Fas-Associated Death Receptor Signaling Evoked by Human Amylin in Islet β-Cells

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OBJECTIVE—Aggregation of human amylin (hA) into β-sheet–containing oligomers is linked to islet β-cell dysfunction and the pathogenesis of type 2 diabetes. Here, we investigated possible contributions of Fas-associated death-receptor signaling to the mechanism of hA-evoked β-cell apoptosis.

RESEARCH DESIGN AND METHODS—We measured responses to hA in isolated mouse islets and two insulinoma cell lines, wherein we measured Fas/Fas ligand (FasL) and Fas-associated death domain (FADD) expression by quantitative RT-PCR, Western blotting, and immunofluorescence staining. We used two anti-Fas/FasL blocking antibodies and the Fas/FasL antagonist Kp7–6 to probe roles of Fas interactions in the regulation of apoptosis in hA-treated β-cells and measured Kp7–6–mediated effects on β-sheet formation and aggregation using circular dichroism and thioflavin-T binding.

RESULTS—hA treatment stimulated Fas and FADD expression in β-cells. Both blocking antibodies suppressed hA-evoked apoptosis but did not modify its aggregation. Therefore, Fas receptor interactions played a critical role in induction of this pathway. Interestingly, hA-evoked β-cell apoptosis was suppressed and rescued by Kp7–6, which also impaired hA β-sheet formation.

CONCLUSIONS—This is the first report linking hA-evoked induction and activation of Fas and FADD to β-cell apoptosis. We have identified a Fas/FasL antagonist, Kp7–6, as a potent inhibitor of hA aggregation and related β-cell death. These results also support an interaction between hA and Fas on the surface of apoptotic β-cells. Increased expression and activation of Fas in β-cells could constitute a molecular event common to the pathogenesis of both type 1 and type 2 diabetes, although the mode of pathway activation may differ between these common forms of diabetes. ***Diabetes* 57:348–356, 2008**

The pathological characteristics of type 2 diabetes include progressive β-cell dysfunction, loss of pancreatic β-cell mass, and islet amyloid deposits formed by the 37–amino acid peptide amylin (known also as islet amyloid polypeptide) (1–4). Amylin is a physiological component of islet β-cell granules, whence it is cosecreted with insulin onto the cell surface via the regulated secretory pathway (5–6). Human amylin (hA), unlike its rodent homologs, has physicochemical properties that predispose it to aggregate and form amyloid fibrils, i.e., it is "fibrillogenic." Rat amylin (rA), which has a different amino acid sequence in the amyloidogenic molecular segment, does not aggregate and exhibits random conformations in physiological solutions (7–10). hA aggregation has been linked to β-cell degeneration in type 2 diabetes (11–13). However, mature amyloid fibrils may not contribute directly to its cytotoxicity, since there is no strong correlation between the extent of amyloid deposits and disease severity (11,14). Misfolding of hA into β-sheet–containing oligomers can kill β-cells via apoptosis originating on the surface or within cells, as reported from studies using cultured β-cells or transgenic animal models susceptible to hA-evoked diabetes (1,11–12,14–17). Cytotoxic hA aggregates have been detected in both intracellular and extracellular spaces in the islets of hA-transgenic mice (11,18–19), but the detailed molecular mechanisms by which such misfolding might trigger apoptosis remain to be fully elucidated. Previous studies have indicated that exposure of cultured islets or β-cells to extracellular aggregation–competent hA can kill β-cells and that direct contact of hA aggregates with cell membranes is required to elicit apoptosis (12,15–17,20–21). hA-evoked membrane instability and leakage through its interaction with cell membranes is considered to be one potential cytotoxic mechanism (22). Other mechanisms include increased cellular pro-oxidant responses, LDL uptake evoked by hA aggregate/cell interactions (23), and activation of the endoplasmic reticulum stress response (24–25). Our previous cell culture studies have demonstrated activation of multiple apoptosis pathways following hA exposure, including the sequential activation of caspase-8 and caspase-3 and the Jun NH_2-terminal kinase (JNK)-1/c-Jun and p38 kinase pathways (16,21,26). Possible approaches for prevention of amylin-evoked β-cell apoptosis include inhibition of cytotoxic hA oligomer formation and suppression of hA-induced death signaling pathways. The suppression of the latter pathways might be achieved either by preventing the interaction between aggregating amylin and the β-cell or by inhibiting intracellular cell-death signaling pathways. Therefore, increasing emphasis is now being placed on this research area, which is currently the subject of intensive investigation.

This study aimed to explore our hypothesis that small hA aggregates interact with β-cell membranes in a specific manner that activates death receptor signaling to trigger apoptosis. Several death receptor–signaling/death receptor–inducing molecules, including Fas and Fas-associated death domain (FADD), have been implicated in β-cell destruction in various cellular and animal models of immune-mediated diabetes and type 1 diabetic individuals

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CD, circular dichroism; FADD, Fas-associated death domain; FasL, Fas ligand; hA, human amylin; HBSS, Hank's balanced salt solution; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; JNK, Jun NH_2-terminal kinase; qRT-PCR, quantitative RT-PCR; rA, rat amylin; ThT, thioflavin-T.

