

# Pancreatic-Derived Factor (FAM3B), a Novel Islet Cytokine, Induces Apoptosis of Insulin-Secreting $\beta$ -Cells

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**PANDER (PANcreatic DERived factor, FAM3B), a newly discovered secreted cytokine, is specifically expressed at high levels in the islets of Langerhans of the endocrine pancreas. To evaluate the role of PANDER in  $\beta$ -cell function, we investigated the effects of PANDER on rat, mouse, and human pancreatic islets; the  $\beta$ -TC3 cell line; and the  $\alpha$ -TC cell line. PANDER protein was present in  $\alpha$ - and  $\beta$ -cells of pancreatic islets, insulin-secreting  $\beta$ -TC3 cells, and glucagon-secreting  $\alpha$ -TC cells. PANDER induced islet cell death in rat and human islets. Culture of  $\beta$ -TC3 cells with recombinant PANDER had a dose-dependent inhibitory effect on cell viability. This effect was also time-dependent. PANDER caused apoptosis of  $\beta$ -cells as assessed by electron microscopy, annexin V fluorescent staining, and flow-cytometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. PANDER did not affect cytosolic  $Ca^{2+}$  levels or nitric oxide levels. However, PANDER activated caspase-3. Hence, PANDER may have a role in the process of pancreatic  $\beta$ -cell apoptosis. *Diabetes* 52: 2296–2303, 2003**

**T**ype 1 diabetes is characterized by autoimmune-mediated destruction of pancreatic islet  $\beta$ -cells (1,2). This destruction is a complex process characterized by cellular and humoral elements of cytotoxicity (3,4). Cytotoxic actions induce at least two morphologic consequences, apoptosis and necrosis. In apoptosis, endonucleases are activated, resulting in DNA fragmentation, nuclear and cytoplasmic condensation, membrane blebbing, and cell shrinkage. In contrast, necrosis is characterized by cellular swelling, lysis, and discharge of intracellular contents (5). In autoimmune

diabetes, islet-infiltrating cells (macrophages, CD4<sup>+</sup> T-cells, and CD8<sup>+</sup> T-cells) are thought to damage and destroy  $\beta$ -cells by producing one or more cytotoxic mediators, such as free radicals, proinflammatory cytokines, Fas ligand, perforin, and granzymes (6,7). The inflammatory cytokines interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , and interferon- $\gamma$  initiate signal pathways in  $\beta$ -cells, resulting in cell death through necrosis and apoptosis (4,8,9). Recent studies have shown that apoptosis is the mechanism of pancreatic islet  $\beta$ -cell death in autoimmune diabetes (10–12), and the inflammatory cytokines have been shown to induce  $\beta$ -cell destruction by apoptosis (13,14). In human islet cells, cytokines were also reported to induce mainly apoptosis and not necrosis (15). However, the mechanisms causing pancreatic islet  $\beta$ -cells damage in autoimmune diabetes are still not fully elucidated.

PANDER (PANcreatic DERived factor, FAM3B) is a novel cytokine that was recently cloned and identified using an algorithm, ostensible recognition of folds (ORF) (16), searching for novel cytokines based on their predicted secondary structure. The rationale for this approach is that the secondary structure of cytokines is highly conserved through evolution. Many cytokines are four-helix bundles with disulfide bridges. This approach has allowed the identification of a novel cytokine family consisting of several genes: 2-19 (FAM3A), EF-7 (FAM3D), FAM3C, and PANDER (FAM3B, previously named 2-21) (17). PANDER is a 235-amino acid protein with a secretion signal peptide. It is known that PANDER is specifically expressed in the islets of Langerhans of the pancreas, the small intestine, the testis, the prostate, and some neurons of the brain (17). In this study, we have evaluated the effects of PANDER on mouse, human, and rat pancreatic islets as well as insulin-secreting  $\beta$ -cell lines.

## RESEARCH DESIGN AND METHODS

PANDER, a novel cytokine identified and characterized by GlaxoSmithKline (King of Prussia, PA), belongs to a new cytokine family and encodes a 235-amino acid protein with a secretion signal peptide (GenBank accession no. AF494379). Our previous study has shown that PANDER is mainly expressed in the pancreas (17). The expression and purification of recombinant murine and human PANDER was performed as previously described (17). Murine PANDER was used in most of the experiments, except for the studies with human islets, which used human PANDER. All other materials were from Sigma unless otherwise indicated.

**$\beta$ -TC3 cell culture.**  $\beta$ -TC3 insulinoma cells were cultured in T175 cm flasks in complete RPMI-1640 (11 mmol/l glucose) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mmol/l L-glutamine at 37°C under conditions of 95% air and 5% CO<sub>2</sub>. The medium was changed twice a week and on the day before the experiment. Cells were trypsinized weekly and were used exclusively between passages 40

