Nodal induces apoptosis through activation of the ALK7 signaling pathway in pancreatic INS-1 β-cells

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Zhao F, Huang F, Tang M, Li X, Zhang N, Amfilochiadis A, Li Y, Hu R, Jin T, Peng C, Wang Q. Nodal induces apoptosis through activation of the ALK7 signaling pathway in pancreatic INS-1 β-cells. Am J Physiol Endocrinol Metab 303: E132–E143, 2012. First published May 1, 2012; doi:10.1152/ajpendo.00074.2012.—We demonstrated previously that the activation of ALK7 (activin receptor-like kinase-7), a member of the type I receptor serine/threonine kinases of the TGF-β superfamily, resulted in increased apoptosis and reduced proliferation through suppression of Akt signaling and the activation of Smad2-dependent signaling pathway in pancreatic β-cells. Here, we show that Nodal activates ALK7 signaling and regulates β-cell apoptosis. We detected Nodal expression in the clonal β-cell lines and rodent islet β-cells. Induction of β-cell apoptosis by treatment with high glucose, palmitate, or cytokines significantly increased Nodal expression in clonal INS-1 β-cells and isolated rat islets. The stimuli induced upregulation of Nodal expression levels were associated with elevation of ALK7 protein and enhanced phosphorylated Smad3 protein. Nodal treatment or overexpression of Nodal dose- or time-dependently increased active caspase-3 levels in INS-1 cells. Nodal-induced apoptosis was associated with decreased Akt phosphorylation and reduced expression level of X-linked inhibitor of apoptosis (XIAP). Remarkably, overexpression of XIAP or constitutively active Akt, or ablation of Smad2/3 activity partially blocked Nodal-induced apoptosis. Furthermore, siRNA-mediated ALK7 knockdown significantly attenuated Nodal-induced apoptosis of INS-1 cells. We suggest that Nodal-induced apoptosis in β-cells is mediated through ALK7 signaling involving the activation of Smad2/3-caspase-3 and the suppression of Akt and XIAP pathways and that Nodal may exert its biological effects on the modulation of β-cell survival and β-cell mass in an autocrine fashion.

activin receptor-like kinase-7; X-linked inhibitor of apoptosis protein; Akt; caspase-3; insulin

THE TGF-β SUPERFAMILY consists of a wide range of factors including TGF-β proteins, activins, bone morphogenetic proteins, differentiation factors, and Nodal and its related proteins (4, 17, 26). Members of the TGF-β superfamily regulate diverse cellular processes including cell growth, cell differentiation, extracellular matrix modification, immunosuppression, apoptosis, and other functions (3, 39). A TGF-β protein exerts its biological function through interactions with type I and type II transmembrane serine/threonine kinase receptors on the cell surface (44). Five members of type II and seven members of type I receptors [activin receptor-like kinase (ALK)1–7] have been characterized in mammals (44). Upon ligand binding, type II receptor recruits and phosphorylates a type I receptor to form a ternary ligand-receptor complex (13) and initiates the downstream signaling cascades by phosphorylating the Smad proteins (61, 62). Typically, the phosphorylated receptor-regulated Smads (R-SMAD) can bind to the common mediator Smad (co-SMAD) to form Smad protein complexes, which are then translocated into the nucleus to act as transcription factors to participate in the regulation of targeted gene expression (44).

ALK7, initially isolated from rat brain as an orphan receptor, is known to be expressed in several tissues including adipose tissue, gut, and pancreas (7, 9, 39, 49). Upon heterodimerization with type II (ActRII) B, ALK7 confers responsiveness to activin-A, activin-AB, and Nodal and its related proteins (47). An in vitro study has demonstrated that ALK7 is specifically expressed during the late phase of adipocyte differentiation (31). Recent studies in rodents and human tissue have shown that the expression levels of ALK7 are negatively correlated with the markers of metabolic syndrome, implying a potential role of ALK7 in the regulation of metabolism and adipose tissue function (2, 31, 34). ALK7 is specifically detected in insulin-producing β-cells (52, 64). A study by Wawenable et al. (60) suggested that Nodal and activin AB could signal through the ALK7/Smad2/3 pathway to regulate PDX-1 (pancreatic duodenal homeobox-1) and proinsulin gene activity. Mice lacking ALK7 displayed islet enlargement, enhanced capacity of insulin secretion, progressive hyperinsulinemia, and impaired glucose tolerance, suggesting that ALK7 plays a negative regulatory role in the modulation of β-cell function (7).