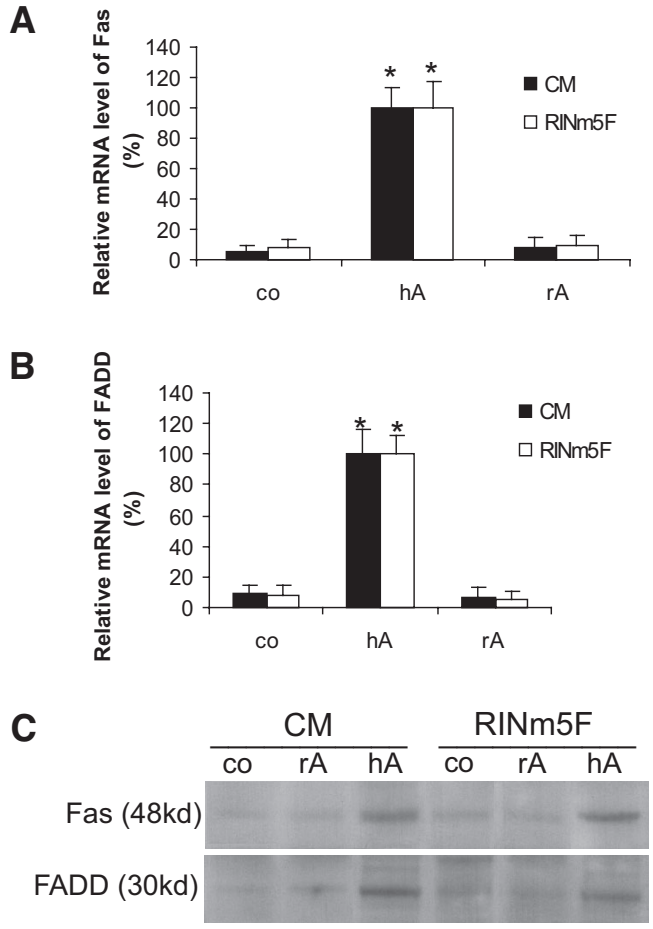

FIG. 1. Increased mRNA and protein expression of Fas and FADD in cultured RINm5F and CM cells. Total RNA was isolated from cells treated with 10 μmol/l hA, rA, or vehicle control (co). Relative Fas (*A*) and FADD (*B*) mRNA levels were determined after 8-h hA exposure by qRT-PCR normalized to 18S rRNA (endogenous control) and expressed relative to levels in hA-treated samples (100%). Fas and FADD mRNA were detected at cycles between 29 and 32 (Ct value). Results are the mean ± SE of three independent experiments, each performed in triplicate. **P* < 0.05 vs. control. *C*: Western blot analysis of Fas and FADD protein expression. Equivalence of loading and blotting was verified by Ponceau S staining. Results are representative of three independent experiments.

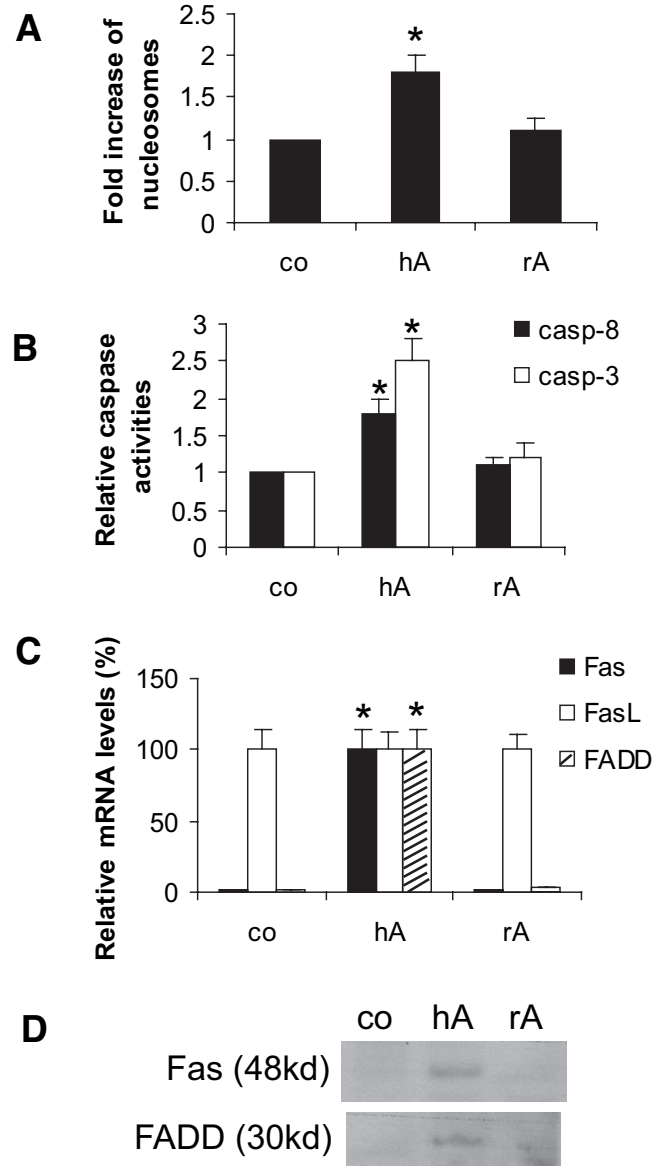

FIG. 2. Human amylin treatment–induced apoptosis is associated with increased Fas and FADD expression and caspase activation in cultured murine islets. *A*: Isolated islets were cultured and exposed to 40 μmol/l hA, and apoptosis was measured after 24 h as fold increase in nucleosomes (enzyme-linked immunosorbent assay) and expressed relative to controls (set at 1). Values are the mean ± SE of three independent experiments, each performed in duplicate. **P* < 0.05 vs. control. *B*: Caspase-8 (casp-8) and caspase-3 (casp-3) activities were determined after 16-h hA exposure using synthetic fluorogenic oligopeptide substrates z-LETD-AFC and z-DEVD-AFC, respectively (excitation, 400 nm; emission, 540 nm). Values are relative to untreated control (co) and represent mean ± SE of four independent experiments, each performed in duplicate. **P* < 0.05 vs. control. *C*: Relative mRNAs corresponding to Fas and FADD were determined after 8-h hA exposure by real-time qPCR, with normalization to 18S rRNA (endogenous control), and presented relative to hA-treated samples (100%). Values are the mean ± SE of three independent experiments, each performed in triplicate. **P* < 0.01 vs. control. *D*: Western blot analysis of Fas and FADD protein expression. Results are representative of three independent experiments.

(27–31). Islet β-cells normally express Fas ligand (FasL) constitutively but not the Fas receptor; however, cytokine exposure can lead to β-cell apoptosis and elevated Fas expression (27,32). Increased β-cell Fas expression has also been reported in type 2 diabetic individuals (33–34). These data are consistent with a major effector role for the Fas/FasL interaction in the mechanism of β-cell destruction in both major classes of diabetes.

Here, we first determined whether Fas/FasL expression changed in cultured rat and human insulinoma cells and isolated murine islets following treatment with extracellular hA. We next measured FADD protein expression in these cells. Third, we used specific Fas/FasL blocking antibodies to probe the role of Fas-associated death receptor signaling and used a selective and specific Fas/FasL antagonist in parallel experiments. We then characterized the effects of the Fas/FasL blocking antibodies and antagonist on β-sheet and fibril formation by hA. We now report that the cell-surface death receptor Fas and the related death domain protein FADD are important contributors in the pathway by which hA evokes β-cell apoptosis. We expect these data will provide better understanding of the relationship between amylin aggregation and β-cell degeneration and their possible contribution to the pathogenesis of type 2 diabetes as well as other related diseases caused by protein aggregation.