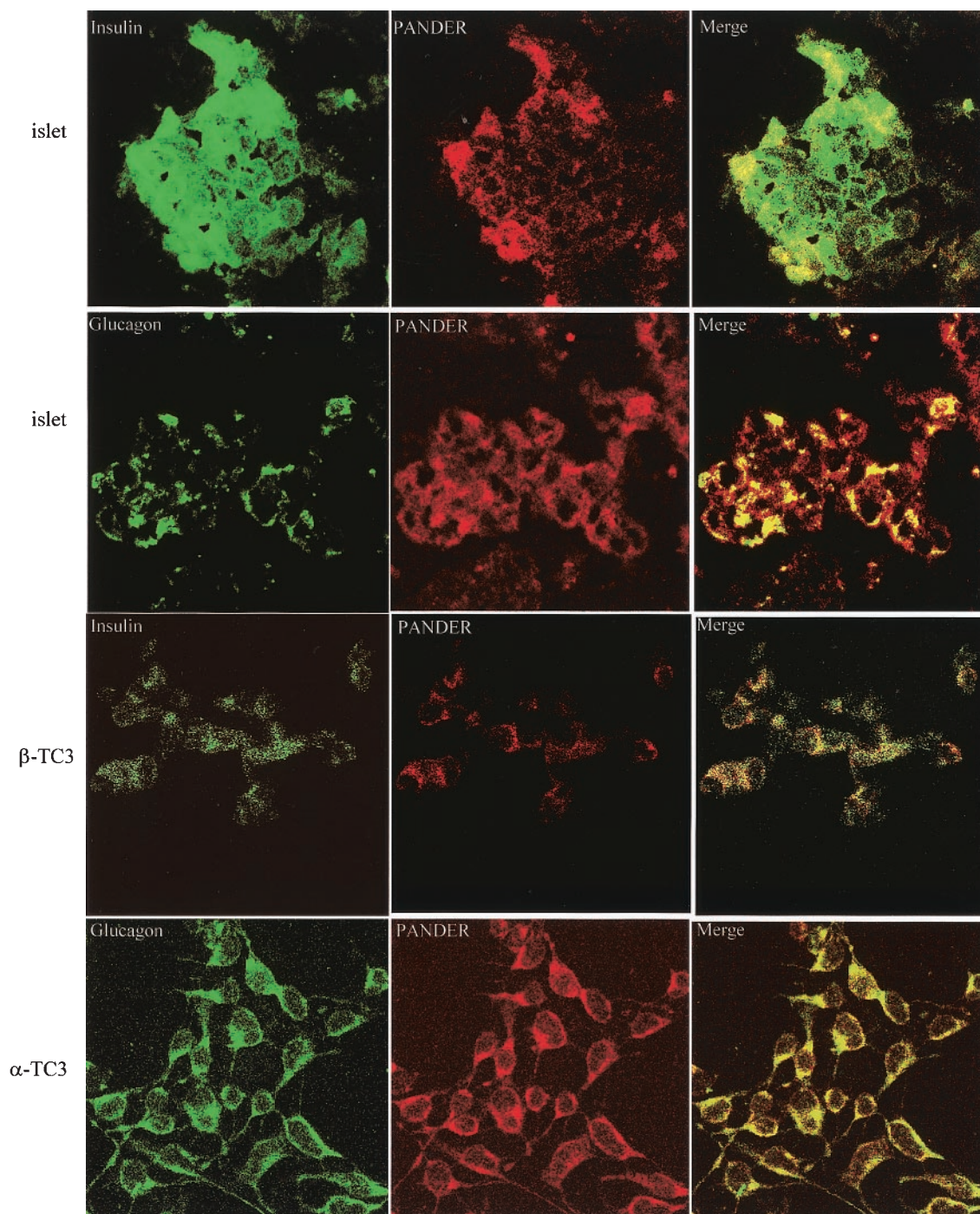
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DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; MTT, 3-(4,5-dimethylthiazol-2-yl) tetrazolium bromide; NO, nitric oxide; PI, propidium iodide; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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**FIG. 1.** Expression of PANDER in human islets,  $\beta$ -TC3 cells, and  $\alpha$ -TC3 cells. Human islets were gently pelleted and cut into 4- $\mu$ m sections.  $\alpha$ -TC3 and  $\beta$ -TC3 cells were cultured in the chambers of poly-L-lysine-coated Lab-Tek Chamber slide system. Sections were treated with appropriate antibodies as described in RESEARCH DESIGN AND METHODS and analyzed by fluorescent confocal microscopy. Immunostaining of insulin (green) and PANDER (red) or glucagon (green) and PANDER (red) were used to localize PANDER in normal human islets (images of rows 1 and 2),  $\beta$ -TC3 cells (row 3), and  $\alpha$ -TC3 cells (row 4). Results are representative of three independent experiments.

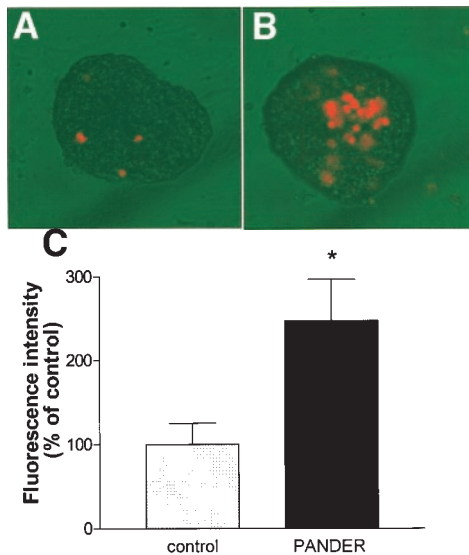
and 53. For PANDER studies,  $\beta$ -TC3 cells were fed for 24 h before use and washed with prewarmed RPMI-1640 (3 mmol/l glucose, 1% FBS) twice. Cells were then cultured in RPMI-1640 (1% FBS, 3 mmol/l glucose) supplemented with PANDER as indicated. Vehicle-treated cells served as controls.

**$\alpha$ -TC3 cell culture.**  $\alpha$ -TC3 cells were cultured in T175 cm flasks in complete Dulbecco's modified Eagle's medium (DMEM) (25 mmol/l glucose) supplemented with 15% FBS, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mmol/l L-glutamine at 37°C under conditions of 95% air and 5% CO<sub>2</sub>. The medium was changed twice a week and on the day before the experiment. Cells were trypsinized weekly and were used exclusively between passages 37 and 41. For PANDER studies,  $\alpha$ -TC3 cells were fed for 24 h before use and

washed with prewarmed DMEM (25 mmol/l glucose, 5% FBS) twice. Cells were then cultured in DMEM (1% FBS, 25 mmol/l glucose) supplemented with PANDER as indicated.

**Isolation of pancreatic islets.** Male Sprague-Dawley rats or C57 black mice (Charles River Laboratories, Boston, MA) were injected with Nembutal. After the rats were anesthetized, the bile duct was cannulated, and the pancreas was inflated with ~20 ml Hank's balanced buffer. The inflated pancreas was removed and cleaned of its lymph nodes, fat, blood vessels, and bile duct. Tissue was digested with collagenase P (Roche Molecular Biochemicals) as previously described (18,19) and purified on a discontinuous Ficoll gradient. Isolated islets were washed and cultured in complete CMRL-1066 (supple-

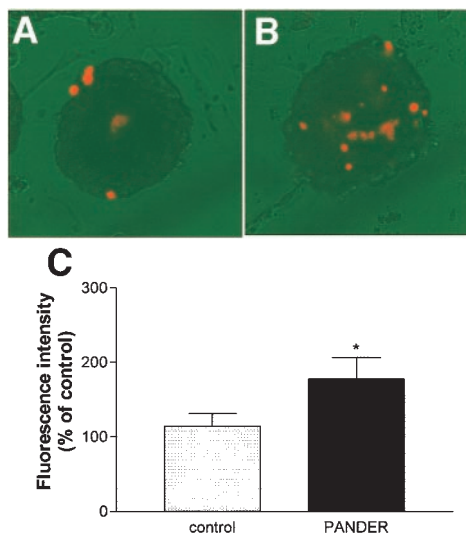




**FIG. 2.** Cytotoxic effects of PANDER on rat islets. Rat islets were placed into 6-well plates and cultured in complete CMRL-1066 with vehicle (A) or with 4 nmol/l PANDER (B) for 7–9 days. Islet cell viability was determined by incubation with PI as described in RESEARCH DESIGN AND METHODS, followed by confocal microscopy analysis (A and B). Total fluorescence intensity of the islets in each well was quantitated by a fluorometer (C). Results are expressed as the means  $\pm$  SE of three independent experiments. \* $P < 0.05$ .

mented with 10% FBS, 5.6 mmol/l glucose, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin culture medium at 37°C (95% air and 5% CO<sub>2</sub>). Human islets were obtained from the Islet Isolation Core of the Juvenile Diabetes Foundation–Penn Islet Transplantation Center. Human islets were cultured in CMRL (lot no. 99603007, supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin; Mediatech, Cellgro, VA) at 37°C under 95% air and 5% CO<sub>2</sub>.

