium (Invitrogen) and incubated for 20 min at room temperature. Cells were then incubated at 37 °C for 6 h in OPTI-MEM 1-reduced serum medium containing plasmid DNA and FuGENE 6 reagent mixture and incubated for a further 24 h in complete RPMI 1640 medium prior to exposure to human amylin. Transfection efficiency was normalized by co-transfection with the pEGFP plasmid (Clontech) at a ratio of 3:1. Transfected cells were detected by measuring green fluorescent protein (GFP) expression using the fluorescence plate reader (Spectra Max) at λ = 488-nm excitation and 507-nm emission. Cells were lysed 16 h after human amylin treatment, and caspase activity was measured.

For transfection of sense and antisense jnk oligonucleotides, CM cells were cultured and incubated with 0.2 μg of sense SO-jnk1, ASO-jnk1, SO-jnk2, or ASO-jnk2 in the presence of 10 μg/ml Lipofectin reagent (Invitrogen) in RPMI 1640 medium for 24 h. Cells were then exposed to human amylin for 4 or 16 h, and cell lysate was prepared for Western blot or caspase activity assay, respectively. The sequences of the jnk-specific oligonucleotides used were as follows: jnk1 antisense, 5′-GTCACGCTGCTCTTCTCATGAT-3′; jnk1 sense, 5′-ATGAGCAAGAACGGTGAC-3′; jnk2 antisense, 5′-GTACATTACTACTGTGCTAC-AT-3′; jnk2 sense, 5′-ATGAGCACAGTAAATGTGAC-3′. These oligonucleotides sequences correspond to the first 21 bases of the cDNA sequences for jnk1 and jnk2. All oligonucleotides were phosphorothioate-modified and purified by high-performance liquid chromatography (Sigma).

**Western Blot Analysis**—CM cells were treated with caspase inhibitors as described above. Cells were preincubated with caspase-1, -3, -7 or -8 inhibitors, 30 min before exposure to human amylin for 16 h. Total cellular lysates were prepared as described above. The sequences of the jnk-specific oligonucleotides used were as follows: jnk1 antisense, 5′-GTCACGCTGCTCTTCTCATGAT-3′; jnk1 sense, 5′-ATGAGCAAGAACGGTGAC-3′; jnk2 antisense, 5′-GTACATTACTACTGTGCTAC-AT-3′; jnk2 sense, 5′-ATGAGCACAGTAAATGTGAC-3′. These oligonucleotides sequences correspond to the first 21 bases of the cDNA sequences for jnk1 and jnk2. All oligonucleotides were phosphorothioate-modified and purified by high-performance liquid chromatography (Sigma).

**Expression and purification**—Purified rh-human amylin was expressed and purified by high-performance liquid chromatography (Sigma).

**RESULTS**

**Caspase Activation in Human Amylin-treated Pancreatic Islet β-Cells**—Two insulinoma β-cell lines were used in the current study to investigate the possible involvement of caspase activation in apoptosis induced by human amylin in islet β-cells. It has been previously determined that the calculated EC$_{50}$ value for the concentration dependence of human amylin...
cytotoxicity in islet \( \beta \)-cells was 10 \( \mu \)M (29). Studies of time dependence of cell killing by 10 \( \mu \)M human amylin indicated that cell death reached half-maximal after 24 h. Here, RINm5F (passages 27–35) and CM (passages 6–15) cells were cultured in the presence or absence of 10 \( \mu \)M human amylin for up to 24 h and caspase activities (caspase-1, -2, -3, -6, -8, and -9) determined at specific times during this period, by measurement of optical changes caused by cleavage of specific oligopeptide substrates. Fig. 1 shows that human amylin-evoked apoptosis resulted in time-dependent activation of caspase-8 and caspase-3 in RINm5F and CM cells. Activation of initiator caspase-8 was detected at 30 min after initiation of human amylin treatment (2- to 3-fold increased in RINm5F cells and 4- to 5-fold increased in CM cells), followed by a subsequent decrease in its protease activity by 4 h (Fig. 1, A and B). Activation of effector caspase-3 was detected after 8-h exposure, became maximal at 16 h (4- to 5-fold increased in RINm5F cells and 6- to 7-fold increased in CM cells) and had declined at 24 h (Fig. 1, C and D). These results indicate that caspase-8 acts upstream from caspase-3. In addition, this time-dependent sequential activation of caspase-8 (Fig. 1, A and B) and caspase-3 (Fig. 1, C and D) was blocked both by a selective inhibitor of caspase-8 (z-LETD-FMK) and of caspase-3 (z-DEVD-FMK), which further demonstrated that the caspase activation is a specific response of \( \beta \)-cells to human amylin treatment.