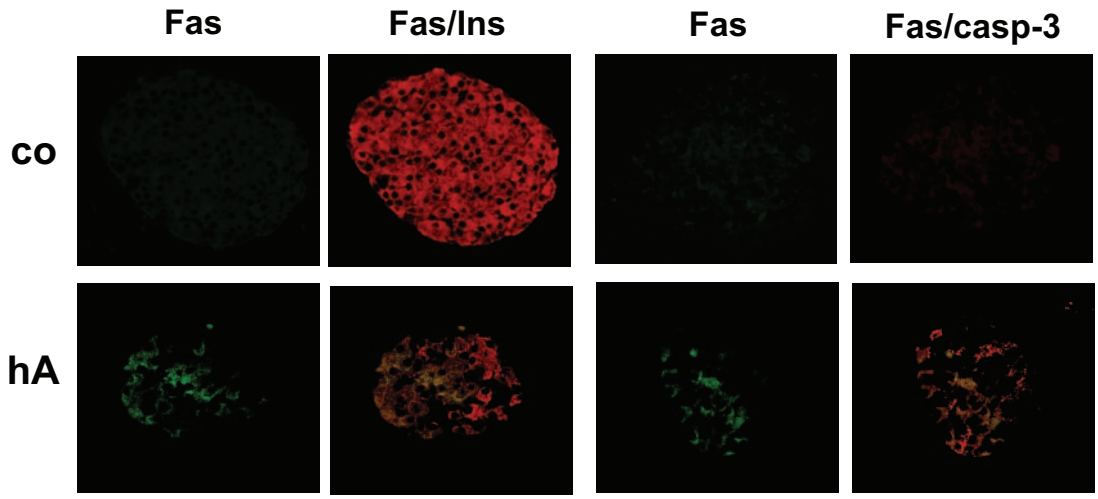

FIG. 3. Representative double-immunofluorescence staining for Fas and cleaved-caspase-3 (casp-3) or insulin (Ins) in cultured mouse islets exposed to hA. Fas was labeled with fluorescein isothiocyanate (green), whereas cleaved caspase-3 and insulin were labeled with rhodamine Red-X (red). Fluorescence-stained islets were viewed and photographed using inverted-phase fluorescence microscopy (Nikon). (Please see http://dx.doi.org/10.2337/db07-0849 for a high-quality digital representation of this figure.)

RESEARCH DESIGN AND METHODS

Cell culture and treatment. Rat and human insulinoma cell lines, RINm5F (passages 28–35) and CM (passages 8–28), were cultured as described (17,26).

For peptide treatments, amylin stock solutions were prepared freshly by dissolving lyophilized hA or rA (Bachem) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) followed by removal of HFIP by evaporation under N_2; residue was then dissolved in water to 500 μmol/l (35). Aliquots were then added to cultures to a final concentration of 10 μmol/l and incubated for stated periods of time. Untreated control cells were similarly treated (17,26).

For antibody blocking experiments, anti-human Fas (ZB4; MBL, Nagoya, Japan) or anti-FasL (Q-20; Santa Cruz, CA) blocking antibodies were applied to CM cells at 500 ng/ml or 10 μg/ml, respectively, 1 h before addition of amylin solutions. For Fas/FasL antagonism experiments, cells were preincubated with Kp7–6 (Calbiochem) at various concentrations for 1 h before amylin addition. Rescue experiments were performed by addition of Kp7–6 to cell cultures after 8-h hA treatment. For JNK inhibition experiments, cells were preincubated with JNK inhibitor-I before exposure to hA as described (16).

Islet isolation and culture. All animal use was approved by the animal ethics committee of the University of Auckland. Islets were isolated from pancreases of 8- to 12-week-old FVB/N male mice using described methods (36) with modifications. Briefly, pancreases were minced with scissors in Hank's balanced salt solution (HBSS) and digested with collagenase-P (Roche, Basel, Switzerland) in HBSS/25 mmol/l HEPES/0.1 mg/ml DNase I for 30 min at 37°C (water bath). Digestion was stopped by adding ice-cold HBSS/10% FBS followed by filtering through a 500-μm mesh screen (Costar, Corning, NY). After three washes, islets were handpicked.

Isolated islets were cultured on extracellular matrix–coated plates (Sigma) in RPMI-1640 medium containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS for 3 days before treatment with 40 μmol/l hA. Preincubation of islets with anti-FasL blocking antibody or Fas/FasL antagonist was performed as described above. Dispersed islet cells were prepared by trypsin incubation and cultured in equivalent medium.

Quantitative RT-PCR. RINm5F and CM cells were cultured in 6-well plates and isolated islets in 12-well plates; both were exposed to hA as described above. Total cellular RNA was isolated using Trizol reagent (Invitrogen, CA) and cDNA prepared using the Superscript-II cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed using a Prism 7900HT Sequence Detection System (ABI, Foster City, CA). A 20-μl reaction mixture containing sample cDNA, TaqMan universal PCR master mix, and TaqMan-MGB probe-expression assay mix (Fas, FasL, or FADD) was incubated at 50°C for 2 min then 95°C for 10 min, followed by amplification for 40 cycles (95°C for 15 s and 60°C for 1 min/cycle). Predeveloped eukaryotic 18S rRNA TaqMan assay (ABI) was used as endogenous control.

Western blot analysis. Cells and isolated islets were cultured in the presence or absence of antagonist or JNK inhibitor-I before exposure to hA as described above. Whole cell lysates were then prepared and protein concentrations determined as described (26). Ponceau S staining was performed to confirm equal loadings on SDS-PAGE. Western blots were performed using rabbit anti-Fas (FL-335), anti-FasL (C-178), anti-FADD (H-181), or anti–caspase-8 (H-134) (Santa Cruz) or rabbit anti–phospho-JNK and anti-cleaved caspase-3 (Cell Signaling, MA). Specific immune-complex signals were detected as described (26).

Immunofluorescence staining. Isolated islets treated or untreated with hA were fixed in 4% (vol/vol) phosphate-buffered paraformaldehyde for 30 min at room temperature and embedded in paraffin. Thereafter, 8-μm sections were cut, treated for antigen retrieval, and double stained for Fas and insulin or cleaved caspase-3. Islet sections were first blocked with normal goat serum (10% vol/vol) for 2 h, then incubated with rabbit anti-Fas antibody (Santa Cruz Biotechnology), followed by detection using goat anti-rabbit fluorescein isothiocyanate–conjugated antibody (Jackson ImmunoResearch). Washed sections were then probed with guinea-pig anti-insulin (Dako, Glostrup, Denmark) or rabbit anti-cleaved caspase-3 (Cell Signaling) and detected with rhodamine Red-X–conjugated secondary antibodies (Jackson ImmunoResearch).