**Confocal microscopy.** Human islets were centrifuged at 600g for 5 min, and the pellet of islets was embedded with frozen tissue embedding media (Fisher Diagnostic). The islet mass was then cut into sections of 4  $\mu$ m and stored at  $-80^{\circ}\text{C}$  until use.  $\alpha$ -TC3 cells and  $\beta$ -TC3 cells were seeded and cultured in the chambers of a poly-L-lysine-coated Lab-Tek chamber slide system (Nalge Nunc). On the day of the experiment, sections were washed twice with cold



**FIG. 3.** Cytotoxic effects of PANDER on human islets. Human islets were placed into 6-well plates and cultured in complete CMRL-1066 with vehicle (A) or with 4 nmol/l PANDER (B) for 7–9 days. Islet cell viability was determined by incubation with PI as described in RESEARCH DESIGN AND METHODS, followed by confocal microscopy analysis (A and B). Total fluorescence intensity of the islets in each well was quantified by a fluorometer (C). Results are expressed as the means  $\pm$  SE of three independent experiments. \* $P < 0.05$ .

PBS, fixed with acetone at  $-20^{\circ}\text{C}$  for 5 min, and then incubated with 5% BSA in PBS for 30 min at room temperature. Sections were then treated with a mixture of antibodies of goat anti-insulin (1:50 dilution; Santa Cruz) and rabbit anti-PANDER (1:50 dilution; GlaxoSmithKline) or a mixture of goat anti-glucagon (1:50 dilution; Santa Cruz) and rabbit anti-PANDER (1:50 dilution; GlaxoSmithKline) for 2 h at room temperature. The sections were washed and treated with a mixture of donkey anti-goat fluorescein isothiocyanate (FITC; 1:100 dilution) and donkey anti-rabbit rhodamine (1:200 dilution; Jackson ImmunoResearch, Chester, PA) in 2% normal donkey serum in PBS for 1 h in the dark. The sections were washed with ice-cold PBS again and mounted with PermaFluor reagent (Immunon). Samples were visualized by fluorescent confocal microscopy (Leica). Images were captured using Leica TCS SP2 fluorescent microscopy using a HeNe-argon laser as a light source. Leica confocal software was used to operate the microscopy and laser source. Each image was collected with excitation at 488 nm, emission at 520 nm (green, FITC), and with excitation at 543 nm, emission at 610 nm (red, rhodamine), concurrently. The images were analyzed using Leica confocal software.

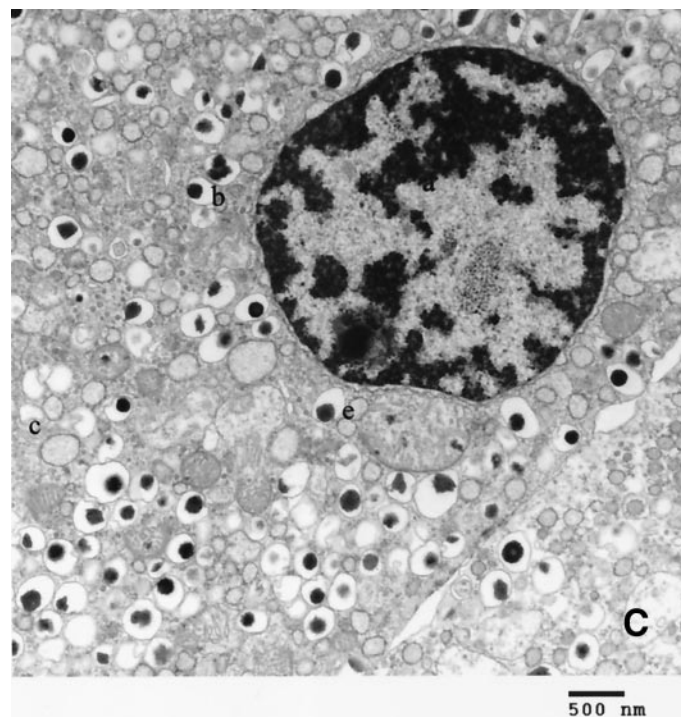
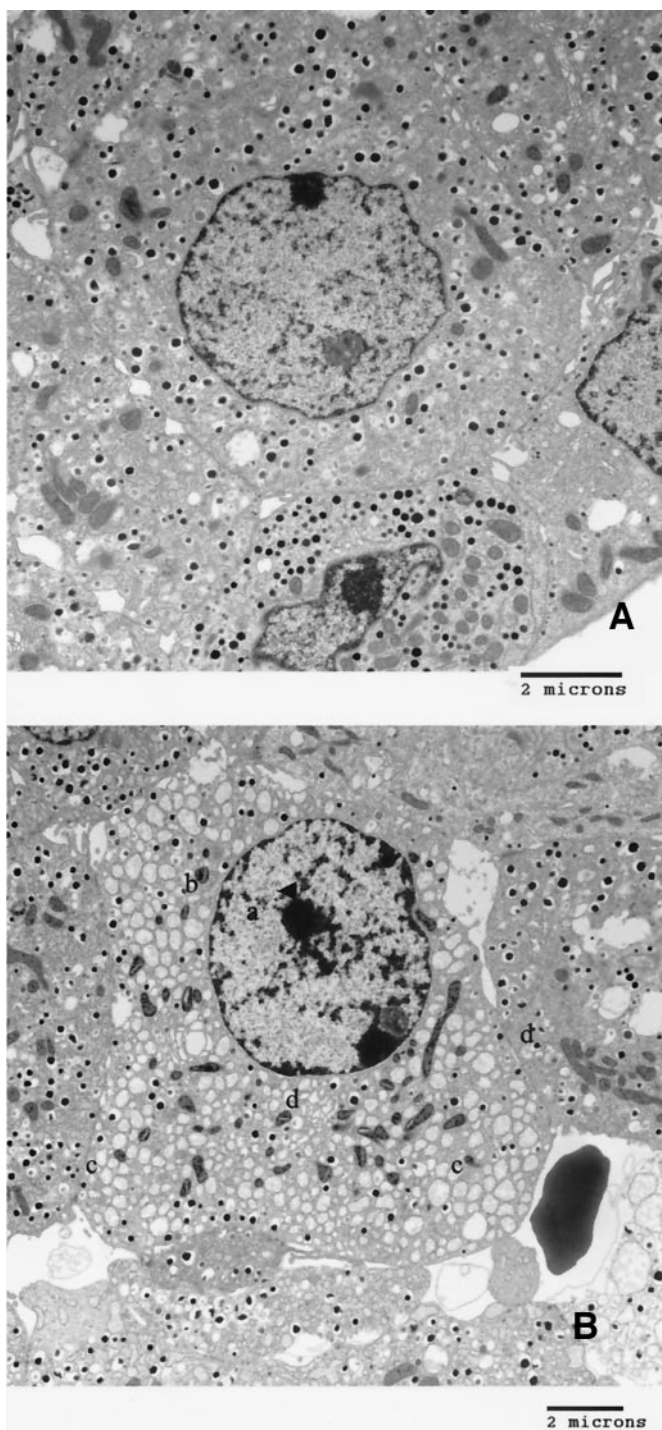
#### Cell viability determination