In contrast, we observed very little change in protease activity for caspases-1, -2, -6, and -9 in response to human amylin treatment (data not shown), suggesting that the contribution of these caspases to the induction of \( \beta \)-cell apoptosis is likely to be insignificant. Fig. 2 shows time-course studies of caspase activation in RINm5F and CM cells treated with rat amylin and two human amylin variants, \([25,28,29\text{triprolyl}]\)human amylin and \(8–37\)human amylin, which served as peptide treatment comparisons. Non-fibrillogenic rat amylin elicited neither apoptosis nor activation of caspase-8 or caspase-3, thus indicating that the sequence differences between human and rat amylin and the fibrillogenic property of the human peptide are necessary for caspase activation. The substitution of prolyl residues in the human amylin analogue is thought to suppress the \( \beta \)-sheet formation and fibrillogenic propensity of the wild-type protein (21). Indeed, we detected no fibril formation by this synthetic homologue of human amylin under the experimental conditions tested (data not shown). Here, we assessed apoptosis in \( \beta \)-cells treated with \([25,28,29\text{triprolyl}]\)human amylin and \(8–37\)human amylin using a quantitative cell death-detection ELISA, by measuring the enrichment of mono- and oligonucleosomes in the cytoplasm of apoptotic cells. \([25,28,29\text{triprolyl}]\)human amylin did not induce time-dependent caspase activation or apoptosis in either RINm5F or CM cells (Figs. 2 and 3), indicating that formation of \( \beta \)-sheet structure is important for
human amylin-induced caspase activation. By contrast, human amylin, which lacks the NH₂-terminal seven amino acids and the Cys²-Cys⁶ intramolecular disulfide bond, did evoke activation of caspase-8 and caspase-3 (Fig. 2) and showed cytotoxicity similar to that of wild-type human amylin (Fig. 3). Thus, the NH₂-terminal sequence and intramolecular disulfide bond are not essential to trigger caspase activation and apoptosis. This observation contrasts with the activation by amylin of responses mediated through its physiological receptor, for which these molecular structures are essential (47–49). In addition, this truncated human amylin exhibits in vitro kinetics of fibril formation that are similar but not identical to those of human amylin (16).

Activation of caspases was also examined in RINm5F and CM cells exposed to different concentrations of human amylin (5, 10, 20, or 30 μM, respectively). Our results showed that both caspase-8 and caspase-3 can be activated in a dose-dependent manner (data not shown). In addition, activation of caspase-3 was further demonstrated by immunocytochemical studies. The results, shown in Fig. 4, demonstrated a significant increase in antibody staining for cleaved caspase-3 at 16 h after exposure of CM cells to full-length human amylin and 8–37 human amylin, whereas cells treated with [25,28,29triprolyl]human amylin exhibited no increase in cleaved caspase-3 immunoreactivity compared with untreated controls. Fig. 4 also confirms the occurrence of changes in cell morphology, such as cell shrinkage, following human amylin treatment, as previously described (11). Furthermore, the effect of human amylin on the expression of pro-caspase mRNAs was examined using Northern blot analysis. The results revealed that the level of

**Fig. 2. Time-course studies of caspase-8 and caspase-3 activation in apoptosis evoked by amylin variants.** Caspase-8 (A and B) and caspase-3 (C and D) activation in RINm5F (A and C) and CM (B and D) ß-cells treated with vehicle control (co, ●), rat amylin (rA, ■), 8–37 human amylin (hA8–37, ★) or [25,28,29triprolyl]human amylin (Tripro-hA, ◊). Caspase activities were measured using synthetic fluorogenic oligopeptide substrates z-LETD-AFC for caspase-8 and z-DEVD-AFC for caspase-3. Fluorescence was measured at excitation λ = 400 nm and emission = 540 nm. All values are mean ± S.E. of four independent experiments, each performed in duplicate. Data were analyzed by ANOVA; *, p < 0.01; +, p < 0.05 versus control.