Circular dichroism spectroscopy. hA stock solution (500 μmol/l) was prepared freshly in HFIP and diluted to 10 μmol/l in 10 mmol/l phosphate buffer (pH 7.4) with or without antibodies or antagonist. Circular dichroism (CD) spectra were recorded, and molar ellipticity (θ) was determined as described (35).

Thioflavin T fluorescence assay. hA stock solution (500 μmol/l) was prepared as described above and diluted to 10 μmol/l in 10 mmol/l phosphate buffer (pH 7.4) containing 5 μmol/l thioflavin-T (ThT), with or without antibodies or antagonist. Fluorescence was then measured as described (35).

Caspase activity assay and cell death detection enzyme-linked immunosorbent assay. Isolated islets and cells were cultured in the presence or absence of Fas/FasL blocking antibodies or antagonist before exposure to hA as described above. Caspase activity and apoptosis were measured after 16 or 24 h, respectively, as described (16,26).

Statistical analysis. Data are presented as means ± SE. Differences between preplanned experimental treatments were analyzed using unpaired two-tailed Student's t test or ANOVA as appropriate, with significance at $P < 0.05$.

RESULTS

Induced expression of death receptor Fas and FADD in cultured β-cells following hA treatment. Dysregulation of death receptor signaling, allowing either too much or too little apoptosis, can lead to various disorders. Using our previously established cell culture model for hA-induced β-cell apoptosis, we sought to investigate the possible roles of the Fas-associated receptor pathway in the control of apoptosis in hA-treated β-cells. Changes in mRNA expression of Fas/FasL and FADD were examined by real-time quantitative RT-PCR (qRT-PCR) (Fig. 1*A* and *B*). The level of Fas mRNA was very low but reproducibly detectable in unstimulated RINm5F and CM cells. We detected significant increases in the level of Fas and FADD

mRNA after hA treatment compared with control cells. By contrast, nonfibrillogenic, noncytotoxic rA did not induce either Fas or FADD transcription (Fig. 1*A* and *B*). Changes in corresponding protein levels of Fas and FADD were determined by Western blotting: hA exposure led to or caused increased Fas and FADD protein expression in both RINm5F and CM cells, whereas rA was again without effect (Fig. 1*C*). By contrast, FasL mRNA and protein expression was unaffected by hA, although FasL was constitutively expressed at low levels (data not shown).

To substantiate these studies in clonal β-cell lines, we also studied hA-induced Fas receptor signaling in ex vivo islets from normal FVB/N mice. We first established that hA evoked apoptosis in the isolated islets (Fig. 2*A*). We next determined the effects of hA on Fas and FADD expression and caspase activation in this tissue. Fas expression was not detectable in normal mouse islets by either qRT-PCR or Western blotting, but hA exposure evoked both Fas and FADD gene transcription and protein expression (Fig. 2*C* and *D*), as well as caspase-8 and -3 activation (Fig. 2*B*). Human amylin did not modify FasL expression in cultured islets. In addition, hA also induced Fas and FADD expression in cultured dispersed islet cells (data not shown). These findings were all consistent with parallel results from the β-cell lines. By contrast, rA did not elicit expression of Fas, FasL, or FADD; therefore hA's aggregation propensity was necessary for stimulation of their mRNA and protein expression.

In addition, double-immunofluorescence staining confirmed that β-cells in normal islets, which did not constitutively express Fas, become strongly Fas-positive following hA exposure. In hA-treated islets, most Fas-positive cells were also positive for both insulin and cleaved caspase-3 (Fig. 3); therefore Fas was mainly colocalized with these proteins. Thus, upregulation of Fas contributed to β-cell death in hA-treated islets.

Suppression of hA-evoked β-cell apoptosis by Fas/FasL blocking antibodies. We next used anti-Fas and anti-FasL blocking antibodies to probe the role of the Fas-receptor interaction in hA-evoked β-cell apoptosis. CM cells were preincubated with anti-Fas (ZB4) or anti-FasL (Q-20) blocking antibody for 1 h before hA exposure, with assessment of caspase-3 activation and apoptosis after 16 or 24 h. Both ZB4 and Q-20 blocking antibodies strongly suppressed hA-evoked apoptosis and caspase-3 activation, demonstrating that Fas-receptor interaction, at least in part, elicits hA-induced β-cell apoptosis (Fig. 4*A* and *B*). Q-20 pretreatment also downregulated hA-evoked apoptosis in ex vivo cultured islets and in cultured dispersed islet cells (Fig. 4*C*), whereas control experiments with nonimmune mouse/rabbit IgG or antibodies alone were without effect. Inhibition of apoptosis was thus by specific neutralization of Fas/FasL molecules.

We next determined whether inhibition of apoptosis by the Fas/FasL blocking antibodies might be mediated through interference with hA β-sheet and fibril formation or direct action on cellular Fas receptor interaction. We analyzed effects of blocking antibodies on β-sheet and fibril formation by hA with CD spectroscopy and ThT binding assay. Human amylin in solution underwent conformational transition from its mainly unordered state at time 0 into predominantly β-sheet conformer 3 h later, and this conformational transition was unmodified in the presence of either antiapoptotic or control antibodies (Fig. 5*A*). Furthermore, ThT binding assays indicated that neither ZB4 nor Q-20 significantly delayed or modified the kinetics of fibril formation by hA ($P > 0.05$, Fig. 5*B*). Taken together, suppression of hA-induced β-cell apoptosis by these anti-Fas/FasL blocking antibodies was not mediated through interference with β-sheet or fibril formation, confirming that their blocking effects were on the Fas receptor pathway itself.