**C,N-diphenyl-N'4-5-dimethyl thiazol-2-yl tetrazolium bromide assay.** The C,N-diphenyl-N'4-5-dimethyl thiazol-2-yl tetrazolium bromide (MTT) assay is an indirect measure of  $\beta$ -cell viability (20). The assay is based on the ability of viable cells to reduce MTT to insoluble colored formazan crystals.  $\beta$ -TC3 cells were plated in 24-well plates ( $1 \times 10^6$ ) on the day before the experiment. After culture with or without PANDER, cells were washed twice with Krebs-HEPES buffer (115 mmol/l NaCl, 24 mmol/l NaHCO<sub>3</sub>, 5 mmol/l KCl, 1 mmol/l MgCl<sub>2</sub>, 2.5 mmol/l CaCl<sub>2</sub>, 25 mmol/l HEPES, and 1% BSA, pH 7.4) with no glucose and incubated in 1 ml Krebs-HEPES buffer (0 mmol/l glucose) with 0.5 mg/ml MTT for 60 min at 37°C. The supernatant was discarded, and cells in each well were lysed with 500  $\mu$ l of 2-propanol and incubated for 1 h at room temperature. The optical density of the resultant colored 2-propanol was measured at 560 nm on a MicroKinetics plate reader.

**Annexin V and propidium iodide staining.** Annexin V is a Ca<sup>2+</sup>-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS). In normal cells, PS is located on the cytoplasmic surface of the cell membrane. In apoptotic cells, PS is translocated from the inner to the outer surface of the cell membrane. Annexin V labeled with a fluorophore can identify apoptotic cells by binding to PS exposed on the outer leaflet.  $\beta$ -TC3 cells were treated with vehicle or with 4 nmol/l PANDER for 2 days and then washed once with ice-cold PBS followed by annexin V binding buffer (10 mmol/l HEPES, 140 mmol/l NaCl, and 2.5 mmol/l CaCl<sub>2</sub>, pH 7.4). Cells were then resuspended with annexin V binding buffer and incubated with 25  $\mu$ l annexin V/100  $\mu$ l binding buffer and 10  $\mu$ g/ml propidium iodide (PI) for 15 min at room temperature in the dark. Cells were washed again with annexin V binding buffer and analyzed by confocal microscopy with excitation at 488 nm (green, annexin V) and 543 nm (red, PI), concurrently.

**Quantitative assay of PI nuclear staining.** PI is a highly polar dye that penetrates cells with damaged membranes and stains the nuclei red. After  $\beta$ -TC3 cells were cultured with PANDER, PI solution was added to each well (final 10  $\mu$ g/ml) and incubated for 15 min at room temperature in the dark. Islets were stained with 15  $\mu$ g/ml PI for 45 min at room temperature. Cell images were visualized by a Nikon fluorescence microscope (Diaphot) connected with a Hamamatsu digital camera (C4742-95) at 40 $\times$  magnification and were analyzed with SimplePCI software (Compix Imaging Systems, Cramberry, PA) with excitation at 488 nm and emission at  $>510$  nm. For each well, three or more random pictures (at 40 $\times$ ) were acquired under bright and fluorescence microscopy. Apoptotic cells were detected by their fragmented red nuclei, whereas necrotic cells were detected by their intact red nuclei. The percentage of apoptotic and necrotic cells was determined by counting all of the red-stained cells divided by all of the cells in the bright-field pictures. A minimum of 500 cells was counted per well. For quantitative analysis of islet viability, the PI fluorescence intensity of the total islets in each condition was measured with a fluorometer (Victor 2000; PerkinElmer).

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay.** An in situ cell death detection kit (Boehringer Mannheim, Indianapolis, IN) was used to quantify apoptotic cells. In this method, terminal deoxynucleotidyl transferase was used to catalyze the polymerization of fluorescein-labeled nucleotides to free 3-OH termini of DNA strand breaks. After 2 days culture with vehicle or with 4 nmol/l PANDER,  $\beta$ -TC3 cells were trypsinized and washed twice with cold PBS/1% BSA. Cells were then fixed with 200  $\mu$ l 4% paraformaldehyde and incubated 30 min at room temperature. After rinsing with PBS, cells were resuspended with 250  $\mu$ l of permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) and incubated on ice for 10 min. Then, 50  $\mu$ l of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction mixture was added to samples and the positive controls (50  $\mu$ l of label solution only was added to the negative controls), and cells were incubated at 37°C for 1 h. Apoptotic cells were



**FIG. 4. Apoptotic effects of PANDER on mouse islets.** Mouse islets were cultured in CMRL-1066 (10% FBS). Islets were treated with vehicle (A) or with 4 nmol/l PANDER (B and C) for 4–5 days. Cell apoptosis was measured by electron microscopy as described in RESEARCH DESIGN AND METHODS. Apoptotic cells in islets were detected by nuclear condensation (a), irregular dilation of the nuclear envelope (b), granule dilution (c), clumped and condensed mitochondria (d), and flocculent density of the mitochondria (e). Results are representative of two independent experiments.

hyde and 2% paraformaldehyde in sodium cacodylate buffer for 4 h. The islets were collected from the dish to make a pellet. After washing, osmication, and dehydration with ethanol and propylene oxide, the pellets were embedded in EM-Bed 812 and polymerized at 70°C for 48 h. Semithin sections (1  $\mu$ m) were stained with Toluidine blue to screen general cell morphology. Ultrathin sections (80 nm) were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEOL-1010 TEM (transmission electron microscopy) operated at the accelerated voltage of 80 kV.