The homeostatic control of β-cell mass is based on the balance in survival and death of the insulin-secreting cells (28). Progressive loss of β-cell mass due to β-cell destruction is implicated in the pathogenesis of type 1 and type 2 diabetes (14, 20, 28, 36). Under pathological conditions, inflammation, lipotoxity, and glucotoxity are the three main stimuli that trigger β-cell apoptosis (28). These stimuli can potentially initiate β-cell apoptosis by activating death receptors at the cell surface, including Fas-Fas ligand interactions (37, 40). Increased expression and activation of Fas in β-cells appear to comprise a molecular event common to the pathogenesis of both type 1 and type 2 diabetes (65). Furthermore, activation of granzymes, or various proinflammatory cytokines, as well as a high concentration of glucose, induces the production of free radicals and oxidative stress, which results in the release of...
cytochrome c and cell death (1, 28). Islet amyloid formation also plays a role in β-cell apoptosis (11, 28, 38).

We (64) demonstrated previously that endogenous ALK7 levels were increased during the induction of β-cell apoptosis by high glucose and/or fatty acid. Elevation of ALK7 by induction with adenovirus-carrying expression vector coding for active ALK7 resulted in a remarkable inhibition of β-cell proliferation and the acceleration of β-cell apoptosis (64). In the β-cells, activation of ALK7-induced inhibition of cell proliferation and increased apoptosis appeared to be mediated by the Smad2-caspase-3 signaling pathway(s), as demonstrated in many other cell lineages (30, 41, 46, 63), and the suppression of Akt activation (64). Previous in vitro and in vivo studies showed that activins or Nodal may interact with ALK7 to exert downstream events (7, 27, 41, 47).

Here, we show that Nodal is specifically expressed in the pancreatic INS-1 β-cell line and in rodent islet β-cells. Upregulation of Nodal expression increased β-cell apoptosis, which is associated with increased ALK7 expression, the activation of Smad2/3 signaling pathways and the inhibition of Akt activation, along with reduced X-linked inhibitor of apoptosis protein (XIAP) expression. ALK7 knockdown using siRNA strategy significantly attenuated Nodal-induced apoptosis of INS-1 cells. Our results suggest that Nodal, in an autocrine fashion, downregulates β-cell function through the ALK7 and downstream signaling pathways involving the activation of Smad2/3-caspase-3 signaling and the suppression of Akt and XIAP signaling pathways.

MATERIALS AND METHODS

Plasmids, reagents, cells, islets and pancreatic tissue preparation.
The construct pcDNA3 constitutively active (CA) Akt was a gift from J. R. Woodgett (University of Toronto). XIAP was a gift of H. Hiu (University of California, Los Angeles). Expression plasmids for Nodal, wild-type (WT) ALK7, CA (threonine-194 was replaced with aspartic acid) ALK7, dominant negative (DN; lysine-222 was replaced with arginine) ALK7, DN-Smad2, and DN-Smad3 have been reported previously (41, 63). The small interfering (si)RNA reagents (siGENOME SMARTpool targeting rat and mouse ALK7, and Scrambled siRNA) were purchased from Dharmacon RNAi technologies (Chicago, IL). Recombinant mouse Nodal protein was purchased from R&D Systems (cat. no. 1315-ND-025; Minneapolis, MN). Palmitate was dissolved in serum-free RPMI 1640 (Invitrogen, Carlsbad, CA) containing 1% fatty acid-free BSA (Sigma-Aldrich, Oakville, ON, Canada). A cytokine cocktail mixture (IL-1β, 10 ng/ml; TNF-β, 50 ng/ml; and IFN-γ, 50 ng/ml; R&D Systems) was prepared.
in RPMI 1640. Streptozotocin (Sigma-Aldrich) was freshly prepared in RPMI 1640 prior to treatment. INS-1 and MIN6 clonal cell lines were maintained in RPMI 1640 containing 10 mmol/l HEPES, 10% fetal bovine serum, 100 U/ml penicillin G, 100 µg/ml streptomycin, 1 mol/l sodium pyruvate, 50 µM 2-mercaptoethanol, and 10 mol/l NaOH, as described previously (64).