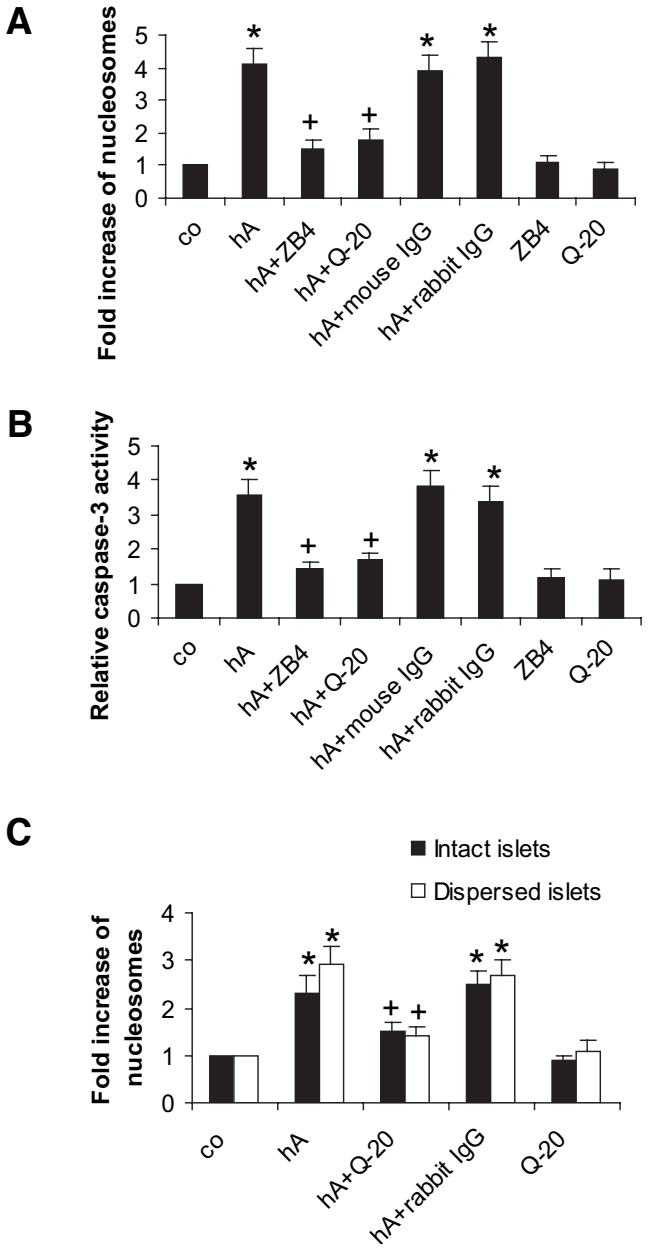

FIG. 4. Effects of Fas/FasL blocking antibodies on hA-induced apoptosis and caspase-3 activation. *A*: Suppression of apoptosis in CM cells preincubated with or without anti-Fas blocking antibody (ZB4) or anti-FasL blocking antibody (Q-20) before exposure to hA. Results represent increased nucleosomes (fragmented DNA). *B*: Caspase-3 (casp-3) activities in CM cells determined after preincubation with blocking antibody followed by 16-h hA exposure using synthetic fluorogenic oligopeptide substrate z-DEVD-AFC (otherwise as in Fig. 2.). *C*: Suppression of apoptosis in isolated islets and dispersed islet cells preincubated with or without Q-20 before hA exposure. Data are relative to untreated control (co) and represent the mean ± SE of four independent experiments, each performed in duplicate. *$P < 0.01$ vs. control. +$P < 0.05$ vs. hA-treated cells.

Suppression of hA-evoked β-cell apoptotic signaling by a Fas/FasL antagonist. To further characterize the Fas-receptor interaction and its contribution to hA-

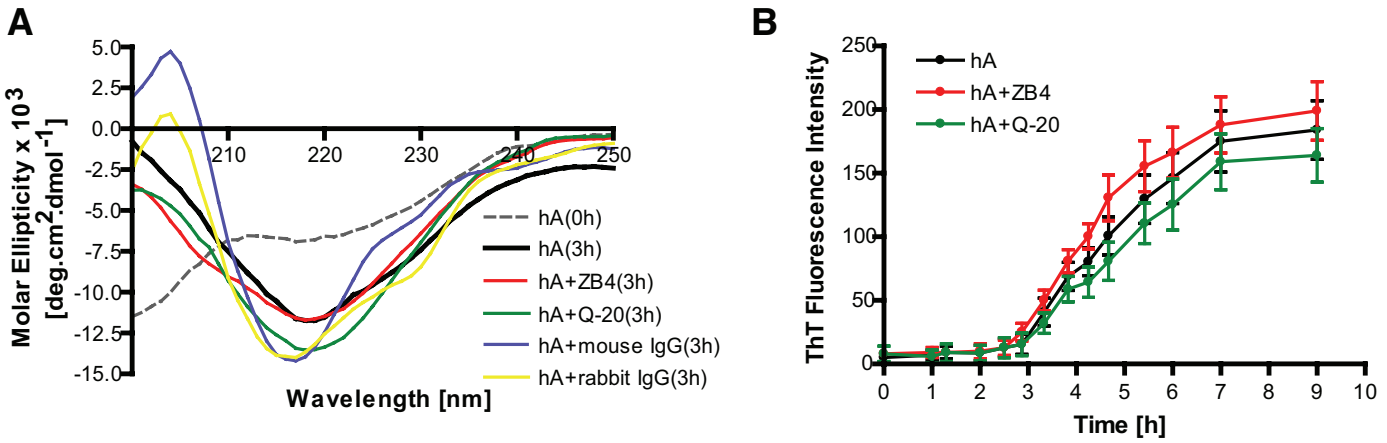

FIG. 5. Effects of Fas/FasL blocking antibody on β-sheet and fibril formation by hA. *A*: CD analysis of hA aggregation into β-sheet (at 0 and 3 h) in the presence or absence of blocking or control antibodies, with results in molar ellipticity (θ). Interpretation was performed according to generally accepted guidelines for the prediction of protein secondary structure from CD spectra (57). Spectra are representative of three independent experiments. *B*: ThT binding assays were performed to quantitatively monitor ThT-detectable fibrils formed by hA over time. hA was incubated with or without blocking or control antibodies (1:10 molar ratio) in phosphate buffer containing ThT, a fluorescent dye that preferentially binds amyloid fibrils, and fluorescence was measured (black plastic microtiter plates). Results are the mean ± SEM of three independent experiments, each performed in triplicate.

induced apoptosis, we investigated whether the peptidic Fas/FasL antagonist could protect against hA-evoked apoptosis. Kp7–6, originally derived from Fas, is a small exocyclic cystine-knot peptide mimetic that contains an intramolecular Cys-Cys disulfide bridge and bears structural resemblance to hA's eight NH_2-terminal residues (Fig. 6*A*). Prior Kp7–6 treatment dose-dependently suppressed hA-evoked apoptosis in isolated islets as well as RINm5F and CM cells, and Kp7–6 was shown to be more protective toward cultured cell lines than isolated islets (Fig. 6*B*).