**Nitrate/nitrite assay.** A Cayman chemical nitrate/nitrite assay kit was used to detect the sum of both nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ). Two hundred islets were transferred to each well in a 96-well plate with 200  $\mu$ l of complete CMRL, and they were incubated with the reagents indicated for 2 days. Then, 100  $\mu$ l of cell-free culture media was mixed with 100  $\mu$ l of Greiss reagent and incubated for 15 min, and the absorbance was determined at 540 nm with a Microkinetics plate reader (Fisher) (21).

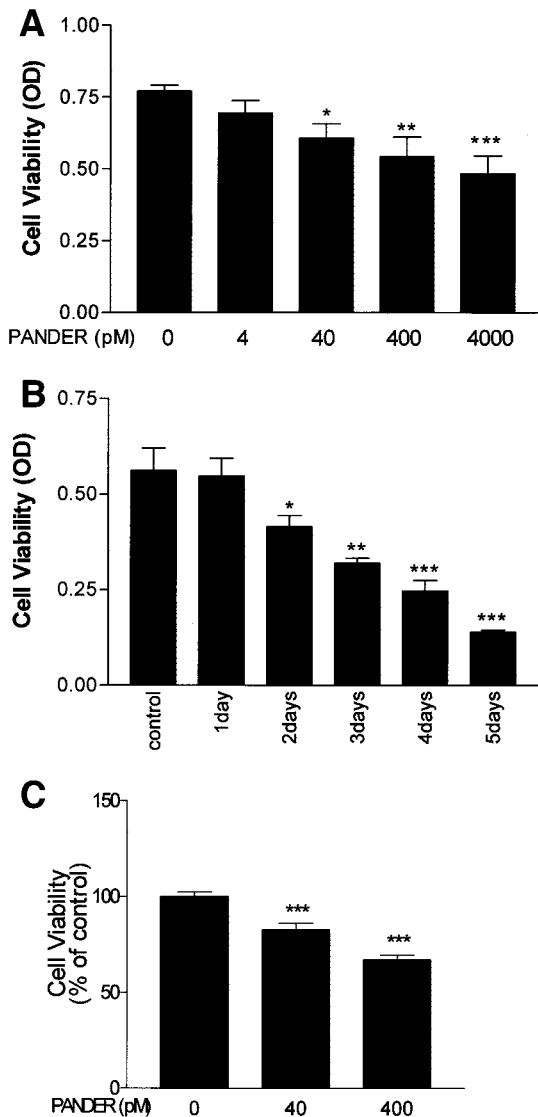
**Cytosolic free  $\text{Ca}^{2+}$  measurement.**  $\beta$ -TC3 cells were plated on microscope glass coverslips in 6-well plates 2 days before the experiment and then cultured in RPMI-1640 supplemented with 1% FBS and 3 mmol/l glucose with vehicle or with 4 nmol/l PANDER. On the day of the experiment, cells were loaded with 2.5  $\mu$ mol/l fura-2 (Molecular Probes, Eugene, OR) at 37°C and 5%  $\text{CO}_2$  for 30 min. The coverslip with the cells was then mounted in a perfusion chamber placed on a homeothermic platform set at 37°C and visualized by a Nikon fluorescence microscope (Diaphot) connected with a Hamamatsu digital camera (C4742-95), at 20 $\times$  magnification. The cells were superfused with Krebs-HEPES buffer at 37°C. Fura-2 was excited at 340 nm and 380 nm and emitted at 510 nm. The fluorescence images were analyzed by SimplePCI software (Compix Imaging Systems). The concentration of  $\text{Ca}^{2+}$  was calculated by comparing the ratio of fluorescence at each pixel to an in vitro two-point calibration curve. The  $\text{Ca}^{2+}$  concentration is presented by averaging the values of all pixels of a single cell.

**Immunoblotting.**  $\beta$ -TC3 cells were cultured in 10-cm dishes in RPMI-1640 supplemented with 1% FBS, 3 mmol/l glucose, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mmol/l L-glutamine at 37°C under conditions of 95% air and 5%  $\text{CO}_2$ . Cells were treated with vehicle or with 4 nmol/l PANDER for 20 h. Cell lysates were prepared and extracted with SDS sample buffer. Samples were analyzed by 15% SDS-PAGE and electrotransferred to Hybond

identified by FITC staining and analyzed by flow cytometry. A Coulter EPICS Elite flow cytometer (Beckman-Coulter, Hialeah, FL) equipped with a 5-W argon laser operated at 488 nm and 260 mW output was used for all studies. Monomeric forms of the  $\beta$ -TC3 cells were electronically gated based on forward- and side-scatter measurements to exclude cell aggregates from evaluation. Fluorescence signals were collected with a photomultiplier tube configured with 550 nm dichroic and 525 nm band pass filters. A total of 10,000 events were collected into a 4-decalog single-parameter histogram for each sample. Percent positive cells were determined based on the evaluation of cells treated with TUNEL reagents lacking TdT (terminal deoxynucleotidyl transferase) using a cursor setting that yielded <2% positive cells.