Rat pancreatic islets were isolated from male Sprague-Dawley rats (Charles River Canada, Montreal, QC, Canada), as described previously (64). Islets were maintained in RPMI 1640 medium for 16 – 24 h prior to the experiments. Mouse pancreata were isolated from CD1 mouse (Charles River Canada), processed, and paraffin-embedded as described previously (50, 57). All procedures complied with guidelines approved by St. Michael’s Hospital’s animal care committee.

RT-PCR. Total cellular RNA was extracted from rat islets, brain tissues or cultured cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA). Nodal mRNA transcripts were detected using a one-step RT-PCR kit (Qiagen). Briefly, 100 ng of total RNA was used in 25-µl one-step RT-PCR reactions containing 0.4 mmol/l dNTPs and 0.6 µmol/l of each primer. The template was omitted for the negative control. Thermocycler conditions were 50°C for 30 min, 95°C for 15 min, and 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. This was followed by a 10-min extension at 72°C. The RT-PCR products were separated on a 1% agarose gel and visualized with ethidium bromide. The Nodal gene-specific primers used were as follows: forward 5’-CAGAAGCCAACCTATGTAGGAGGGTCA-3’ and reverse 5’-CGGGAGCACA GCATGTAGAAGGAAC-3’.

Fig. 2. Pathophysiologica®l stimuli increase Nodal expression, associated with elevated ß-cell apoptosis. INS-1 cells were incubated with medium alone or with 30 mM glucose (HG) or 0.4 mM palmitate (Pal) or a cytokine cocktail (Cyto; IL-1β 10 ng/ml, TNF-α 50 ng/ml, and IFN-γ 50 ng/ml), or streptozotocin (STZ; 0.5 mg/ml) for 24 h. A: representative images show DAPI nuclear staining. Arrowheads indicate typical condensed and fragmented nuclei of apoptotic cells; bar graphs represent means ± SE (n = 3). B: cell lysates (25 µg protein) were subjected to Western blotting using active caspase-3 antibody and GAPDH antibody as loading control, or using Nodal antibody (C). Isolated rat islets (30 islets) were treated with medium alone or with HG or Pal for 24 h, and cell lysates were subjected to Western blotting using active caspase-3 antibody and GAPDH antibody as loading control (D), or Nodal antibody (E). Data shown represent 3–5 separate experiments.
**Immunostaining.** Cells grown on coverslips were fixed using 4% paraformaldehyde and permeabilized using 0.1% Triton X-100 in PBS and then blocked (3% bovine serum albumin in PBS, 1 h) prior to overnight incubation with the appropriate antibodies: mouse or goat anti-Nodal IgG (1:500; R&D Systems) and guinea pig anti-insulin (1:1,000; Dako, Mississauga, ON, Canada). The corresponding FITC- or Cy3-conjugated secondary antibodies were used. Pancreatic sections (5 μm) were dual-stained for Nodal [1:100, rabbit polyclonal antibody, Millipore (cat. no. AB4334), Temecula, CA] and insulin (guinea pig anti-insulin, 1:1,000) or glucagon (rabbit anti-glucagon, 1:1,000; Dako) and detected with biotinylated secondary antibodies followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories) before chromogen staining of DAB (Sigma-Aldrich) or Fuchsin red (Dako), as described previously (50, 51, 57).