Next, we investigated whether Kp7–6 might interfere with hA misfolding by measuring its effects on β-sheet and fibril formation. Kp7–6, at a 1:10 molar ratio to hA, suppressed β-sheet formation and enabled hA to retain its soluble unordered conformation for 24 h (Fig. 7*A*). Spectra of hA/Kp7–6 mixtures deviated markedly from the summation of those of hA and Kp7–6 alone (Fig. 7*B*), showing that Kp7–6 attenuated β-sheet formation by interacting with hA. Results of ThT binding were consistent, indicating that Kp7–6 suppressed hA-fibril formation. Kp7–6, at 0.1 and 1 mmol/l, markedly decrease ThT fluorescence (Fig. 7*C*) and prolonged the lag time to 5 h, as compared with 3 h in solutions of hA alone. Thus, Kp7–6 strongly attenuated early hA-nucleation and aggregation into ThT-detectable fibrils. Inhibition of hA fibril formation by Kp7–6 was further demonstrated by transmission electron microscopy (Fig. 7*D*).

We further investigated whether Kp7–6 could rescue β-cells following apoptosis induction by adding it in increasing doses to β-cells and islets, 8 h after initial hA-exposure, and measuring apoptosis 26 h later (Fig. 6*C*). Indeed, Kp7–6 (5–10 mmol/l) effectively inhibited apoptosis in all three β-cell systems, and its rescue effects appeared to be comparable between cultured cell line and islets. In a parallel control experiment, hA was incubated in cell-free culture medium for 8 h before addition to cells. In that case, hA became non- or only minimally cytotoxic (data not shown), indicating that it was not active at the time when Kp7–6 was added. This experiment indicates that Kp7–6 rescued apoptosis mainly via acting on a Fas receptor interaction, rather than simply by interfering with cytotoxic aggregate formation. Taken together, our results indicate that Kp7–6 can prevent and rescue hA-evoked β-cell apoptosis, not only by impairing Fas receptor interaction but also by interfering with β-sheet and fibril formation.

We further probed the functional interactions between Fas/FasL and the downstream signaling pathway through which it mediates hA-evoked apoptosis using combinations of Kp7–6 and a JNK inhibitor. Both pre- and postincubation of murine islets with Kp7–6 (5 mmol/l) inhibited downstream caspase-8, caspase-3, and JNK activation (Fig. 8*A*). Interestingly, JNK inhibitor-I also suppressed upregulation of Fas and FADD protein (Fig. 8*B*), indicating the existence of an auto-stimulatory loop and auto-feedback regulation mechanism.

DISCUSSION

Apoptosis contributes to β-cell destruction in diabetes (1,28); therefore, improved understanding may enable development of therapeutic strategies for preventing β-cell loss and diabetes. To our knowledge, evidence for the involvement of specific death-receptor signaling such as that elicited via Fas, in the process of hA-evoked β-cell death, has not previously been reported. Our hypothesis of hA-evoked Fas-associated death-receptor signaling is based on several lines of evidence. First, it is becoming clear that multiple pathways are activated during hA-evoked β-cell apoptosis, including caspase-8 and the JNK and p38 kinase pathways (16,21,37). Second, the Fas death receptor pathway has been associated with caspase-8 activation and JNK/p38 mitogen-activated protein kinase regulation (27,38–41). Caspase-8 is the most upstream caspase in the Fas apoptotic pathway; therefore, its activation in cultured β-cells and mouse islets following hA treatment further supports a role for Fas in hA-evoked apoptosis (16). Third, formation of clathrin-coated pits has been observed on the surface of cultured β-cells exposed to hA (42), and such structures are normally indicative of receptor clustering in the plasma membrane and endocytotic uptake of protein into mammalian cells (43). Fas/FasL functions as a trimer or multimer of trimers, and Fas-FasL interactions lead to oligomerization and clustering of receptor molecules and initiation of apoptotic

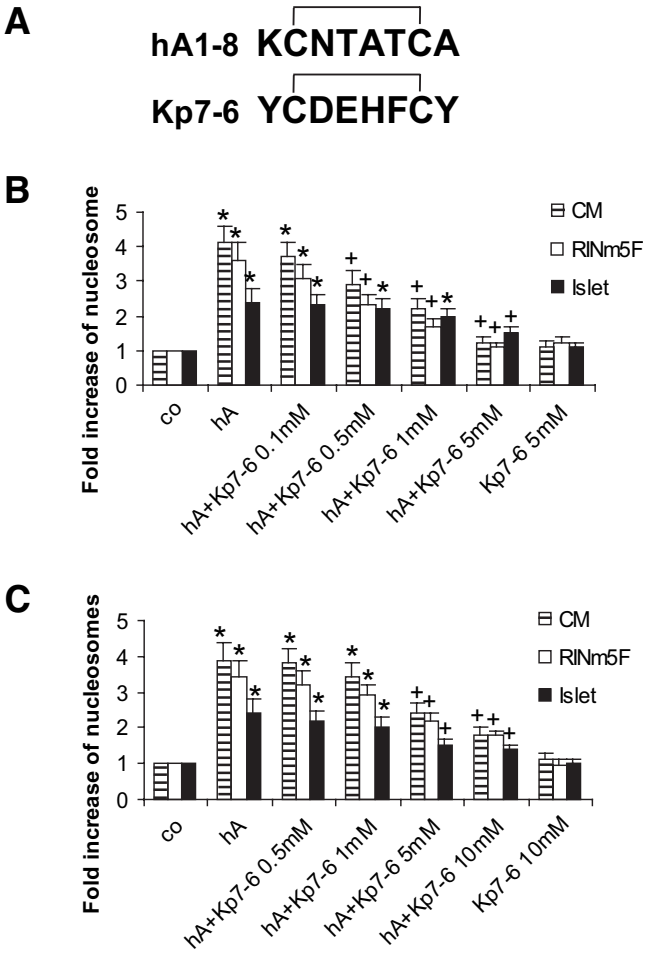

FIG. 6. Effects of Fas/FasL antagonist Kp7–6 on hA-evoked apoptosis. *A*: Comparison of primary structures of Kp7–6 and hA_{1-8} showing the Cys2-Cys7 disulfide bridges. *B*: Suppression of apoptosis measured in isolated islets, RINm5F, and CM cells preincubated with or without various concentrations of Kp7–6 before hA exposure. Kp7–6 (0.5–1 mmol/l) partially but significantly suppressed apoptosis in both cell types, whereas it did not measurably effect apoptosis in islet β-cells. A total of 5 mmol/l of Kp7–6 completely suppressed apoptosis in both cultured cell types but incompletely inhibited islet β-cell apoptosis; thus, Kp7–6 was more potently protective in cultured cell lines. *C*: Rescue of β-cell apoptosis measured in isolated islets and RINm5F and CM cells postincubation with/without Kp7–6 after 8-h hA exposure. Results represent fold increases in nucleosomes. The rescue effect of Kp7–6 was similar in both cell lines and islets β-cells. Data are relative to untreated control (co) and represent the means ± SE of four independent experiments, each performed in duplicate. *$P < 0.01$ vs. control. +$P < 0.05$ vs. hA-treated cells.

signaling (44–45). Finally, hA elicits oxidative stress (23); its cytotoxicity is associated with reduced cellular redox potential, and Fas is redox responsive (38–39,46).