**Electron microscopic determination of cell apoptosis in islets.** Mouse islets were cultured in complete CMRL-1066 in 6-cm dishes with vehicle or with 4 nmol/l PANDER for 4–5 days. Islet preparations were then washed briefly with prewarmed serum-free medium and fixed with 2.5% glutaralde-





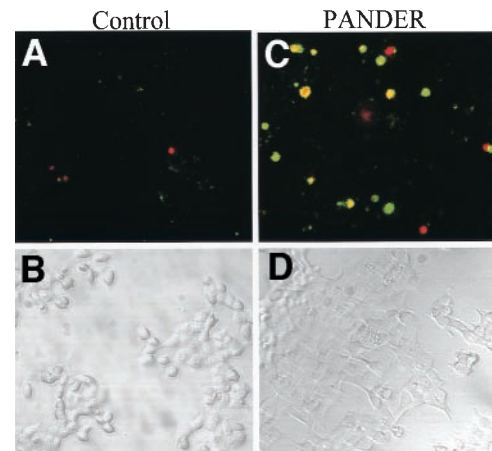
**FIG. 5.** PANDER affects  $\beta$ -TC3 cell line viability. **A:** Dose-dependence of PANDER on  $\beta$ -TC3 cell viability.  $\beta$ -TC3 cells ( $1 \times 10^6$ ) were plated in 12- or 24-well plates and cultured for 48 h in RPMI-1640 (1% FBS plus 3 mmol/l glucose) with different concentrations of PANDER. Cell viability was measured by MTT metabolism as described in RESEARCH DESIGN AND METHODS. **B:** Time-dependence of PANDER on  $\beta$ -TC3 cell viability.  $\beta$ -TC3 cells were cultured as above without (control) or with 4 nmol/l PANDER for 1–5 days. **C:** Dose-dependence of PANDER on  $\alpha$ -TC3 cell viability.  $\alpha$ -TC3 cells were plated in 24-well plates and cultured for 48 h in DMEM (1% FBS plus 3 mmol/l glucose) with different concentrations of PANDER. Cell viability was measured by MTT metabolism at each time point. Results are expressed as the means  $\pm$  SE of optical density (OD) of five independent experiments in triplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

nitrocellulose membranes, which were then probed with antibodies to cleaved caspase-3 (Cell Signal) and PANDER (GlaxoSmithKline). For PANDER expression, only the control lysates were used. Immunoblot analysis was performed using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

**Statistical analysis.** Data are presented as the means  $\pm$  SE. Statistical significance of differences between groups was analyzed by unpaired Student's *t* test or by one-way ANOVA when more than two groups were compared.

## RESULTS

PANDER was clearly localized to the islets of Langerhans of human pancreas and colocalized with insulin as well as glucagon (Fig. 1), suggesting that PANDER is expressed in

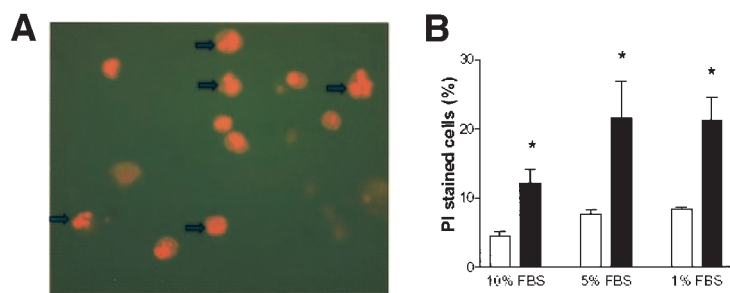


**FIG. 6.** Annexin V and PI staining of  $\beta$ -TC3 cells treated with PANDER.  $\beta$ -TC3 cells ( $1 \times 10^6$ ) were plated in 10-cm dishes and cultured in RPMI-1640 (1% FBS). Cells were treated with vehicle (**A** and **B**) or with 4 nmol/l PANDER (**C** and **D**) for 48 h, incubated with annexin V and PI, and analyzed by confocal microscopy. Apoptosis was measured by annexin V staining (green). Late apoptosis or necrosis were detected by PI staining (red nuclei). Results are representative of three independent experiments.

pancreatic  $\beta$ - and  $\alpha$ -cells in the human islets. Immunofluorescent double-staining of insulin and PANDER in  $\beta$ - and  $\alpha$ -TC3 cells (Fig. 1) also demonstrated the colocalization. A protein band around 23 kDa, the expected mass of PANDER, was detected in Western blots of lysates from insulin-secreting cell lines ( $\beta$ -TC3) and blotted with anti-PANDER antibody (Fig. 9A).

The effect of PANDER on rat and human islet-cell viability was measured by confocal microscopy analysis and quantitative assay of fluorescence intensity after PI staining. After 7–9 days culture with 4 nmol/l PANDER, there was an increase in dead cells in the PANDER-treated rat and human islets (Figs. 2B and 3B) compared with vehicle-treated islets (Figs. 2A and 3A). As shown in Figs. 2C and 3C, the increase in PI-staining cells was significantly higher in PANDER-treated rat and human islets compared with control. Electron microscopy analysis of PANDER-treated mouse islets showed that PANDER induced different stages of apoptotic morphologic changes in the cells, which included condensation in the nuclei, granule dilution, clumped and condensed mitochondria, and nuclear envelopes with irregular dilation in the early stage (Fig. 4B). PANDER also induced flocculent density of mitochondria, more condensation in the nuclei, and more diluted granules in the late stage (Fig. 4C).

To determine the dose-dependency of PANDER's cytotoxic effects, insulin-secreting  $\beta$ -TC3 cells were incubated in RPMI-1640 with increasing amounts of PANDER for 48 h and then evaluated for cell viability with the MTT assay. As shown in Fig. 5A, a low dose of 4 pmol/l PANDER had no effect on  $\beta$ -TC3 cell viability, but a dose-dependent inhibitory effect on cell viability was observed with PANDER concentrations  $>40$  pmol/l. The optical densities in the MTT assay were  $0.70 \pm 0.05$  (vs. control  $0.77 \pm 0.05$ ,  $P > 0.05$ ),  $0.61 \pm 0.05$  (vs. control  $P < 0.05$ ),  $0.54 \pm 0.05$  (vs. control  $P < 0.01$ ), and  $0.48 \pm 0.05$  (vs. control  $P < 0.001$ ) for 4, 40, and 400 pmol/l and 4 nmol/l of PANDER, respectively. Furthermore, exposure of  $\beta$ -TC3 cells to 4 nmol/l PANDER for 24 h to 5 days



**FIG. 7.** PI staining of control and PANDER-treated  $\beta$ -TC3 cells.  $\beta$ -TC3 cells ( $1 \times 10^6$ ) were plated in 24-well plates and cultured in vehicle or with 4 nmol/l PANDER (A) for 48 h. Cell viability was determined with DNA-binding dye PI as described in RESEARCH DESIGN AND METHODS. The nuclei of the cells with damaged cell membranes are stained by PI and emit red fluorescence. Apoptotic cells were detected by fragmented nuclei (indicated by green arrows), whereas necrotic cells were detected by intact red nuclei. B: An estimate of the percentage of red-stained cells. A minimum of 500 cells was counted in at least three random fields for each condition. Results are expressed as the means  $\pm$  SE of nine independent experiments.  $\square$ , vehicle;  $\blacksquare$ , PANDER. \* $P < 0.05$ .

induced a time-dependent inhibitory effect on  $\beta$ -TC3 cell viability. Cell viability was inhibited by 27, 43, 55, and 75% at 2, 3, 4, and 5 days, respectively, even though PANDER had no significant effect at 24 h (Fig. 5B).