**DNA transfection.** Cells were transfected with or without indicated plasmid DNA, siRNA constructs, or scrambled siRNA using lipofectamine 2000 according to the manufacturer’s instructions. The transfected cells were allowed to grow in Complete medium for 24–72 h posttransfection before treatment with various stimuli as indicated.

**Western blot analysis.** Cells were lysed in RIPA lysis buffer containing protease inhibitors phenylmethylsulfonylfluoride (PMSF) (1 mol/l) and EDTA (1 mol/l), Na3VO4 (1 mol/l), and NaF (1 mol/l). Pancreatic tissue (50–100 mg total wet wt) was minced and homogenized in RIPA lysis buffer; 25 μg of protein was loaded and resolved by SDS-PAGE followed by semidry transfer (Bio-Rad Laboratories) to nitrocellulose membranes. The membranes were probed with primary poly- or monoclonal anti-ALK7 antibodies (1:1,000; R&D Systems), anti-phospho-Amad3 (rabbit monoclonal, cat. no. 9520; Cell Signaling), anti-Nodal antibodies (1:500, R&D Systems), anti-XIAP (1:1,000, R&D Systems), anti-Akt (1:1,000) and anti-phospho-Akt (1:1,000; Cell Signaling), and anti-cleaved caspase-3 (1:1,000; Cell Signaling) or anti-β-actin or GAPDH (1:30,000; Abcam, Cambridge, MA) and visualized with horseradish peroxidase-conjugated secondary antibodies using ECL Plus detection (Amersham, Mississauga, ON, Canada). The protein levels were quantified by densitometry analysis using ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD). The graphs were made after normalization with the protein loadings.

**Proliferation and cell death assays.** Cell proliferation was measured in INS-1 cells treated with or without Nodal protein using [3H]thymidine (PerkinElmer, Boston, MA) incorporation by incubating the cells with the culture medium containing [3H]methylthymidine for 4 h, as described previously (64). Cell apoptosis was measured by 4’-6-diamidino-2-phenylindole (DAPI) nuclear staining or terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit and TMR red (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions, as described previously (59, 64).

**Statistical analysis.** All data are presented as means ± SE. Statistical analysis was done with Student’s t-test or ANOVA with Tukey’s post hoc test as appropriate. Significance was assumed at a P value of <0.05.

**RESULTS**

**Nodal expression was detected in pancreatic β-cells.** RT-PCR detected the Nodal transcripts in rat islets and insulin-secreting β-cell lines INS-1 and MIN6 (Fig. 1A). Nodal mRNA was also detected in clonal acinar AR42J cells or in the brain as control but not in clonal glucagon-secreting α-TC cells (Fig. 1A). Nodal protein was detected by immunocytochemistry in INS-1 cells (Fig. 1B). Mouse pancreatic sections were dual-stained for insulin (red) and Nodal (brown) (Fig. 1C, left), and the adjacent sections were dual-stained for glucagon (red) and Nodal (brown, Fig. 1C, right). The results showed that Nodal expression is localized mostly in the islet β-cells. In contrast, the detection of Nodal protein in the pancreatic acinar or α-cells was at background levels (Fig. 1C).