**Fig. 3. Detection of apoptosis induced by amylin variants.** RINm5F and CM cells were treated with 10 μM rat amylin (rA), full-length human amylin (hA), 8–37 human amylin (hA8–37), or [25,28,29triprolyl]human amylin (Tripro-hA). Apoptosis was assessed using a quantitative cell death detection ELISA at 24 h post-treatment. Results shown represent enrichment of nucleosomes, which have been calculated relative to the untreated control value, which was set at one. All values are mean ± S.E. of three independent experiments, each performed in duplicate. Data were analyzed by ANOVA; *, p < 0.01 versus control.
mRNA for pro-caspase-3 and pro-caspase-8 in both RINm5F and CM cells remained unchanged throughout 24-h exposure (data not shown), indicating that human amylin had no effect on transcriptional expression of pro-caspase-3 and pro-caspase-8.

Effects of Caspase Inhibitors on Human Amylin-evoked Apoptosis—To investigate the effects of caspase inhibitors on human amylin-evoked apoptosis, RINm5F and CM cells were preincubated for 30 min with the selective caspase-1, -2, -3, -6, -8, -9, or pan caspase inhibitors and then treated with human amylin or 8–37 human amylin for 16 h. Caspase activity was measured as described above. Apoptosis was detected in parallel experiments using a quantitative cell death detection ELISA. The results, shown in Fig. 5, demonstrate that pretreatment of CM cells with peptidase caspase inhibitors before exposure to human amylin or 8–37 human amylin, result in substantial inhibition of oligonucleosomal DNA fragmentation. In the absence of inhibitor, human amylin induced significant apoptosis accompanied by sequential activation of caspase-8 and caspase-3 after 16 h of exposure, whereas most but not all cells were protected against apoptosis in the presence of 1 μg/ml caspase inhibitor. We found that the selective caspase-8 inhibitor, which blocks caspase-8 activation, inhibited apoptosis incompletely, whereas the general caspase inhibitor and the selective caspase-3 inhibitor suppressed both caspase-3 activation and apoptosis to a greater extent. Fig. 5 also shows that preincubation with selective caspase inhibitors of CM cells subsequently treated with 8–37 human amylin, resulted in a similar degree of protection shown by cells treated with full-length human amylin. In addition, lesser concentrations of the inhibitor caused a lower level of protection (data not shown), indicating that the protective effect of caspase inhibitors was dose-dependent. It is further shown in Fig. 5 that the selective caspase-1 inhibitor (z-WEHD-FMK) could protect β-cells against human amylin-evoked apoptosis, although no change in caspase-1 activity was detected in our previous experiments. The protective effect of the caspase-1 inhibitor was noticeably less effective than the other protective inhibitors, whereas no equivalent protective effect was detected for inhibitors of caspase-2, -6, and -9. We then further investigated the effect of the selective caspase-1 inhibitor on activation of caspase-3, because studies by other researchers have shown that caspase-3 can be proteolytically activated by caspase-1, for example in Fas-mediated cell death (50). Fig. 6 shows that activation of caspase-3 in CM cells treated with wild-type human amylin or 8–37 human amylin was suppressed significantly by treatment with the selective inhibitor of caspase-1, indicating that caspase-1 may act upstream of caspase-3 and that it may also be required for amylin-mediated apoptosis in islet β-cells. Our studies thus demonstrate that activation of caspases-8, -1, and -3 are necessary for the induction by human amylin of apoptosis in pancreatic islet β-cells.

Effects of Caspase Inhibitors on Human Amylin-induced Activation of c-Jun—We have previously shown that increased expression and activation of c-Jun is required for human amylin-induced apoptosis (5) wherein c-Jun was activated through site-specific phosphorylation at ser63. Here, we first determined the specific isoform(s) of JNK that catalyze phosphorylation of c-Jun using antisense oligonucleotides specifically targeted to isoforms of jnk1(s). RINm5F and CM cells were treated with antisense or sense oligonucleotides specific for jnk1 with JNK1-specific antisense oligonucleotides (ASO-jnk1) prior to exposure of 10 μM of full-length human amylin (hA) or 8–37 human amylin (hA8–37). Apoptosis was assessed using quantitative cell death detection ELISA at 24 h post-treatment. Results shown represent enrichment of nucleosomes that have been calculated relative to the untreated control value, which was set at one. All values are mean ± S.E. of three independent experiments, each performed in duplicate. Data were analyzed by ANOVA; *, p < 0.01 versus vehicle.
we found no detectable changes in the protein levels of phospho-JNK1(p46) and phospho-JNK2(p54) between cells treated with JNK2-specific antisense and sense oligonucleotides (ASO-jnk2 and SO-jnk2) (Fig. 7A). We next examined the direct activation of c-Jun by JNK1. The same Western blot membrane was stripped and subsequently re-probed with phospho-c-Jun-Ser63-specific antibody. The results show that suppression of JNK1 activation by ASO-jnk1 result in a marked reduction in activation of c-Jun, whereas SO-jnk1 had no effect on c-Jun activation, as compared with the untreated control (Fig. 7B). These results demonstrate that c-Jun is activated by phosphorylation at Ser63 through activation of JNK1.