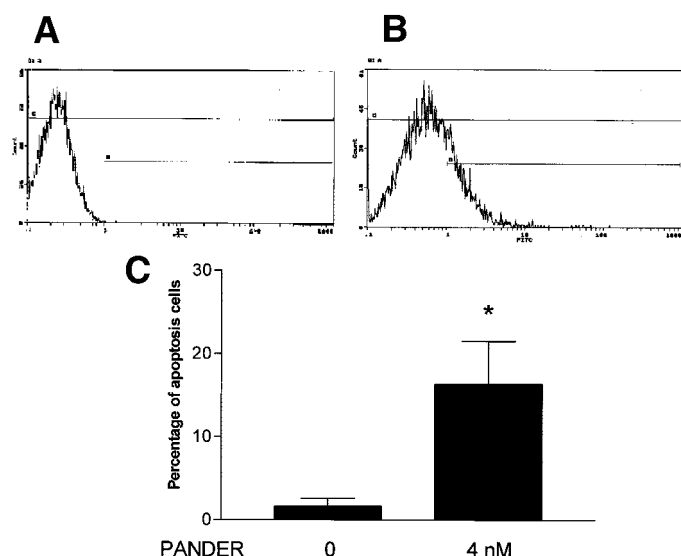
To determine the cytotoxic effect of PANDER on glucagon-secreting  $\alpha$ -cells,  $\alpha$ -TC3 cells were treated with or without 40 or 400 pmol/l PANDER for 48 h before cell viability was measured by the MTT method. Cell viability was decreased to  $82.8 \pm 3.3$  or  $67.0 \pm 3.5\%$  compared with control by 40 or 400 pmol/l PANDER, respectively ( $P < 0.05$ ) (Fig. 5C). These results imply that PANDER is also cytotoxic to  $\alpha$ -cells.

Both annexin V fluorescence staining and PI fluorescence staining were used to analyze the morphologic changes of  $\beta$ -TC3 cells treated by PANDER. After a 48-h culture with 4 nmol/l PANDER or with vehicle (control), PANDER-treated cells had a much higher percentage of annexin V-positive apoptotic cells (green membrane fluorescence) and PI-positive necrotic cells (red nuclei fluorescence) than vehicle-treated cells, confirming the findings of the MTT assay (Fig. 6). To obtain a quantitative assessment, PANDER-treated  $\beta$ -TC3 cells were stained with PI and counted. Compared with control, there was an almost threefold increase of necrotic cells in the PANDER-treated cells than the control cells. The presence of different concentrations of FBS (1–10%) did not prevent PANDER-

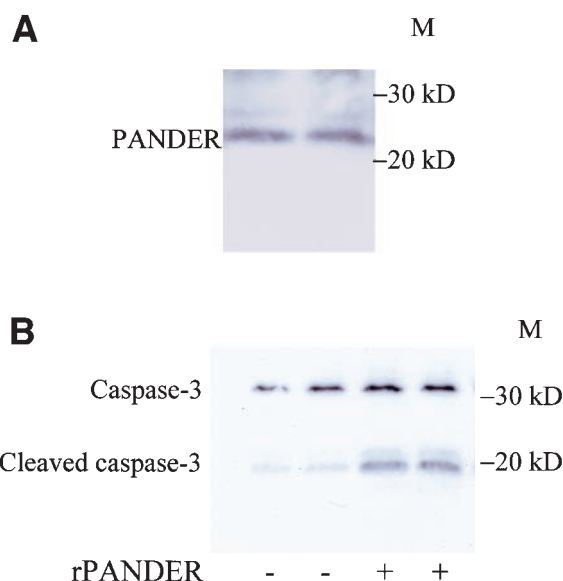
induced toxicity (Fig. 7). Several of the PANDER-treated  $\beta$ -TC3 cells showed condensation and fragmentation of the nuclei, suggesting that PANDER induced apoptosis in these cells (Fig. 7).

To further analyze PANDER-induced apoptosis in  $\beta$ -TC3 cells, DNA fragmentation was measured by TUNEL labeling of  $\beta$ -TC3 cells. The TUNEL method was first validated using negative and positive controls. First, the number of positive cells increased to  $94.1 \pm 1.3\%$  after DNase treatment of  $\beta$ -cell DNA compared with  $1.3 \pm 0.2\%$  in the negative control. Second, after  $H_2O_2$  killing of  $\beta$ -cells, the number of positive cells increased to  $65.9 \pm 18.2\%$ . As shown in Fig. 8, PANDER (48-h treatment) caused a significant right shift of TUNEL labeling of  $\beta$ -TC3 cells analyzed by flow cytometry, resulting in a highly significant 7.5-fold increase in apoptotic  $\beta$ -TC3 cells compared with vehicle ( $16.4 \pm 5.1$  vs.  $2.2 \pm 1.0$ ,  $n = 7$ ,  $P < 0.05$ ).

The level of cleaved caspase-3 was significantly increased in cells treated with PANDER for 20 h compared with control cells, as assessed by immunoblotting. Densitometric analysis of the cleaved caspase-3 bands showed an increase to  $1,029 \pm 34\%$  vs. control  $100 \pm 16\%$  ( $P < 0.0001$ ), with no changes in caspase-3 between the two groups (Fig. 9B). Carbachol (1 mmol/l) induced a rapid



**FIG. 8.** TUNEL assay of  $\beta$ -TC3 cells treated with PANDER.  $\beta$ -TC3 cells ( $1 \times 10^6$ ) were plated in 10-cm dishes and cultured in RPMI-1640 (1% FBS). Cells were treated with vehicle (A) or with 4 nmol/l PANDER (B) for 48 h. Apoptosis was measured by TUNEL flow cytometric analysis as described in RESEARCH DESIGN AND METHODS. Percentage of positive cells in  $10^5$  cells was then counted for each condition (C). Results are expressed as mean  $\pm$  SE of seven independent experiments. \* $P < 0.05$ .