Fig. 3. Pathophysiological stimulus-induced apoptosis is associated with upregulation of Nodal expression and activation of activin receptor-like kinase-7 (ALK7)-Smad3 signaling pathway in INS-1 cells. A: Western blotting using protein extracts (25 μg) from INS-1 cells treated with 30 mM HG or 0.4 mM Pal or Cyto cocktail (IL-1β 10 ng/ml, TNF-α 50 ng/ml, and IFN-γ 50 ng/ml) or STZ (0.5 mg/ml) for 24 h and probed for Nodal, ALK7, phosphorylated Smad3, active caspase-3, or GAPDH as loading control. B–E: bar graphs represent densitometry analysis. Data are means ± SE; n = 3–5; *P < 0.05, **P < 0.01.
Stimuli-induced apoptosis was associated with elevated levels of nodal expression in the clonal β-cells and isolated rat islets. Various stimuli were used for the induction of β-cell apoptosis, including high glucose (30 mM), palmitate (0.4 mM), cytotoxic cytokines (IL-1β 10 ng/ml, TNF-α 50 ng/ml, and IFN-γ 50 ng/ml) and streptozotocin (0.5 mg/ml). Incubation of the INS-1 cells with the stimuli for 24 h increased apoptosis as determined by nuclear staining, with characteristic condensed and fragmented nuclei (Fig. 2A) or by Western blot analysis using pro-caspase-3 antibody (Fig. 2B) or Nodal antibody (Fig. 2C). The results showed that treatment with the stimuli induced upregulation of Nodal protein expression and apoptosis of INS-1 cells. Similar results were obtained in MIN6 cells (not shown) or in isolated rat islets (Fig. 2, D and E). These observations suggest that stimuli-induced apoptosis is associated with elevated Nodal expression in pancreatic β-cells.

Stimuli-induced apoptosis was associated with upregulation of nodal expression and activation of the ALK7-Smad3 signaling pathway. We previously demonstrated that elevation of ALK7 induces β-cell apoptosis (64). To gain insight into the functional roles of Nodal in β-cells, we examined the potential downstream mechanisms involved in the stimuli-induced activation of Nodal signaling. Western blot analysis showed that high glucose-, palmitate-, or proinflammatory cytokine-induced INS-1 cell apoptosis (Fig. 3A) is associated with enhanced Nodal (Fig. 3B), ALK7 (Fig. 3C), and phospho-Smad3 (Fig. 3D) protein expression, along with increased pro-caspase-3 protein expression (Fig. 3E), suggesting the activation of the Nodal-ALK7-Smad3 pathway during the process of pancreatic β-cell apoptosis.

Nodal induced apoptosis and suppressed proliferation of INS-1 cells. To determine whether Nodal exerts direct apoptotic effects on β-cells, we performed Western blotting in INS-1 cells.
cells treated with increased concentrations of Nodal (16 h). As shown in Fig. 4A, Nodal significantly and dose-dependently increased active caspase-3 expression in the INS-1 cells. Consistently, elevation of Nodal protein levels by plasmid transfection resulted in significant and time-dependent increases in pro-caspase-3 levels in INS-1 cells (Fig. 4B). Nodal-induced INS-1 β-cell apoptosis was also verified by the TUNEL-labeling method (Fig. 4C). Furthermore, the effects of Nodal on β-cell proliferation were evaluated by the [3H]thymidine incorporation assay. Nodal treatment (1 μg/ml) significantly inhibited the proliferation of INS-1 cells (Fig. 4D). We therefore suggest that Nodal induces β-cell apoptosis and inhibits β-cell proliferation.

**Nodal upregulated ALK7, suppressed akt activity, and reduced XIAP expression.** To determine whether Nodal affects ALK7 expression, Western blot analysis was performed in INS-1 cells treated with medium alone or with Nodal protein at indicated concentrations for 24 h (Fig. 5A) or in INS-1 cells transfected with Nodal cDNA for the indicated time periods (Fig. 5B). As shown, Nodal treatment or transfection of Nodal-encoding plasmid dose- or time-dependently increased ALK7 expression levels (Fig. 5, A and B). Given that elevation of ALK7, while activating Smad2 signaling significantly suppresses Akt activities (64), Western blotting was also performed to probe for Akt and its potential downstream molecule XIAP (15). The results showed that Nodal treatment or transfection of Nodal-encoding plasmid dose- or time-dependently reduced phospho-Akt levels (Fig. 5, A and B), whereas total Akt protein levels remained unchanged (not shown). Concomitantly, the treatment significantly reduced XIAP expression in the INS-1 β-cells (Fig. 5, A and B). These results suggest that Nodal-induced apoptosis is involved with the elevation of ALK7, suppression of Akt activity, and reduced XIAP expression in pancreatic β-cells.