We showed here that the cell-surface death receptor Fas and its associate FADD are mechanistically important in hA-evoked β-cell apoptosis, although the current data cannot explicitly show that the Fas/FADD pathway is independent of hA-induced endoplasmic reticulum stress, which can provoke apoptosis via cytokine activation, as reported in other studies (24–25). Mouse islets do not normally express Fas, but hA treatment induced Fas and FADD gene transcription and protein expression. Therefore, upregulation of Fas by hA aggregation may activate β-cell destruction independently of the Fas-mediated autoimmune reaction that occurs in type 1 diabetes. These data provide evidence for a convergence in the apoptotic signaling pathways elicited by pathological factors implicated in both type 1 and type 2 diabetes.

Using anti-Fas/FasL blocking antibodies, which exhibited no effects on β-sheet or fibril formation, we showed that hA-induced β-cell apoptosis can be suppressed by anti-Fas/FasL blocking antibody, thus supporting a role for Fas receptor interactions in triggering apoptosis. The inhibitory effect of blocking antibody was less potent in cultured islets than CM cells, perhaps because of more limited access of the macromolecular blocking antibody to the islet center. The lower responsiveness of the preventive and rescue effects of Kp7–6 in cultured islets might be partially explained by the lack of Fas expression in normal islet β-cells. RINm5F and CM cells, which constitutively express low but detectable amounts of Fas, may thus be more sensitive to Kp7–6. To our knowledge, this is the first report linking expression and activation of Fas and FADD with hA-induced β-cell apoptosis. However, there is published evidence that other pathologic processes elicited by amyloidogenic peptides, for example Aβ-evoked neuronal cell death, might also involve activation of the Fas-FADD pathway (47–48).

Substantive evidence indicates that it is small soluble hA oligomers, formed early in the amyloidogenic pathway, that mediate hA cytotoxicity (14–15,20,22). However, the structural properties of the conformers that actually mediate cytotoxicity have yet to be fully elucidated. Here we report that the Fas antagonist Kp7–6 can interact with hA to modify β-sheet and fibril formation, indicating that it may suppress apoptosis, in part, by interfering with its misfolding. Kp7–6 is a small exocyclic cystine-knot peptide that binds specifically to Fas and FasL and modifies their signaling by disabling receptor ensembles (49). The distinct cystine-knot loop structure (eight–amino acid residues with an internal cysteine disulfide bridge) in Kp7–6 was originally derived from a Fas receptor region that is important for Fas receptor recognition and binding (49). Interestingly, this cystine-knot structure bears similarity to amylin's NH_2-terminal ring structure (Fig. 6*A*), which is also essential for its receptor-mediated signaling. The observed Kp7–6-hA interaction implies that hA misfolding could generate aggregates/oligomers with structural features mimicking those of FasL. Moreover, these aggregates could upregulate Fas via cell membrane–based interactions that lead to formation of hA oligomer/Fas receptor complexes that elicit apoptosis and can be blocked by Kp7–6. Kp7–6 may possess dual antiapoptotic actions (prevention and rescue) by interfering with hA aggregation and exclusion of hA binding to upregulated Fas and/or promotion of inactive hA aggregate/Fas signaling complex ensembles. We are currently studying direct and/or indirect molecular interactions between hA aggregates and cell surface Fas receptors.

Molecules that can affect hA aggregation and suppress its cytotoxicity have potential for therapeutic development. Several types of inhibitors of hA fibrillogenicity have been discussed in the literature, including aromatic organic molecules, such as polycyclic compounds, Congo red and rifampicin (20,50–52), and small peptide-based inhibitors in modified or unmodified form derived from the amyloidogenic region and self-recognition sequences (hA_{20-29}) in hA (53–56). Some of these molecules have demonstrated both antiaggregation and anticytotoxic properties. We show here that the Fas/FasL antagonist Kp7–6, which is structurally similar to hA_{1-8}, can suppress both hA fibrillogenesis and toxicity. Kp7–6 may represent