**FIG. 9.** PANDER and caspase-3 levels in  $\beta$ -TC3 cells.  $\beta$ -TC3 cells were plated in 10-cm dishes and cultured in RPMI-1640. Cell lysates were subjected to 15% SDS-PAGE gel. PANDER expression was analyzed by immunoblotting with PANDER antibody (A). Caspase-3 and cleaved caspase-3 levels were analyzed by immunoblotting with anti-cleaved caspase-3 antibody (B) after cells were treated with vehicle or 5 nmol/l PANDER for 20 h. Data are representative of three independent experiments.

and transient increase of cytosolic intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in both vehicle- and PANDER-treated  $\beta$ -TC3 cells. The peak was about twofold higher than the average baseline ( $230.0 \pm 12.6$  vs.  $112.2 \pm 2.7$  nmol/l  $\text{Ca}^{2+}$  in the control cells,  $201.3 \pm 7.3$  vs.  $114.9 \pm 3.8$  nmol/l  $\text{Ca}^{2+}$  in the PANDER-treated cells;  $n = 7$ ). There was no significant difference between the control and the PANDER-treated cells in both the basal condition as well as the carbachol-stimulated condition. Also, potassium induced a rapid and fourfold increase of cytosolic  $[\text{Ca}^{2+}]_i$  in both vehicle- and PANDER-treated  $\beta$ -TC3 cells ( $467.5 \pm 19.6$  vs.  $152.1 \pm 5.3$  nmol/l  $\text{Ca}^{2+}$  in the control cells,  $422.5 \pm 13.7$  vs.  $160.4 \pm 6.3$  nmol/l  $\text{Ca}^{2+}$  in the PANDER-treated cells;  $n = 7$ ). There were also no significant differences between the control and the PANDER-treated cells under basal conditions and after potassium stimulation. To determine whether PANDER cytotoxicity was caused by elevated nitric oxide (NO) production, we measured levels of NO end products in the culture media. Mouse PANDER did not induce NO production at either of the concentrations tested (data not shown).

## DISCUSSION

PANDER is a member of a new cytokine family identified by a search for novel proteins with the unique architecture and topology of the short-chain, four-helix bundle cytokines (17). Although its function is unknown, PANDER was found to be specifically expressed in pancreatic islets of Langerhans, including  $\beta$ -cells as well as  $\alpha$ -cells, and is present in insulin-secreting  $\beta$ -cell lines. To elicit the potential role of PANDER in the pancreatic islets, we investigated whether PANDER affects pancreatic  $\beta$ -cell,  $\alpha$ -cell, and islet viability. We show that treatment of  $\beta$ -cells,  $\alpha$ -cells, and islets with PANDER causes cell death. PANDER inhibits  $\beta$ -cell viability in a dose-dependent as well as time-dependent manner. It induces  $\beta$ -cell apoptosis through caspase-3 activation.

$\beta$ -Cell destruction in type 1 diabetes is the result of a chronic inflammatory lesion mediated by interaction between macrophages,  $\text{CD4}^+$  cells, and  $\text{CD8}^+$  T-cells. The role of the inflammatory cytokines interleukin- $1\beta$ , tumor necrosis factor- $\alpha$ , and interferon- $\gamma$  produced by activated macrophages and  $\text{CD8}^+$  T-cells in this process has been reported by many groups (7,22). Cytokines are important mediators of this process (9,15,23,24). There is extensive evidence that these cytokines cause  $\beta$ -cell destruction through necrosis or apoptosis (13), although apoptosis is considered the main mode of  $\beta$ -cell destruction in immune-mediated type 1 diabetes (10–12). However, the exact mechanisms underlying the apoptotic process are still controversial and obscure (25–29). The increased production of NO may be an important signal in cytokine-induced cell death (9,23). In rat islets, NO can cause  $\beta$ -cell toxicity via different mechanisms (13,30), although in human pancreatic islets, cytokine-induced  $\beta$ -cell death may be independent of NO generation (2,15). PANDER, however, had no effect on NO production, suggesting that its mechanism of action is different from that of cytokines such as interleukin- $1\beta$  and tumor necrosis factor- $\alpha$ .

Intracellular  $\text{Ca}^{2+}$  may have a role in apoptosis of the pancreatic  $\beta$ -cell (31,32). Increases in intracellular  $\text{Ca}^{2+}$  can promote endonuclease activation, mitochondrial de-

polarization, and permeability transition and subsequent cytochrome c release, caspase activation, and apoptotic cell death (33,34). Other intracellular cysteine proteases, such as calpains, can be initiated by  $\text{Ca}^{2+}$  and are involved in apoptosis (32). It has also been reported that high glucose and tolbutamide can induce apoptosis in pancreatic  $\beta$ -cells in a  $\text{Ca}^{2+}$ -dependent manner (35), and a calcium channel blocker can partially inhibit glibenclamide-induced  $\beta$ -cell apoptosis (33). In this study, we examined the effects of PANDER on  $\text{Ca}^{2+}$  homeostasis in  $\beta$ -cells. Neither basic  $[\text{Ca}^{2+}]_i$  nor carbachol- or potassium-induced  $[\text{Ca}^{2+}]_i$  increase was affected by PANDER. Carbachol and potassium utilize different pathways to stimulate cytosolic  $\text{Ca}^{2+}$ . Carbachol releases  $\text{Ca}^{2+}$  from the endoplasmic reticulum, whereas potassium depolarizes the membrane, resulting in  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels (36). Clearly, PANDER does not affect  $\text{Ca}^{2+}$  homeostasis in pancreatic  $\beta$ -cells. Therefore, its pro-apoptotic effects in pancreatic  $\beta$ -cells are  $\text{Ca}^{2+}$  independent.

Caspases are a family of proteases. They are present in most cells and are crucial mediators of apoptosis (37). Caspase-3 is at pivotal junction in the apoptotic pathway (38). It is activated by proteolytic cleavage into subunits of 19 and 17 kDa. Our data has shown that culture of  $\beta$ -TC3 cells with PANDER for 20 h significantly increased the 19- and 17-kDa cleaved caspase-3 subunit, suggesting that PANDER induces  $\beta$ -cell apoptosis via the caspase-3 pathway.

What is the role of PANDER in islet physiology and pathophysiology? PANDER is a novel, secreted cytokine-like protein present in  $\beta$ -cells of mouse, rat, and human pancreatic islets. Our initial observations indicate that recombinant PANDER has potent apoptotic effects on the  $\beta$ -cell that are analogous to those originally described for interleukin-1 (39,40). Although the receptor for PANDER has yet to be identified, the demonstration that it kills  $\beta$ -cells in vitro suggests that the  $\beta$ -cells could be a target for the action of PANDER. Clearly, an understanding of the mechanisms regulating PANDER production and secretion are crucial to understanding how a  $\beta$ -cell-specific secreted protein can induce apoptosis of islets. Whether PANDER is involved in the pathogenesis of diabetes remains to be determined and is currently under investigation.

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