**Fig. 5.** Nodal treatment upregulates ALK7 and decreases active Akt and X-linked inhibitor of apoptosis (XIAP) expression in INS-1 cells. Western blotting used protein extracts (25 μg) from INS-1 cells treated with Nodal protein as indicated for 24 h (A) or from INS-1 cells transfected with Nodal cDNA for indicated time period (B), using relevant antibodies as indicated and GAPDH as loading controls. Bar graphs show densitometry analysis. Data represent means ± SE; n = 3–5. *P < 0.05, **P < 0.01.
Elevation of Akt/XIAP or suppression of ALK7/Smad2 attenuated Nodal-induced β-cell apoptosis. Experiments involving cotransfection combined with Western blotting were conducted to examine the role of ALK7/Smad2/3 and of Akt/XIAP in mediating Nodal-induced apoptosis in INS-1 cells. As shown, the β-cell apoptosis determined by pro-caspase-3 levels was consistently increased in the INS-1 cells cotransfected with Nodal and empty vector (Fig. 6A). Cotransfection of Nodal with DN-ALK7, DN-Smad2, or DN-Smad3 significantly reduced active caspase-3 protein levels in these cotransfected INS-1 cells (Fig. 6A). Furthermore, Nodal-induced apoptosis either by transfection of Nodal cDNA (Fig. 6B) or Nodal protein treatment (Fig. 6C) was dramatically reduced in INS-1 cells cotransfected with expression vectors coding for XIAP or the active form of Akt (Akt-CA). These results collectively suggest that pancreatic β-cell apoptosis induced by Nodal occurs via two distinct signaling pathways: activation of the Smad2/3-caspase-3 cascade and suppression of Akt/XIAP signaling.

Ablation of ALK7 diminished stimulus-induced β-cell apoptosis. To verify the role of Nodal/ALK7 in mediating stimulus-induced β-cell apoptosis, we examined the effect of ALK7 knockdown on high-glucose- or palmitate-induced apoptosis in INS-1 cells by using the siRNA strategy. As shown, transfection of INS-1 cells with ALK7 siRNA resulted in a reduction of 75–80% of ALK7 compared with transfection with scrambled siRNA (Fig. 7, A and B). Induction of apoptosis by 30 mM glucose or 0.4 mM palmitate, which occurred in the control INS-1 cells as determined by active caspase-3 protein, was significantly attenuated in the INS-1 cells with ALK7 knockdown (Fig. 7C). The high-glucose or palmitate treatment enhanced phospho-Smad3 in the control cells but not in the cells transfected with ALK7 siRNA. Interestingly, both high glucose and palmitate enhanced Nodal expression levels.

Fig. 6. Nodal-induced apoptosis is modulated by various signaling molecules. A: Western blotting was performed using protein extracts (25 μg) from INS-1 cells transfected with expression vectors coding for Nodal and either empty vector (EV), or dominant negative (DN)-ALK7, DN-Smad3, or DN-Smad2 for 36 h or from INS-1 cells cotransfected with Nodal cDNA and EV or Nodal cDNA with constitutively active (CA)-Akt or with XIAP (B) or transfected with or without CA-Akt or XIAP and exposed to Nodal protein (1 μg/ml) for 24 h using anti-cleaved caspase-3, anti-Akt, or anti-GAPDH antibodies (C). Data represent 3–4 experiments.
in the INS-1 cells with either the scramble siRNA or the ALK7 siRNA transfections (Fig. 7E).

To further verify the role of ALK7 in mediating Nodal-induced β-cell apoptosis, we conducted ALK7 knockdown in INS-1 cells treated without or with Nodal. As shown in Fig. 8, Nodal-induced β-cell apoptosis, which occurred in the INS-1 cells transfected with the scramble siRNA, was significantly attenuated in the β-cells transfected with the specific ALK7 siRNAs. Of note, ALK7 knockdown also diminished Smad2/3 activation in the presence of Nodal, indicating that Nodal-induced apoptosis in INS-1 cells is partially via activation of the ALK7-Smad2/3 signaling pathway. Collectively, these data suggest that Nodal modulates cell apoptosis via the activation of ALK7 signaling pathway(s) in INS-1 cells.