Furthermore, the relationship between human amylin-induced caspase activation and the JNK pathway was investigated by examining activation of c-Jun in cells treated with various caspase inhibitors. RINm5F and CM cells were preincubated with selective caspase-1, -3, or -8 inhibitors prior to exposure to wild-type human amylin or 8–37 human amylin, as described above. Activation of c-Jun was examined by Western blot analysis using phospho-specific c-JunSer63 antibodies, and Fig. 7C shows the results of a representative study. We found that the protein levels of phospho-c-JunSer63 decreased partially in CM cells preincubated with caspase-8 inhibitor before exposure to human amylin, whereas the Western blot membrane was shifted and subsequently re-probed with phospho-c-Jun-Ser63-specific antibody. The results show that suppression of JNK1 activation by ASO-jnk1 result in a marked reduction in activation of c-Jun, whereas SO-jnk1 had no effect on c-Jun activation, as compared with the untreated control (Fig. 7B). These results demonstrate that c-Jun is activated by phosphorylation at Ser63 through activation of JNK1.

Suppression of c-Jun Activation Inhibits Activation of Caspase-3 but Not of Caspase-8—The interplay between the caspase cascade and the JNK pathway was further studied by measuring caspase activity in β-cells pretreated with a JNK inhibitor (Fig. 8) or transfected with AS-c-jun or ASO-jnk1 (Fig. 9). Fig. 8 shows activity of caspase-3 and caspase-8 in RINm5F and CM cells preincubated with either JNK inhibitor 1 or a JNK inhibitor-negative control molecule at 1 μmol/liter, for 1 h prior to exposure to human amylin. We found that the caspase-3 activity evoked by human amylin was suppressed by JNK inhibitor 1 in both RINm5F and CM cells, whereas suppression of the JNK pathway by JNK inhibitor 1 had no effect on caspase-8 activation. In contrast, the JNK inhibitor-negative control, an inactive analogue of active JNK inhibitor (casp8 inhibitor), caspase-3 inhibitor (casp3 inhibitor), or caspase-1 inhibitor (casp1 inhibitor), before exposure to human amylin for 4 h. Western blotting was performed as described above. All changes of protein levels were calculated based on levels in corresponding human amylin-treated cells, which were set at one. Results shown are the average of three independent experiments.
no effect on caspase-8 activity (Fig. 9). Our data thus demonstrate a link between this caspase cascade and JNK1 in the regulation of apoptosis evoked by human amylin in islet \( H9252 \)-cells.

These results suggest that the initiator caspase-8, which can directly activate the effector caspase-3, may also directly and/or indirectly activate the JNK pathway to trigger apoptosis in human amylin-treated islet \( H9252 \)-cells. However, this does not exclude the possibility that JNK1 could also be activated through a pathway independent of the activation of caspase-8.