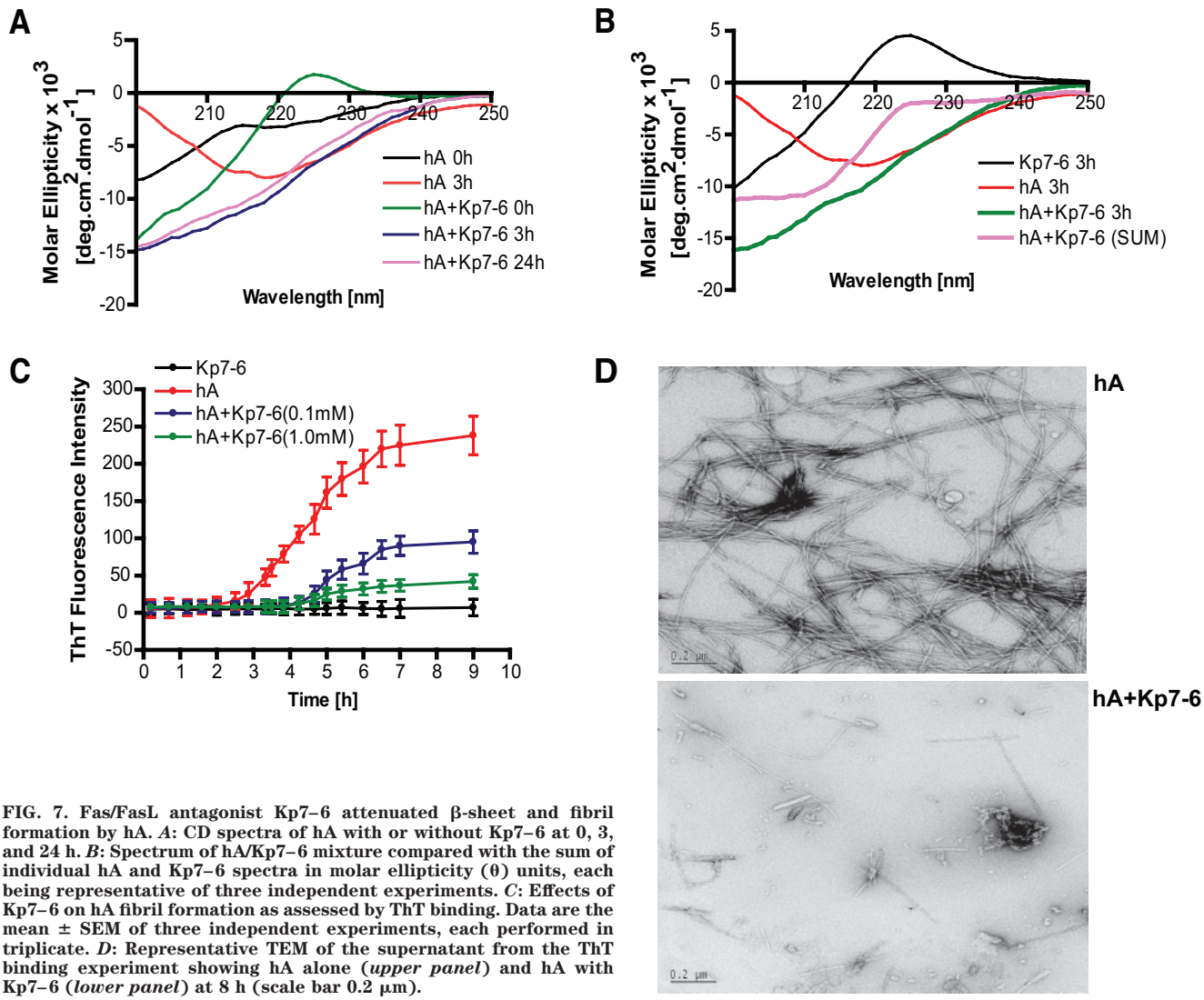

FIG. 7. Fas/FasL antagonist Kp7–6 attenuated β-sheet and fibril formation by hA. *A*: CD spectra of hA with or without Kp7–6 at 0, 3, and 24 h. *B*: Spectrum of hA/Kp7–6 mixture compared with the sum of individual hA and Kp7–6 spectra in molar ellipticity (θ) units, each being representative of three independent experiments. *C*: Effects of Kp7–6 on hA fibril formation as assessed by ThT binding. Data are the mean ± SEM of three independent experiments, each performed in triplicate. *D*: Representative TEM of the supernatant from the ThT binding experiment showing hA alone (*upper panel*) and hA with Kp7–6 (*lower panel*) at 8 h (scale bar 0.2 μm).

another subclass of small peptide-mimetic inhibitor with structural similarity to hA_{1-8}. The detailed biophysical properties of Kp7–6, including its molecular assembly with hA, need to be further characterized. The NH_2-terminal 1– to 8–amino acid sequence and hA_{2-7} ring structure are identical to those from all other nonhuman species studied (5), and our previous studies have demonstrated that this region may not itself be required for cytotoxicity (16). We plan to design and systematically test a series of hA_{1-8}-derived structural derivatives for their ability to inhibit the following processes: β-sheet formation, fibrillar aggregation, and β-cell death. The most effective suppressor of hA aggregation–mediated β-cell apoptosis will be selected for further in vivo study using animal models and human tissues.

In summary, we have shown that hA-evoked β-cell apoptosis is accompanied by increased expression of Fas and FADD. Our data also indicate that anti-Fas/FasL blocking antibodies and a specific Fas/FasL antagonist, Kp7–6, can protect cultured β-cells and murine islet β-cells against hA-induced caspase-8/3 activation and apoptosis. Kp7–6 is the first reported small peptide inhibitor derived from Fas/FasL that interacts with hA to suppress its cytotoxic aggregation. This study provides substantive evidence to support a role for the Fas-associated signaling pathway in hA-mediated β-cell death and suggests that targeting Fas could be of therapeutic value in preventing hA-induced β-cell death in type 2 diabetes.

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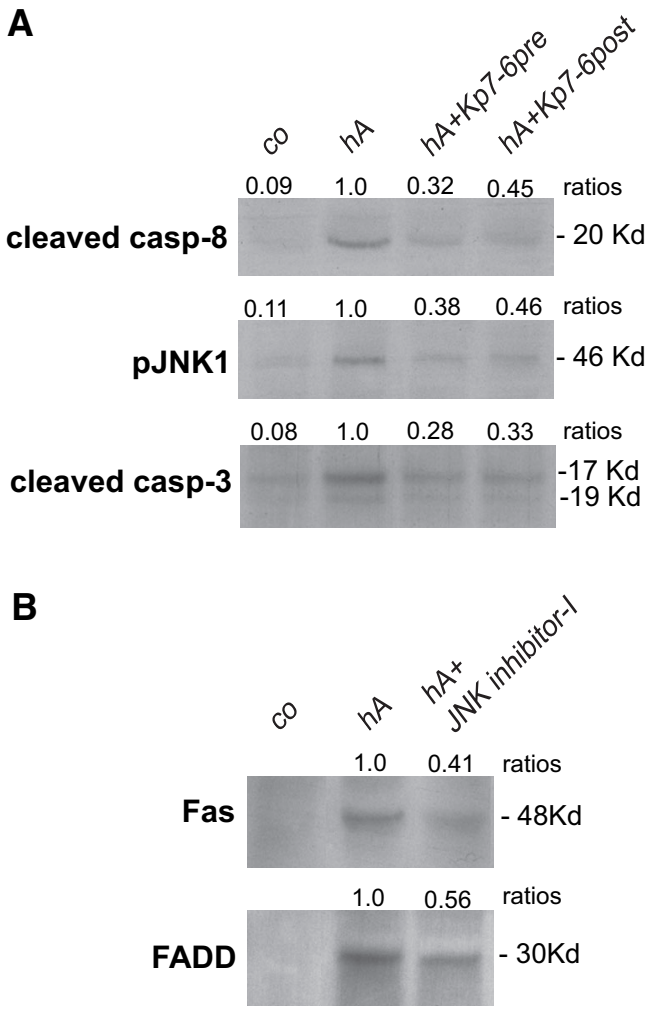

FIG. 8. Representative Western blots showing *A*: Inhibition of cleaved caspase-8 (casp-8), phospho-JNK1 (pJNK1), and cleaved caspase-3 (casp-3) protein expression by Kp7–6. *B*: Inhibition of Fas and FADD protein expression by JNK inhibitor-I. Total protein extracts were prepared from isolated mouse islets pre- or postincubation with Kp7–6 or JNK inhibitor-I before exposure to hA then subjected to Western blotting. Equivalence of loading and blotting was verified by Ponceau S staining. Protein band intensities were determined by scanning autoradiography (imaging densitometer, ScanMark; Microtek). Changes in protein levels (shown as ratios) were calculated based on levels in corresponding hA-treated cells, which were set at 1. All results are representative of three independent experiments.

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