**DISCUSSION**

In this study, we demonstrate that Nodal is expressed in pancreatic cell lines and rodent islets. Immunohistochemistry using mouse pancreatic section localized Nodal protein predominantly in the islet β-cells but not in the islet α-cells or acinar cells. We found that, in a process of β-cell apoptosis induced by stimuli including high glucose, palmitate, and cytotoxic cytokines, the expression levels of Nodal were significantly increased in clonal β-cells and isolated rat islets. Nodal could induce β-cell apoptosis, exemplified by the treatment of INS-1 cells with Nodal protein or Nodal-encoding plasmid transfection. Nodal-mediated β-cell apoptosis was associated with the elevation of ALK7 and phospho-Smad2/3, as well as the reduction in Akt activity and XIAP expression.

We previously demonstrated that ALK7 mediated β-cell apoptosis through suppression of Akt and activation of the Smad2 signaling pathway. Collectively, these data suggest that Nodal modulates cell apoptosis via the activation of ALK7 signaling pathway(s) in INS-1 cells.

Fig. 7. Ablation of ALK7 diminished apoptosis induced by pathophysiological stimuli in INS-1 cells. A: INS-1 cells transfected with scramble siRNA or with ALK7 siRNA constructs were treated with medium alone or with 30 mM glucose or 0.4 mM palmitate for 24 h. Cell lysates were subjected to Western blot analysis using relevant antibodies as indicated. Densitometry analysis for ALK7 (B), active caspase-3 (C), Nodal (D), or phospho-Smad3 (E) is shown. Data represent means ± SE; n = 3. *P < 0.05, **P < 0.01.
critical in early embryonic development (10, 42), its biological function in adult tissue remains largely unknown. The observations from the present study demonstrate that Nodal indeed inhibits β-cell proliferation and induces apoptosis in pancreatic β-cells, consistent with previous findings that Nodal exerts proapoptotic or growth-inhibitory effects in various cell lineages (25, 41, 48, 54, 55), including the clonal pancreatic acinar cells AR42J (67). In ovary and placenta, through the activation of ALK7 (47), Nodal has been reported to induced apoptosis in these reproductive adult tissues (56).

Modulation of β-cell survival is a critical process in maintaining the homeostasis of β-cell mass. The serine/threonine kinase Akt has been implicated in the proliferation and survival of pancreatic β-cells (23, 24). We previously showed that the active Akt content in islet β-cells was significantly reduced during the progression of diabetes in a rat model (68) and that ALK7-induced β-cell apoptosis and suppression of β-cell growth was involved in the suppression of Akt kinase activity (64). We sought to determine whether Nodal-induced apoptosis affects the signaling pathway involving Akt and its potential downstream target XIAP (15). Our results showed that treatment of INS-1 cells with Nodal or Nodal-encoding plasmid transfection dose- and time-dependently reduced levels of phospho-Akt-XIAP survival pathway (12). These data indicate that Nodal downregulates β-cell growth and survival signaling pathways through the suppression of Akt and XIAP.

Our cotransfection studies further characterized the role of the Nodal-ALK7 pathway in β-cells. We observed that elevation of Nodal by transfection or Nodal protein treatment induced apoptosis in INS-1 cells, which was attenuated in the β-cells coexpressing the active Akt or XIAP protein.
diminished apoptosis was also observed in the β-cells cotransfected with dominant negative Smad2 or dominant negative Smad3. These findings suggest that Nodal-induced apoptosis and suppression of proliferation in the β-cells may use intra-cellular machinery that involves the activation of Smad2/3 and the suppression of Akt and XIAP signaling pathways.