### DISCUSSION

It is now widely accepted that aggregation of human amylin can elicit islet \( \beta \)-cell death and may contribute to the development of \( \beta \)-cell failure and the onset of T2DM (2, 12, 13). It has been suggested that overexpression of human amylin in association with the hyperglycemic state might be an important factor that leads to amylin fibril formation. It is also possible that the insulin-resistant stage is associated with hypersecretion of human amylin, which accumulates in the pancreas, thereby initially forming small aggregates containing \( \beta \)-sheet conformers (17), which interact with \( \beta \)-cell membranes in a specific conformation-dependent manner that in turn stimulates intracellular signal transduction pathways leading to apoptosis. We have previously shown that apoptosis evoked by human amylin required activation of the transcription factor c-Jun and the JNK pathway (9), but the detailed roles played by the caspase pathways activated by human amylin are not known. Here, we have identified specific caspases (caspase-1, -3, and -8) that are required for the induction of apoptosis by fibrillogenic amylin in cultured islet \( H9252 \)-cells. Blocking of cascade activation by these caspase inhibitors, significantly suppressed \( H9252 \)-cell apoptosis. We detected activation of caspase-3, which is the common downstream effector caspase. More interestingly, we were able to detect early activation of the initiator caspase-8 in cultured \( H9252 \)-cells following human amylin treatment. Caspase-8, upon activation, can directly or indirectly initiate the proteolytic activities of other effector caspases, including caspase-3 (39). It was expected that blocking of the upstream caspase-8 activation would abort downstream activation of caspase-3, as was indeed found. However, we also observed an inhibitory effect of caspase-3 inhibitor on caspase-8 activation; this is probably due to the fact that z-DEVD-FMK is a selective inhibitor of the CPP32/Apopain/Yama/caspase-3 sub-family whose effects also include inhibition of MACH/FLICE/caspase-3 (53). Alternatively, this result may indicate a self-
feedback regulation of the caspase cascade in our β-cell system. In addition, a significant protective effect of caspase-1 inhibitor can be detected in the apoptosis of human amylin-treated β-cells, although the change in caspase-1 activity was not measurable. This may be because the activation of caspase-1 is transient or too low to be detected. It should also be noted that human amylin is produced endogenously in the human β-cell line CM and secreted into the culture medium. However, we have previously measured the basal level of endogenous human amylin in the cell (16 ± 4.2 pm) and the basal concentration of secreted human amylin in the culture medium (6.8 ± 2.6 pm) by radioimmunoassay.² Our results indicated that both levels of endogenous human amylin are too low to affect the levels of exogenously applied synthetic human amylin (10 μM) and, therefore, that it is unlikely to influence the data obtained.

Recent studies have indicated that each of the three isoforms of JNK may elicit distinct effects on various signal transduction pathway to regulate cellular responses, including growth, differentiation, and apoptosis (54, 55). We show here that it is the specific JNK1 isoform that activates c-Jun by phosphorylation at Ser⁶³. Furthermore, our studies on the interplay between the caspase cascade and the JNK pathway revealed that JNK1 mediates human amylin-evoked apoptosis by activating caspase-1 and caspase-3. The JNK pathway may be activated partially through activation of caspase-8 but may also be activated through a pathway independent of caspase-8. It is more likely that caspase-1 and caspase-3 are activated indirectly by c-Jun, because we found the levels of mRNA for these pro-caspases are not affected. That activated c-Jun has an indirect effect on these caspases was also supported by the finding that c-Jun and JNK1 are activated maximally within 4 h following initiation of human amylin treatment (Fig. 7) (9), whereas caspase-3 activity is not maximal until 16 h (Fig. 1). These results have demonstrated a functional interaction between a caspase cascade and JNK1. Caspase-8 has been linked to death receptor-mediated apoptosis and, by binding to the receptor adaptor protein, it connects receptor signaling to the downstream apoptotic effector machinery (35). In addition, studies have shown that the JNK pathway can also be activated by stimulated death receptors that act through a mechanism that is as yet unknown (41, 56). Our current findings of sequential activation of caspase-8, caspase-1, and caspase-3 and the link to activation of JNK1 in human amylin-treated β-cells, imply that fibrillogenic amylin may induce apoptosis through a receptor-mediated cell surface death pathway. Further evidence for a possible involvement of a surface receptor in amylin-evoked apoptosis was also furnished by our previous electron microscopy studies of apoptotic β-cells, where we demonstrated the development in cell membranes of invaginations with subadjacent electron-dense structures, only a few minutes after exposure to human amylin (11). These observations strongly suggested the formation of clathrin-coated pits, which are normally indicative of receptor clustering in the plasma membrane and endocytotic uptake of protein into animal cells. Taken together, our data indicate that human amylin could initiate apoptotic signaling through a β-conformer-specific interaction with the β-cell membrane that results in receptor-mediated activation of initiator caspase-8 and subsequent effector caspases, such as caspase-1 and caspase-3. It should be noted that β-conformers of human amylin may not necessarily directly interact with a β-cell surface receptor, however. The interaction may instead cause conformational changes in membrane proteins or internal rearrangement of the lipid bilayer in the β-cell membrane that, in turn, can activate death receptors. It is also possible that the death receptor could subsequently activate the JNK pathway independent of caspase-8 activation, because we found that suppression of caspase-8 activity can only partially inhibit c-Jun activation. Therefore, human amylin may induce multiple apoptotic pathways, in which caspase-3 acts as a common downstream effector.

Studies on caspase knockout mice have revealed that two major apoptotic signaling pathways emanate from the activation of caspase-8 (35, 39). Activated caspase-8 can either activate downstream effector caspases directly by substrate cleavage or indirectly by its cleaving of Bid, which in turn evokes release of cytochrome c from mitochondria (39). The second signaling process, which in turn activates caspase-9, is controlled by the expression of Bcl-2 family members (35, 38); activated caspase-9 then cleaves and activates downstream effector caspases. However, in the current study, we observed very little change in caspase-9 activity in β-cells following human amylin treatment. The selective caspase-9 inhibitor also failed to suppress amylin-evoked apoptosis. We also found in a previous study that human amylin had no effect on the expression of bcl-2 mRNA (10). Our current data, taken together with these previous results, indicate that human amylin-elicted apoptotic signaling probably bypasses caspase-9 and bcl-2.

It has been previously discussed that certain conformers, associated with the propensity of human amylin to form aggregated states containing β-pleated sheet structures, are likely to underlie its ability to trigger β-cell apoptosis (9, 10, 13). The one or more specific cytotoxic forms of aggregated human amylin remain to be defined. In our current studies, where we have compared apoptosis induced by wild-type human amylin versus substituted [25,28,29]triprolyl] and truncated 8–37human amylin, we found that disruption of aggregation and/or fibril formation by substitution of the three prolyl residues (which suppress β-sheet formation) in the amyloidogetic region, completely abolished apoptosis and caspase activation. Our data further demonstrate that structures in the NH₂-terminus region of the human amylin peptide are not required for induction of apoptosis, because similar caspase activation and cytotoxicity are elicited by 8–37human amylin. This molecule forms polymorphic, higher order fibrillar structures similar to those of full-length human amylin, although there are some differences between the kinetics of aggregation by the two peptides (16). Together, these results indicate that the NH₂-terminus 1–7 region of the human amylin peptide, which influences the relative frequencies of the various higher order fibril types and thereby the overall kinetics of fibril formation (16), is not required for its cytotoxic properties. Moreover, 8–37amylin can antagonize amylin-mediated hormonal effects (49), and the NH₂-terminus ring structure is essential for the receptor-mediated hormonal signal transduction of amylin (47, 48). This indicates that the apoptotic cell death evoked by human amylin is not mediated by the same receptor that mediated the hormonal signaling of amylin. Therefore, it is more likely that it is the overall context of the full-length sequence that determines its fibrillogenicity and cytotoxicity. Non-cytotoxic [25,28,29]triprolyl]human amylin contains three prolyl substitutions in the 20–29 region, which is necessary for the transition of native and soluble human amylin into amyloid fibrils. These substitutions inhibit the overall formation of amylin fibrils (21) by suppressing its propensity to self-aggregate and precipitate, while enhancing its stability and retaining biological activity (21). Interestingly, this molecule, also known as pramlintide, has been employed to deliver therapeutic amylin supplementation in the clinical treatment of type-1 and type-2 diabetes.

a S. Zhang, J. Liu, and G. J. S. Cooper, unpublished data.
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(57). Pramlintide, as an adjunct to insulin therapy, has been shown to prevent the abnormal postprandial rise in plasma glucagon, improve postprandial glucose excursions, and improve long-term overall glycemic control in human diabetes (58–60). We did not detect fibril formation by this molecule under the experimental conditions employed here. Our current data provide information that further defines the relationship between structure, amyloidogenesis, and amylin by human amylin. We plan to employ these findings in the development of blocking molecules designed to suppress amylin fibril formation and \( \beta \)-cell death in \textit{vivo}. We expect that these insights into the detailed molecular mechanisms by which human amylin fibrils evoke \( \beta \)-cell death will lead to greater understanding of the molecular basis of amyloid formation and ultimately the mechanism of \( \beta \)-cell failure in T2DM.

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