Akt plays an important role in the promotion of cell survival and the inhibition of apoptosis induced by apoptotic stimuli (19). Modulation of the PI3K/Akt survival pathway by TGF-β superfamily protein has been demonstrated (12). Furthermore, it has been shown that activation of the TGF-β heteromeric receptor complexes at the cell surface could initiate the Smad-independent PI3K/Akt signaling cascade (5, 17) and the Smad-dependent pathway through direct interaction between Smad molecule with Akt (12). Taking into account that upregulation of Akt activity could prevent Nodal- (this study) and ALK7-induced β-cells death in the absence of ligand through over-expression of constitutively active ALK7, as we reported previously (64), it could not, however, attenuate ALK7-induced Smad2 phosphorylation (64). It is possible that Nodal/ALK7-Akt signaling is distinct from the ALK7-Smad2/3 pathway.

The observation that the elevation of Akt or XIAP expression attenuates Nodal-induced apoptosis in pancreatic β-cells is consistent with previous findings by other groups, which demonstrates that upregulation of XIAP by induction with adenovirus-carried vector coding for XIAP improved β-cell survival against cytokine attacks in isolated human islets and partially restored insulin secretion in murine islet allografts (29, 45). It has been presumed that, while antiapoptotic effects of Akt occur through inactivating various proapoptotic factors (19), the antiapoptotic effects of XIAP may occur through direct binding and inhibition of caspase activity (18). Furthermore, it has been previously shown that XIAP could also act as a cofactor in the TGF-β signaling pathway (8). Indeed, the interaction between Akt and XIAP has also been suggested by recent studies in various cell lineages (12). Particularly, it has been shown that Akt could directly interact with and phosphorylate XIAP (16), and the phosphorylation of XIAP by Akt could prevent XIAP degradation and thus confer resistance to caspase-3 activation and apoptosis (16). On the other hand, in vitro and in vivo studies by Van Themsche et al. have recently suggested that XIAP can promote Akt activity through the downregulation of PTEN (53), a dual-specificity phosphatase that negatively regulates Akt activity (12, 29). Moreover, our data suggest an important role of Akt-XIAP in modulating β-cell apoptosis mediated by Nodal-ALK7 activation. Further studies are warranted to investigate mechanistically how Nodal/ALK7 signaling modulates the function of Akt and XIAP in β-cells.

The biological relevance of Nodal-ALK7 signaling in β-cells is largely unknown. Identification of Nodal in adult rodent islet β-cells but not in the α-cells suggests an autocrine role of Nodal in the regulation β-cell function. Within the pancreatic islets, insulin is an important autocrine regulator of β-cell growth and survival (6, 32, 35, 58). Such autocrine regulation appears to be critical in regulating the functional plasticity of pancreatic β-cell mass, such as the promotion of islet β-cell growth and the compensation for peripheral insulin resistance (33, 43). We recently demonstrated that the β-cell autocrine trophic effects converged on Akt to regulate β-cell mass (51). It is probable that Nodal in the islet β-cells acts as an antagonist factor to the β-cell growth signaling pathway. The notion that Nodal is a negative modulator in the regulation of islet β-cell mass is in part supported by the recent findings in mice lacking ALK7, which displayed enlargement of islet β-cells, hyperinsulinemia, reduced insulin sensitivity, and impaired glucose tolerance (7). A recent study demonstrated that Nodal exerted inhibitory effects in the process of pancreatic growth and in a murine model of islet regeneration (67). Consistent with this notion, our unpublished data (not shown) showed that the islet Nodal content was remarkably reduced in pancreatic sections from mice which had undergone pancreatectomy.

Within an islet, the negative short-loop insulin-β-cell feedback mechanism appears to be important for maintaining insulin secretion at appropriate levels (66), since inadequate feedback suppression is found in obese subjects and may partly account for their prevailing hyperinsulinemia (22). It is possible, although highly speculative, that Nodal acts as a negative regulator to induce β-cell apoptosis whenever it is necessary, for example, deleting those β-cells that are injured or with misplaced functions to permit a process of islet β-cell self-duplication (21). Nevertheless, further study is needed to investigate whether or not the dysregulation of Nodal/ALK7 signaling that leads to excessive loss of islet β-cells, contributing to the onset of diabetic hyperglycemia, is warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


